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Development of novel wine yeast strains using adaptive evolution

By

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Thesis Summary

Throughout the course of the wine fermentation, yeast are exposed to several simultaneous and sequential stresses, including hyperosmotic shock, nutrient limitation and ethanol accumulation, which are thought to contribute to the development of stuck or sluggish fermentations. Apart from the extended processing time, fermentations in this state can also produce unfavourable aromatic and flavour compounds, collectively leading to increased manufacturing costs and reduced quality. As a consequence there is demand from winemakers for yeast which can overcome these problems, by assuring the rapid completion of fermentation with minimal production of undesirable metabolites. The aim of this study was to produce and characterise more robust strains and address this demand.

Utilising a sequential batch fermentation system we have used adaptive evolution to generate variants of the commercial *Saccharomyces cerevisiae* wine strain L-2056 and a haploid derivative thereof, C911D, which have superior fermentative performance and/or altered metabolite yields. Thus, both evolved cultures produce more glycerol than the respective parent, with the evolved haploid (FM5) also producing a reduced final ethanol concentration and the evolved diploid (FM16) being able to complete fermentation within a shorter duration. Ten clonal isolates were randomly chosen from each of the haploid and diploid evolved cultures and examined in order to determine the degree of heterogeneity of the evolved cultures. In both cases, 9 of the 10 clonal isolates produced similar fermentation phenotypes to the mixed culture from which they were derived.

Transcriptome analysis of mixed cultures and clonal cultures revealed large changes in transcript abundance (magnitude and number of genes) at several time points across fermentation in reference to the parent strains. A commonality noted in a high proportion of genes found to be differentially expressed was the occurrence of a *GCR1* binding site in their promoter regions. Sequencing of the *GCR1* promoter region in the evolved clone FM16-7

revealed several base pair differences compared to the parent L-2056. We propose that these changes may have led to an altered pattern of expression of *GCR1*-regulated genes and in turn the improved fermentation phenotype evident in the evolved diploid cultures.

To further investigate the significance of alterations in the expression of a specific selection of these genes, deletion strains were constructed in the appropriate parental strain background. Fermentation trials conducted with these deletants revealed fermentation phenotypes which approximated those of the evolved cultures. That is the deletion of either *HXT1*, *HXT3*, *GLC3*, *ALD6* and *INO1* in C911D or *ALD6*, *GPH1* and *RCK1* in L-2056 yielded phenotypes similar to the evolved haploid and diploid respectively. No one deletant strain completely matched the magnitude of the improvement in fermentation of the respective evolved culture. As such it is postulated that a number of mutations are responsible for the acquired phenotypes in the evolved cultures.

Declaration of authorship

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

This thesis may be made available for loan or photocopying.

Colin M. McBryde
November 2006

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This thesis is dedicated to Will Gardner.

Under his tall trees,

A true enthusiast works,

Giant hands, deep heart.

15/10/1938 – 11/10/2006

Abbreviations

ACDH	acetaldehyde dehydrogenase
ACS	acetyl CoA synthase
°C	degrees centigrade
cAMP	adenosine-2',3' cyclic monophosphate
CDGJM	chemically defined grape juice media
cDNA	complimentary deoxyribonucleic acid
CO_2	carbon dioxide
DAP	diammonium phosphate
DIG	digoxigenin-11-dUTP
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
FAN	free amino nitrogen
g	grams
x *g*	x gravity
G6P	glucose 6-phosphate
GABA	4-amino butyrate
GR	glutathionc reductase
HAP	6-N-hydroxylaminopurine
HMG CoA	3 hydroxy 3-methylglutaryl coenzyme A
HMGR	3 hydroxy 3-methylglutaryl coenzyme A reductase
HOG	high osmolarity glycerol
HPLC	high performance liquid chromatograghy
l	litre
LB	Luria-Bertani
M	molar
min	minute
mg	milligram
ml	millilitre
mM	millimole
MIP	mitogen activated proteins
mRNA	messenger ribonucleic acid

nM	nanomole
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide reduced form
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate reduced form
OD	optical density
ORF	open reading frame
pM	picomole
PCR	polymerase chain reaction
PDC	pyruvate decarboxylase
PDH	pyruvate dehydrogenase
PKA	protein kinase A
PM	phenotype microarray
QRT-PCR	quantitative real time polymerase chain reaction
RWD	reference window of divergence
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS	sodium dodecyl suplfate
STRE	stress responsive element
TCA	tri-caboxylic acid
T6P	trehalose 6-phosphate
U	units
UAS	upstream activating sequence
UFA	unsaturated fatty acid
UV	ultra violet
µl	microlitre
µM	micromolar
v/v	volume per volume
YEPD	yeast extract peptone dextrose

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Chapter 1 Literature review

1.1 Introduction

The Australian wine industry has grown aggressively over the past decade, reaching and surpassing all major growth, export and sales targets (Woods, 2004). The development of wine yeast strains, optimised to cope under the stresses imposed throughout fermentation and thereby ensuring the best possible final product will help to sustain this competitive international standpoint. Through this project we sought to evaluate the suitability of the use of adaptive evolution for the development of improved wine yeast strains.

Fermentation by yeast is one of the most important processes in wine production. Yeast are responsible for the biochemical conversion of monosaccharides (glucose and fructose) into ethanol, carbon dioxide, and a number of minor compounds, of which some are sensorily significant (for example higher alcohols, organic acids and esters). Consequently, fermentation substantially influences the quality of the final product (Romano *et al.*, 1998; Lambrechts and Pretorius, 2000). Traditionally, the mixture of yeast genera that are present on the grapes and cellar equipment, are involved to varying degrees with fermentation. These yeast include strains from the *Saccharomyces*, *Hanseniaspora*, *Metschnikowia* and *Pichia* genera (Granchi *et al.*, 2002). While many yeast genera may be present at the start of fermentation, as the ethanol content approaches 5 % and above, most progressively die off leaving the more ethanol tolerant *Saccharomyces cerevisiae* to dominate and complete fermentation (Bely *et al.*, 1990b; Fleet, 1990; Boulton *et al.*, 1996; Pretorius, 2001).

Modern winemaking practices utilise purposeful inoculation with selected *S. cerevisiae* strains with favourable oenological characteristics (Mortimer *et al.*, 1994; Pretorius, 2000). While such cultures offer winemakers increased control over fermentation with reduced risk of production of fault or taint organoleptic properties, their use does not guarantee that fermentation will proceed without difficulties. Sluggish or stuck fermentations which result from an attenuation of

yeast sugar catabolic capacity and/or restricted biomass formation (Salmon, 1996; Bisson and Butzke, 2000) are common. In fact, even with the use of preventative treatments approximately 5 – 10 % of industrial fermentations experience some degree of sluggishness or become stuck (N. Bourke, Some Young Punks and T. Adams, Tim Adams Wines; pers. comm.). Factors thought to contribute to the occurrence of stuck fermentations include; restricted oxygen availability, improper inoculum preparation, high ethanol concentrations, low nutrient availability, excessive clarification and/or pH and temperature extremes (Houtman and du Plessis, 1986; Kunkee, 1991; Boulton *et al.*, 1996; Sablayrolles, 1996; Alexandre and Charpentier, 1998; Salmon *et al.*, 1998; Bisson, 1999). Regardless of the cause, stuck fermentations can result in financial losses, because incompletely fermented juice is at risk of microbial spoilage and oxidation, or wine with residual sugar may fall outside of the specifications for a determined market (Henschke, 1997; Iland and Gago, 2002). As a consequence there is a demand from industry to overcome some of the problems surrounding stuck and sluggish fermentations.

While no definitive solution has been found, these problems have also been compounded in recent years as wine styles, and hence the physiochemical parameters of industrial fermentations, have changed. For example, as the Australian wine industry strives for greater complexity and intensity of aroma and flavours, the fruit used for wine production is being allowed to ripen further. The over accumulation of sugar which typically is concomitant with increased flavour ripeness translates to grape juices with higher osmolarity, extended fermentations and higher potential ethanol yields (Day *et al.*, 2002).

Given the multitude of combinations of stresses that a yeast might encounter during fermentation coupled with the requisite to maintain the ability to make positive sensory contributions to the wine, it is clear that no single strain will be suitable for all situations. For this reason there is a growing demand for strains that are tailored to increasingly specialised applications.

Strain improvement broadly seeks to firstly, improve fermentation efficiency and secondly, improve product quality, for example through the generation/isolation of yeast which confer desirable organoleptic properties (Dequin, 2001; Pretorius, 2002). There are several methods which can be utilised for the development of optimised yeast strains. Examples of such include, clonal selection with or without mutagenesis, hybridisation between strains or species, directed genetic alteration (Kielland-Brandt *et al.*, 1983; Snow, 1983) and more recently the use of adaptive evolution (Zelder and Hauer, 2000; Querol *et al.*, 2003). With the current predominance of consumer opposition to the use of genetically modified organisms (GMOs) in food production, strains produced by recombinant strategies have a distinct disadvantage despite the precision of these methodologies (Pretorius and Bauer, 2002). Therefore the remaining methods are enjoying greater attention from researchers.

The primary focus of this project was to test the suitability of generating novel wine yeast strains using adaptive evolution. This technique is essentially a variation of long-standing clonal selection strategies and is used to increase the frequency of fitter phenotypes over time, through the use of an environment that elicits a selective pressure (Hall, 1991; Shapiro, 1997; Foster, 1999; Foster, 2000). The main benefit of this approach is that the extended period of selection allows for the appearance of phenotypes that have a more complex basis, that is, those involving modification of multiple genes compared with clonal selection strategies that tend to produce/isolate point mutations in single or a few genes.

1.2 Fermentation

Generally, industrial grape musts will have a sugar concentration between 200 and 300 g l^{-1}. Under these circumstances yeast cultures will undergo alcoholic fermentation to generate energy for survival (Lagunas, 1979). As the initial concentration of the fermentation medium exceeds the threshold concentrations for sugar signalling cascades (Meijer *et al.*, 1998; Meneses and Jiranek, 2002), the main glucose repression pathway and the Ras/cAMP/PKA pathway are

triggered. Activation of these pathways has three main consequences: repression of respiration, arrest of the consumption of other carbohydrates, and loss of cellular stress resistance (Verstrepen *et al.*, 2004). The expression of many genes is induced particularly by the presence of glucose. Of importance here are those encoding glycolytic enzymes as well as hexose transporters (*HXT*) (Muller *et al.*, 1995; Ozcan and Johnston, 1995).

The regulation of glycolysis is dependent on three systems that involve; 1) a transcriptional repressor Rgt1p (Ozcan *et al.*, 1996b), 2) a multiprotein complex (SCF^{Grr1}) that inhibits Rtp1p repressor function (Li and Johnston, 1997; Kishi *et al.*, 1998), and 3) glucose sensors in the membrane that generate an intracellular glucose signal (Snf3p and Rgt2p) (Ozcan *et al.*, 1996a). In the absence of glucose, the zinc-finger-containing Rgt1p repressor binds to *HXT* promoters and represses their transcription, probably by recruiting the general repressors Ssn6p and Tup1p (Ozcan *et al.*, 1996b). When glucose is added to cells, it binds to glucose sensors located in the cell membrane and generates an intracellular signal that causes the SCF^{Grr1} complex to inactivate the Rgt1p repressor, thereby derepressing *HXT* gene expression and enabling glucose transport. The transport of glucose is regulated by the membrane bound glucose sensors (Snf3p and Rgt2p) that generate an intracellular glucose signal upon binding extracellular glucose (Johnston, 1999). Both are required for induction of *HXT* expression by glucose (Figure 1.1) (Ozcan and Johnston, 1995; Ozcan *et al.*, 1996a).

Upon availability of sugar in the cell, glucose is converted to glucose 6-phosphate by hexokinase, which is in turn is interconverted into fructose 6-phosphate by phosphoglucoisomerase (Pritchard and Kell 2002). The glycolytic pathway is then catalysed by phosphofructokinase (Pfk) (Figure 1.2). Pfk activity increases as a result of rising concentrations of its substrate, fructose 6-phosphate (F6-P), and its main allosteric activator, fructose 2,6-bisphosphate (F2,6-BP) (Gancedo and Serrano, 1989). Activation of the Pfk reaction increases the concentration of its product, fructose 1,6-bisphosphate (F1,6-BP), which is, in turn, an allosteric activator of pyruvate kinase (Pyk),

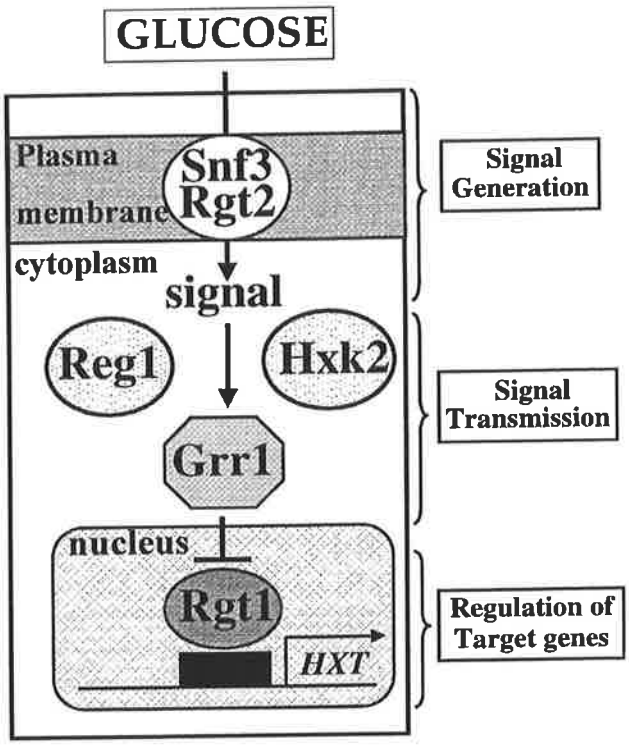


Figure 1.1 The glucose induction pathway of the *HXT* genes and its components. The arrow implies positive regulation; a line with a bar denotes negative regulation (Ozcan and Johnston, 1999).

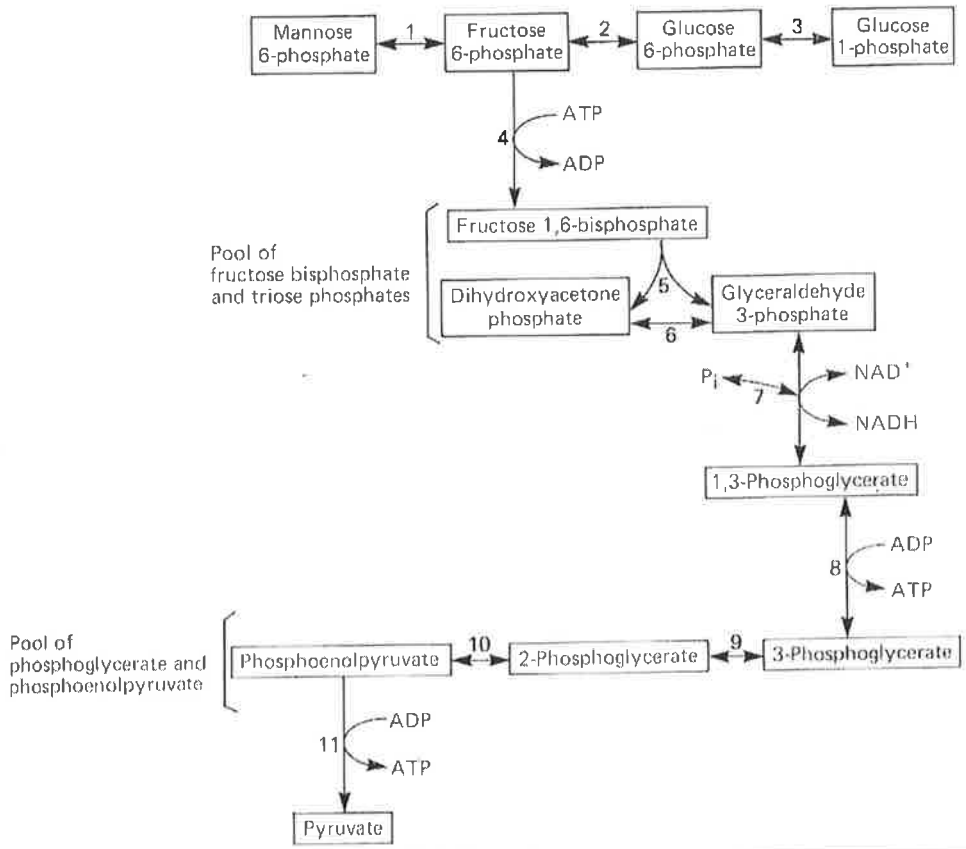


Figure 1.2 The common glycolytic pathway. Enzyme notations 1, phosphomannose isomerase; 2, phosphoglucose isomerase; 3, phosphoglucomutase; 4, phosphofructokinase; 5, aldolase; 6, triosephosphate isomerase; 7, glyceraldehyde 3-phosphate dehydrogenase; 8, phosphoglycerate kinase; 9, phosphoglycerate mutase; 10, enolase; 11, pyruvate kinase (Taken from Gancedo and Serrano, 1989).

the next enzyme in the glycolytic pathway. As such glycolysis is progressively activated by increasing concentrations of enzyme substrates and allosteric activators (Gancedo and Serrano, 1989). F1,6-BP is then cleaved by aldolase to form either dihydroxyacetone phosphate or glyceraldehyde 3-phophate, which interconvert by the action of triosephosphate isomerase. Glyceraldehyde 3-phosphate dehydrogenases and phosphoglycerate kinases catalyse the conversion of triose phosphates into the next pool of intermediates. This pool of intermediates constituted by 3-phosphoglycerate, 2-phosphoglycerate and phosphoenol pyruvate are interconverted by phosphoglyceratemutase and enolase. Pyruvate kinase catalyses the last step in the common glycolytic pathway giving rise to pyruvate. Pyruvate decarboxylase catalyses the decarboxylation of pyruvate, before a final reduction, catalysed by alcohol dehydrogenase to ethanol. During alcoholic fermentation nicotinamide adenine dinucleotide (NAD^+) is reduced from NADH (the reduced form of nicotinamide adenine dinucleotide) in terminal steps during the conversion of glyceraldehyde 3-phosphate to pyruvate. The central role of redox couples NAD^+/NADH and $NADP^+$/NADPH (nicotinamide adenine dinucleotide phosphate and its reduced form) in the metabolism of sugar by yeasts and directly relates to energy metabolism and product formation. NADH is regarded as a predominantly catabolic reducing equivalent, whereas NADPH is mainly involved in anabolic processes (van Dijken and Scheffers, 1986). A means of replenishing NAD^+ in the pathway is the production of glycerol, where dihydroxyacetone becomes the preferred hydrogen acceptor forming glycerol 3-phosphate before formation of glycerol. It is important to note that the regeneration of NAD^+ is necessary to maintain the redox balance to prevent the stalling of a multitude of cellular activities, including glycolysis (Walker, 1998).

1.3 Stresses encountered during wine fermentation

Dried industrial wine yeast preparations, or so called Active Dried Wine Yeasts (ADWY) are rehydrated prior to inoculation. The rehydration step is required to re-establish functional membranes and metabolic activity, since the water content in dried yeast is too low to maintain any of these processes (Boulton *et*

al., 1996). Winemakers commonly rehydrate yeasts in warm (35 – 40°C) water, a procedure that is thought to trigger a heat shock and hypotonic shock, and the inclusion of sucrose or must/juice under such conditions can also lead to a hyperosmotic shock (Rossignol *et al.*, 2006).

Following the inoculation of yeast into grape must or juice, initial stresses may include heat and/or hyperosmotic shock. Furthermore, the acidity (pH 2.8 – 4.0) of the must may represent another challenge to the yeast. The severity of these stresses is dependent on pre-culture treatment, for example dilution of inoculum with juice and gradual adjustment of the inoculum temperature to that of the juice. As fermentation progresses, the biological activity of yeast leads to further adverse conditions, including nutrient limitation, rising ethanol concentrations along with subsiding glucose toward the end of fermentation (Puig *et al.*, 2000; Carrasco *et al.*, 2001). Finally, yeast are exposed to inhibitors, some of which can accumulate over the period of fermentation, including sulfur dioxide and yeast derived killer factors (D'Amore, 1992). Such conditions may lead to the arrest of active growth through a variety of physiological, biochemical and morphological cellular changes, but largely are a result of alterations to the cellular membrane (Werner-Washburne *et al.*, 1996). The most influential fermentation stresses are highlighted in Table 1.1.

Exposure to environmental stress may lead to the disruption of several intracellular processes. Cells can respond to environmental stress by activating a specific set of genes, referred to as heat-shock genes, which encode a set of heat-shock proteins (Hsps). These genes, in addition to being activated in response to elevated temperatures, are induced in response to other stresses, such as ethanol and nutrient-limiting conditions (see review by Lindquist and Craig, 1988). Hsps play important roles in the repair of intracellular damage caused by exposure to stress through their biological activities as molecular chaperones (Bond, 2006). Further to this, environmental stress also activates stress signalling cascades, which are mediated by positive transcriptional control elements such as stress signalling stress responsive elements (STREs)

Table 1.1 Stresses encountered by yeast during fermentation (adapted from Boulton *et al.*, 1996).

Type of Stress	Cause
Physical/Chemical	Temperature shock
	Osmotic shock
	pH
Nutrient Limitation	Macronutrient
	Nitrogen
	Phosphate
	Micronutrient
	Vitamins
	Minerals
	Deficiency of survival factors
	Sterols, unsaturated fatty acids
	Deficiency of oxygen
Inhibitor Accumulation	Organic acids
	Acetate
	Propionate
	Butyrate
	Fatty acids
	cis rather than trans
	Medium chain (C_6-C_{10})
	Microbial toxins
	Yeast killer factors
	Ethanol
	Excess sulfur dioxide

and activating protein 1 (AP1) responsive elements (Ruis and Schuller, 1995). These mediate the activation of other stress responsive genes.

Glycogen and trehalose are the two major storage carbohydrates in the yeast *S. cerevisiae* and can represent up to 25 % of cell dry mass (Lillie and Pringle, 1980). Glycogen and trehalose accumulate under conditions of stress (Attfield, 1987; Hottiger *et al.*, 1987; Parrou *et al.*, 1997; Alexandre *et al.*, 1998). The major function of glycogen is thought to be to provide carbon and energy for maintaining cellular activities when nutrients are scarce. Trehalose protects the cell from autolysis, thought to function through the capacity to protect membranes from desiccation (see review by Francois and Parrou, 2001). The accumulation of these storage carbohydrates, particularly trehalose, is thought to stabilise cellular structures under stress conditions (Crowe *et al.*, 1984), thus preserving cell integrity during the drying process allowing for greater viability upon inoculation.

Glycogen synthesis is initiated by the gene products of *GLG1* and *GLG2* (Cheng *et al.*, 1995), which catalyses the formation of α (1,4) glucosyl primer from UDP-glucose. The primer is elongated by either of two isoforms of glycogen synthase *GSY1* and *GSY2* (Farkas *et al.*, 1991), before branching, mediated by *Glc3p* (Rowen *et al.*, 1992; Thon *et al.*, 1992), which introduces α (1,6) branches (Parrou *et al.*, 1997). Glycogen degradation occurs by amylolysis catalysed by α-glucosidases, producing glucose or by phosphorlysis and debranching activities releasing glucose 1-phosphate and glucose (Francois and Parrou, 2001).

Trehalose is synthesised from UDP-glucose involving trehalose 6-phosphate synthase encoded by *TPS1*-encoded trehalose 6-phosphate synthetase and converted to trehalose by the *TPS2*-encoded trehalose 6-phosphate phospahtase (Bell *et al.*, 1992; Vuorio *et al.*, 1993). Degradation of treholase occurs through hydrolysis by neutral (Nth1p and Nth2p) or acid (Ath1p) trehalases.

1.3.1 Nutrient limitation

Nutrient availability plays an integral role in yeast performance during fermentation. Nutrients considered important include zinc, magnesium, nitrogen and oxygen, and limitations of these often lead to altered fermentation dynamics with negative repercussions (Dombek and Ingram, 1986; Bromberg *et al.*, 1997; Bauer and Pretorius, 2000). Zinc is an essential nutrient, which acts as a ubiquitous structural or catalytic cofactor. Zinc deficiency causes pervasive changes in the gene expression pattern of yeast (Lyons *et al.*, 2000). Deficiency has been shown to be a major contributor to retarded yeast fermentations in the brewing process (Bromberg *et al.*, 1997). Magnesium is important for many metabolic and physiological functions in yeast (Walker, 1994). It is involved in cell integrity, generally by stabilizing nucleic acid, proteins, polysaccharides and lipids and Mg^{2+} also plays a key role in metabolic control, growth and cell proliferation.

Nitrogen is often limiting in grape juice (Henschke and Jiranek, 1993; Bell and Henschke, 2005). Under oenological conditions, the maximum fermentation rate is closely related to the amount of assimilable nitrogen present in the must (Cantarelli, 1957; Agenbach, 1977; Bely *et al.*, 1990a). Nitrogen is present in the grape must as a complex range of compounds, of which the majority of the yeast assimilable portion is made up of amino acids and ammonium (Ingledew *et al.*, 1987; Spayd *et al.*, 1994; Spayd and Andersen-Badge, 1996). A primary concern for winemakers during fermentation is nitrogen deficiency, which results in down-regulation of protein synthesis, inhibition of glucose transport, and subsequently a reduced fermentation rate (Lagunas *et al.*, 1982; Busturia and Lagunas, 1986; Salmon, 1989). Other problems associated with inadequate nitrogen content of grape must include the formation of reduced-sulphur compounds, in particular hydrogen sulphide (Vos and Gray, 1979; Jiranek *et al.*, 1995b; Spiropoulos and Bisson, 2000). There are currently two basic strategies to circumvent these problems: prevention of nitrogen deficiency in grape juice by optimising the nitrogen status of the vineyard or supplementation of the fermentation with ammonium salts such as diammonium phosphate (DAP). However, supplementation such as this often

contravenes the wine industry's desire to minimise its use of additives while producing high quality wines (Pretorius, 2000). Moreover, excessive use of inorganic nitrogen sources can result in high levels of residual nitrogen, which can in turn lead to microbial instability and the potential for ethyl carbamate (Monteiro *et al.*, 1989; Ough, 1991; Pretorius, 2001) and phosphate (in the case of DAP) accumulation in wine (see review by Bell and Henschke, 2005).

The concentration of oxygen in the initial culture medium is considered as an important parameter for successful completion of fermentation. Yeast require oxygen for biosynthetic reactions of oxygenase enzymes such as those involved in the synthesis of sterols and unsaturated fatty acids (Andreasen and Stier, 1953; Andreasen and Stier, 1954; ter Linde *et al.*, 1999; Julien *et al.*, 2000). The dissolved oxygen concentration in grape juice is dependent on grape variety, juice handling methods and temperature (Fornairon-Bonnefond *et al.*, 2003). It has been elucidated that the dissolved oxygen content in reductively handled musts, where sparging and blanketing with CO_2 are employed, could be considered as less than 1 % of oxygen saturation (< 1 mg l^{-1} (Julien *et al.*, 2000)) at the time of inoculation. Non-reductive handling can yield around 42 % of saturation (> 30 mg l^{-1} (Schneider, 1998)), however, this can be depleted within 4 hours of inoculation (Poole, 2002). Consequently, oxygen is typically in limited supply during fermentation.

The presence of molecular oxygen is necessary for the biosynthesis of sterols of which 90% are accounted for by ergosterol in *Saccharomyces* (Arnezeder and Hampel, 1991). The rate of sterol production is thought to be determined by the activity of HMG CoA (3 hydroxy 3-methylglutaryl coenzyme A) reductase (HMGR), responsible for the conversion of 3 HMG CoA to mevalonic acid and dependent on the availability of oxygen (Casey *et al.*, 1992; O'Connor-Cox *et al.*, 1993). HMGR is active early in the ergosterol pathway and is critical for supplying metabolic intermediates to both ergosterol synthesis and others (O'Connor-Cox *et al.*, 1993). Furthermore, oxygen is required for several biochemical reactions in the synthesis process, such as the cyclisation of squalene to form the first sterol, lanosterol, as well as several

demethylation and desaturation reactions (Parks, 1978; Strydom *et al.*, 1982; Casey *et al.*, 1992) . Oxygen is also vital for the biosynthesis of unsaturated fatty acids (UFAs), which are essential for growth. The synthesis of UFAs involves an oxidative desaturation catalysed by the acyl CoA desaturase encoded by *OLE1*, to introduce double bonds into saturated fatty acids (Rosenfeld and Beauvoit, 2003). The key UFAs, which play an important role in yeast structure and function (e.g. membrane integrity and ethanol tolerance) include palmitoleic (16:1) and oleic (18:1) acids. Together these constitute approximately 70 % of total fatty acids in *S. cerevisiae* cell membranes (Walker, 1998). Unless molecular oxygen is available, this transition from saturated to unsaturated fatty acids is not possible, thus membrane composition and functionality is not ideal. Consequently, oxygen, through its influence over the synthesis of lipids is critical to fermentation.

Lipids are not directly responsible for the formation of new cells, but concerned primarily with the physiological state of the cell, permitting it to maintain a higher level of fermentative activity (Mauricio, 1991; Valero *et al.*, 2001) and therefore the activity of the membrane-associated enzymes (Rogers and Stewart, 1973; Aries and Kirsop, 1977; Aries and Kirsop, 1978; Alexandre and Charpentier, 1998). Low membrane contents of sterols and unsaturated fatty acids are proven to cause stuck and sluggish fermentations (Sablayrolles, 1996). Higher concentrations of ergosterol lead to functional plasma membranes, with heightened tolerance to the stressful conditions that prevail through fermentation. Additionally, a rapid onset of fermentation is ensured thereby permitting the yeast to complete fermentation in the presence of higher sugar and ethanol concentrations (Larue *et al.*, 1980; Strydom *et al.*, 1982; Valero *et al.*, 2001).

For these reasons, yeast with a reduced dependency on oxygen would be highly beneficial to the wine industry. Such yeast would help to ensure the completion of fermentation and minimise the risk of stuck fermentations through enhanced membrane functionality.

1.3.2 Ethanol toxicity

One cause of inhibition of fermentation activity of *S. cerevisiae* is altered membrane permeability, which results in slower sugar transport (Larue *et al.*, 1980). This can be associated with ethanol toxicity, particularly apparent during the latter stages of fermentation. Ethanol is highly toxic to yeast growth and metabolism, and when concentrations rise above approximately 14 % (v/v) these effects become particularly deleterious (Sablayrolles, 1996; Puig and Perez-Ortin, 2000; Blateyron and Sablayrolles, 2001). The damaged and more permeable membranes are the result of altered membrane organization (Beavan *et al.*, 1982; Sajbidor and Grego, 1992; Kunkee and Bisson, 1993; Alexandre *et al.*, 1994; Alexandre and Charpentier, 1998). For these reasons, lipids of yeast are important in the adaptation by cells to growth in the presence of ethanol. Lipids ensure adequate cell division when the fermentation becomes anaerobic, allowing prolonged membrane function and nutrient uptake, thus preserving viability and decreasing cell death especially toward the end of fermentation (Eglinton and Henschke, 1991).

1.4 Oenologically important metabolites

A highly important parameter in winemaking is the organoleptic property of the finished product (Pretorius, 2000). The perceived flavour profile of a wine is the result of specific ratios of many compounds (Noble, 1994). In wine the major products of yeast fermentation which influence the olfactory profile include esters and alcohols, with glycerol also being noteworthy, despite its aroma neutrality.

Esters are a by-product of fermentation, of which alcohol acetates and $C_4 - C_{10}$ fatty acid ethyl esters are found in the highest concentration in wine (Nykanen, 1985; Stashenko *et al.*, 1992; Herraiz and Ough, 1993). The concentrations produced during fermentation are dependent on several variables including yeast strain, fermentation temperature, skin contact, must/juice pH and sulfur dioxide concentration (Houtman *et al.*, 1980). Major esters and higher alcohols found in wine are listed in Table 1.2.

Table 1.2 Major esters and alcohols reported in wine with relative concentrations and flavour descriptors (adapted from Clarke and Bakker, 2004).

Compound	Reported concentration in wine (mg l^{-1})	Aroma/flavour Characteristics
Ethyl ethanoate	4.5 - 180.0	Etheral/fruity
Ethyl n-butanoate	0.04 - 1.00	Banana/pineapple
Ethyl octanoate	1.1 - 5.1	Soapy/candlewax
Ethyl decanoate	0.6 - 4.0	Oily/fruity/floral
Ethyl mono-hydroxy propanoate	3.8 - 17.0	Mild/buttery/fruity
n-propylethanoate	0.00 - 0.04	Pear
Hexyl hexanoate	Trace - 1.3	Herbaceous
Acetaldehyde	0.00 - 1000	Pungent/suffocating (high). Fresh green apples (low)
Propan-1-ol	11 - 68	Fruity alcoholic
Butano-1-ol	1.4 - 8.5	Alcoholic
Pentan-1-ol	<0.4	Fusel
2-Methyl butan-1-ol	19 - 96	Earthy/musty/fruity
Hexan-1-ol	0.5 - 12	Fatty/fruity
2-Phenyl ethan-1-ol	~50	Floral/woody
Glycerol	4.2 - 10.4	Mouthfeel/perceived sweetness

Higher alcohols found to contribute wine aroma and flavour are characteristically pungent. At high concentrations (>300 mg l^{-1}) these are negative quality factors, but at lower concentrations add to the desirability and complexity of wine (Clarke and Bakker, 2004).

As previously mentioned glycerol does not directly impact on the aroma of a wine, however it is considered important texturally as a function of its viscous nature, it also offers a slightly sweet taste (Scanes *et al.*, 1998), with its threshold being reported as 5.4 g l^{-1} (Noble and Bursick, 1984). Glycerol is produced as a redox sink during fermentation, its formation is dependent on the several factors, such as yeast strain, inoculum density, aeration and fermentation temperature (Scanes *et al.*, 1998; Remize *et al.*, 1999). The concentrations found in wines range from 4.2 to 10.4 g l^{-1} (Radler and Schutz, 1982) and are typically higher in red compared to white wine (Remize *et al.*, 1999). In addition to a possible sensory effect on wine, the over-production of glycerol offers a mechanism of reduced ethanol concentration in wine. This is seen as highly beneficial, as some consumers deliberately avoid highly alcoholic wines and ethanol can often mask aromas and flavours as well as contribute to an undesirable perception of heat on the back palate (Iland and Gago, 2002). This is particularly relevant in new world winemaking countries where fruit is being allowed to accumulate higher concentrations of sugar for flavour ripeness.

1.5 The development of optimised wine yeast strains

Oenologists have come to recognise the importance of specific *S. cerevisiae* starter culture strains for producing particular types and styles of wine. Consequently, there is a current demand for new and improved wine yeast strains, highly tailored for specific functions. Starter culture strains of *S. cerevisiae* must now possess a range of distinctive properties as listed in Table 1.3. General targets for strain optimisation include improved sugar uptake and assimilation, improved ethanol tolerance, improved nitrogen assimilation, formation of flavour compounds and enhanced resistance to microbial metabolites and toxins (Pretorius, 2000; Pretorius, 2001). Development of such

Table 1.3 Targets for the genetic improvement of wine yeast strains (Pretorius, 2002).

Desirable Properties	Focus Area
Improved Fermentation Performance	
Improved stress tolerance	Stress response, sterol, glycogen and trehalose accumulation
Improved efficiency of sugar utilisation	Hexose transporters, hexose kinases
Improved nitrogen assimilation efficiency	Utilisation of less efficient N sources
Improved ethanol tolerance	Sterol formation, membrane ATPase activity
Increased tolerance to antimicrobial compounds	Resistance to killer toxins, sulphur dioxidem agrochemicals
Improved Processing Efficiency	
Improved protein clarification	Proteases
Improved polysaccharide clarification	Glucanases, pectinases, xylanases
Controlled cell sedimentation and flocculation	Cell wall hydro-phobic properties
Improved Biological Control of Wine Spoilage Microorganisms	
Wine yeast producing antimicrobial enzymes	Lysozyme, glucanases, chitinases
Wine yeast producing antimicrobial peptides	Bacteriocins
Improved Wine Wholesomeness	
Decreased level of alcohol	Carbon flux, glycerol metabolism and glucose oxidation
Descreased urea content	Complete utilisation of arginine nitrogen, urease enzymes.
Increased resveratrol content	Phenolics antioxidative substances
Improved Wine Flavour and Other Sensory Qualities	
Enhanced liberation of grape terpenoids	Glycosidases, gluconases, arabinofuranosidases
Enhanced production of desirable volatile esters	Esterases
Optimised fusel oil production	Amino acid metabolism
Enhanced glycerol production	Glycerol metabolism
Bioadjustment of wine acidity	Malo-ethanolic and malolactic fermentation, lactic acid production

strains caters for the need for yeast that are better adapted to regional differences in grape varieties, viticultural practices and wine styles. It is generally accepted that a range of yeasts is required as it is highly unlikely that a single strain would suit all applications (Pretorius, 2000).

Obviously there are numerous attributes that a wine yeast enhancement program could target. Two are considered particularly important to the basic need for fermentation reliability. A reduction in the dependency for oxygen by wine yeast and their ability to utilise nitrogen more efficiently. A yeast with reduced oxygen dependency could be speculated to arise in a number of ways: either by being able to pre-accumulate lipids during the aerobic propagation of the starter culture or alternatively through changes which minimise the impact that a low level of aerobically synthesised membrane components has on yeast growth and fermentation. In either case, the risk of a stuck fermentation should be reduced. Nitrogen efficient yeast would overcome the risk of declining protein synthesis and subsequent stuck fermentations that occur in nitrogen-limited juices. Construction of yeast able to effectively utilise proline or arginine during fermentation could be another way of enhancing nitrogen efficiency.

1.5.1 Methods of strain improvement

It is recognised that an urgent need exists to modify wine yeast strains in order to meet the needs of winemakers, and to satisfy the demands of increasingly sophisticated wine consumers. This directed strain improvement is achievable via several mechanisms, including genetic engineering, clonal selection, mutation and selection, hybridisation, protoplast fusion and adaptive evolution.

1.5.1.1 Genetic engineering

To apply genetic engineering techniques, an efficient transformation system is required to introduce recombinant DNA into a host organism. An ideal gene transfer system for industrial yeasts often requires, among other things, a selectable marker and methods yielding high transformation efficiency to allow

construction of stable strains by chromosomal integration (Puig *et al.*, 1998). For commercial reasons, it has been recommended that transformants; 1) maintain the same useful properties as the parental strain, 2) be free of non-essential foreign DNA (for example antibiotic resistant genes), 3) have the minimal amount of synthetic DNA linkers, and 4) do not express any fusion protein that may arise as a consequence of the genomic integration events (Linko *et al.*, 1997).

Wach *et al.* (1994) have designed a yeast transformation protocol that utilises a *KanMX* expression module that confers kanamycin or G418 resistance for the integration of linear DNA fragments into desired sites of the yeast genome. The integrative cassette includes the kan^R selectable marker (gene from the *Escherichia coli* transposon Tn903). Targeting of the integrative event is achieved by way of the two flanking "arms" that are complementary to the integration site. Importantly, there is also a version of the cassette (pFA6-kanMX3) that permits subsequent loss of the marker by homologous recombination and loop-out of intervening sequences. The method allows a yeast gene to be disrupted or an exogenous gene with minimal foreign sequence to be inserted into the chosen site (Puig *et al.*, 1998). This technique was used in this laboratory to engineer a haploid wine yeast strain (Walker *et al.*, 2003).

Puig *et al.* (1998) used this cassette to disrupt the two *URA3* alleles of the wine yeast T_{73} strain, obtaining a stable recombinant strain that retained all of its useful winemaking properties. Similar transformation methods have been used to express the malolactic gene of *Lactococcus lactis (mleS)* in *S. cerevisiae* strains V5 and OL1 to produce yeast that yield significant amounts of L-lactate during fermentation. Additionally, when the malate permease from *Schizosaccharomyces pombe* (*mae1*) was co-expressed in such strains, malolactic fermentation was conducted in 4 days, without the need for the presence of malolactic bacteria under oenological conditions (Ansanay *et al.*, 1996; Bony *et al.*, 1997; Volschenk *et al.*, 1997). The strain V5 was also engineered to over-express the *GPD1* gene, encoding glycerol-3-phosphate

dehydrogenase, resulting in increased glycerol production and a slight decrease in ethanol formation under conditions simulating wine fermentation (Remize *et al.*, 1999). It was ascertained that the engineered yeast strains exhibited a significant increase in catabolism of sugars during stationary phase, thereby reducing fermentation duration. Recently, Husnik *et al.* (2006) engineered a commercially available malolactic wine yeast, integrating a linear cassette containing *S. pombe* malate permease *mae1* and the *Oenococcus oeni* malolactic gene (*mleA*) under the control of the *S. cerevisiae PGK1* promoter, and which has 'generally regarded as safe' status from the Food and Drug Administration in the USA.

Thus, while recombinant strategies are not as well developed for use in homothallic wine yeasts as they are for laboratory strains, these approaches have nonetheless enjoyed some success. However, given current scepticism by consumer groups about genetic modification of organisms, the feasibility of using this technology for the production of optimised wine yeast strains is reduced (Pretorius and Bauer, 2002). Therefore, non-recombinant techniques warrant serious attention at this time. The main approaches to non-recombinant strain improvement are outlined below along with a summary of their main benefits and disadvantages.

1.5.1.2 Clonal selection

The selection of variants from a diverse naturally occurring population is a simple, direct means of strain development that depends on genetic variation normally present in all populations of organisms, including wine yeast strains (Pretorius *et al.*, 1999). The practicality of this method is dependent on the availability of an effective selection or screening method since large numbers of clones derived from single cells of the parental strain must be evaluated. This method of strain improvement has been used for decades with success, resulting in such attributes as low foaming mutants of sake yeast (Ouchi and Akiyama, 1971). However, dramatic improvements in wine traits are generally

not expected using this method (Snow, 1983) and the necessity to evaluate large numbers of candidate strains can prove resource intensive.

1.5.1.3 Mutation and selection

Methods of mutation of a cell population followed by selection for a particular phenotype have been published as logical approaches to strain development wherein a large number of performance parameters are kept constant with a single variable (Kielland-Brandt *et al.*, 1983). This approach is often used when the biochemical or genetic basis for the phenotype is not clear. The most frequently used mutagens for this technique are ultra violet (UV) radiation or chemicals, such as ethylmethylsulfonate or nitrosoguanidine (Barre *et al.*, 1993). The mutation of commercial yeasts has lead to improvement of certain traits, for instance the isolation of auxotrophic diploid sake yeast strains following UV mutagenesis (Hashimoto *et al.*, 2005). It has also been used to produce auxotrophs for valine, leucine and isoleucine (Rous *et al.*, 1983), which give reduced production of isobutyl, active amyl and isoamyl alcohols during fermentation, a desirable outcome if a preference for neutral flavour contribution from yeast was desired. However, since mutagenesis is a random approach the debilitation of other important characteristics is very possible and in fact not uncommon (Pretorius and van der Westhuizen, 1991).

1.5.1.4 Hybridisation

Ideally, hybridisation involves a mating between two heterothallic strains of opposite mating types, which can thus produce stable hybrid lineages. Two homothallic strains can also be used, on the condition that the cultures can efficiently sporulate and that fusion can occur between germination and re-diploidisation (Barre *et al.*, 1993). However, wine yeasts are generally diploid and homothallic making mating reactions rare and hybridisation in such programs are further complicated by their frequent poor sporulation and spore viability (Snow, 1983; Walker *et al.*, 2002; Walker *et al.*, 2003). Some more advanced methods are beginning to be used to facilitate isolation of hybrids from amongst mixtures of poorly sporulating or mating parents. For example,

flow-cytometry assisted cell sorting (FACS) analysis has been used to collect multi-dyed putative hybrids from mating populations of differentially pre-dyed parent cell populations (Bell *et al.*, 1998).

Hybridisation has often been used to eliminate an undesirable property from a strain or to confer a particular attribute (Barre *et al.*, 1993). Both Thornton (1985) and Shinohara *et al.* (1997) achieved this objective by introducing flocculent properties into non-flocculent wine yeast where improved hybrids were isolated and shown to demonstrate practical flocculation and desirable fermentation properties. In other work, Zambonelli *et al.* (1994) performed crosses between *S. cerevisiae* strains that produced different amounts of glycerol. The glycerol production of the resultant hybrids was highly variable, and poorly predicted from the behaviour of the parental strains. Such findings confirm that traits such as glycerol production are the result of complex biological pathways and therefore difficult to modify predictably by non-specific methods such as hybridisation. Nevertheless, in recent work, the Australian Wine Research Institute has had success in a hybridisation project with particular focus on the *sensu stricto* group of yeasts (J. Bellon, pers. comm.). The objective here however was to generate novel yeasts, with distinct properties or combinations of properties compared to the parents, with regard to a fairly broad set of oenologically important attributes.

1.5.1.5 Protoplast fusion

Another method that has allowed the creation of novel strains of yeast that display enhanced biotechnological potential is protoplast or spheroplast fusion (Kavanagh and Whittaker, 1996). The isolation of protoplasts from yeast cells involves the total digestion of the cell wall with lytic enzymes. Traditionally, a preparation derived from the digestive juices of the snail, *Helix pomatia* is used. Enzymes of microbial origin, are also capable of digesting the yeast cell wall, the most relevant being zymolyase (lyticase, a glucanase isolated from *Arthrobacter luteus*) (Peberdy, 1980; Kavanagh and Whittaker, 1996). When the cell wall is removed, the plasma membrane is left surrounding the

cytoplasm and these cells are subsequently referred to as spheroplasts. To encourage fusion the spheroplasts are washed and then mixed in equal numbers and treated with fusogen (polyethylene glycol and calcium ions in buffer), before the suspension is plated out and products are recovered on a selective medium (Stewart, 1981; D'Amore, 1992). This technique has been applied, with varying degrees of success. It has been used to manipulate a number of xylose-fermenting yeasts with the aim of optimising the efficiency of pentose fermentations (Johannesen *et al.*, 1985). In this experiment cultures derived from fusion products enhanced the rate of production, but not the final yield, of ethanol from xylose. Consequently, the application of protoplast fusion in the construction of novel yeast strains allows the combination of characteristics present in the parents, although isolation of a strain displaying specific traits could be regarded as difficult (Kavanagh and Whittaker, 1996).

1.5.1.6 Adaptive evolution

Adaptive evolution can be defined as the process by which a biological system evolves and therefore adapts under various environmental conditions. Because of the numerous advantages of using microorganisms as model systems for studying evolution (Elena and Lenski, 2003), the technique of adaptive evolution has recently grown into a standard tool in the laboratory for studying evolutionary processes of microbial communities in a controlled manner (Helling *et al.*, 1987; Wood and Ingram, 1992; Lenski *et al.*, 1998; Massey *et al.*, 1999; Cooper *et al.*, 2001; Shaver *et al.*, 2002; Riehle *et al.*, 2003). We hypothesise that continued propagation under controlled conditions (essentially an adaptive evolution system), offers a versatile model for the development of optimised wine yeast.

Most research on adaptive evolution has utilised *Escherichia coli* (Helling *et al.*, 1987; Lenski *et al.*, 1998; Papadopoulos *et al.*, 1999; Cooper *et al.*, 2001; Riehle *et al.*, 2003). For example strain FC40, which is unable to catabolise lactose (Lac^-), is able to revert to lactose utilisation (Lac^+) when cultured on media where lactose is its sole carbon and energy source. Consequently this

can be considered as an adaptive mutation through selective growth (Rosche and Foster, 2000).

As an example of an evolution experiment as applied to *S. cerevisiae*, albeit laboratory strains, Ferea and coworkers (1999) cultured three replicate populations of a single ancestral diploid strain in chemostats with continuous aerobic growth in glucose-limited, minimal medium. The three fermentations were cultivated for either 250 or 500 generations. It was found that all three experimental populations exhibited significant phenotypic adaptation to the glucose-limited environment. For example, each adaptively evolved mutant produced approximately three-fold greater biomass per gram of glucose than the parental. Additionally the growth characteristics of the three independently evolved strains were very alike.

There has been a great deal of conjecture about the model which best describes the mechanism of adaptive mutation with several general models being postulated. Thus mutations may be targeted toward genes in which a mutation occurring as a direct result of the selective pressure relieves the stress (directed mutation model). Alternatively, mutation rates may increase genome wide such that both adaptive and non-adaptive mutations are stimulated (hypermutation model). Finally, mutations may arise infrequently or not at all, but DNA replications might let normal mutation rates acting on multiple DNA copies give the appearance of an enhanced mutation rate, and possibly even of mutations of selected genes (cryptic growth model) (Hersh *et al.*, 2004).

The incidence of mutation is another fact to bear, be they replication independent mutations or proliferation dependent mutations (Heidenreich *et al.*, 2003). The dependence of mutational events on the cell cycle highlights the fact that mutations may not only arise during replication, but also during cell cycle arrest. Given that a large portion of the yeast cell cycle is spent in a resting state during an oenological fermentation, the contribution from replication independent mutations may be significant to these populations.

Marini *et al.* (1999) postulated that the frequency of advantageous mutation is much higher than the frequency of "neutral" mutations, which are of no advantage to the cell. However, it was further reported that prolonged starvation in a yeast culture strongly increases the mutation rate in stationary cells in a non-adaptive manner (Marini *et al.*, 1999). This was determined through monitoring the frequency of reversion in a histidine-requiring mutant of *S. cerevisiae* during histidine starvation. The use of a base analogue and very powerful mutagen, 6-N-hydroxylaminopurine (HAP) was successfully employed to induce mutation in growing cells, yet proved completely ineffective when added to stationary cells. The finding strongly suggests that starvation-induced stress significantly increases the mutation frequency in yeast. Given that a similar increase was noted for both the selected markers and neutral markers, it was concluded that mutations arising in stationary cells during starvation are not adaptive. This is again of particular relevance considering that two-thirds of the wine fermentation process is carried out by cells in the stationary stage (Salmon, 1996). These findings differed to the results published by Hall (1992) who stated that starvation induced adaptive mutation in the absence of a mutagen was without any increase in the frequency of neutral mutation.

It is thought that diverse mutations emerge in evolution experiments, including point mutations, small insertions and deletions that cause frame shifts, and larger rearrangements (Elena and Lenski, 2003). It is postulated that these rearrangements usually involve transposable elements that generate insertions as well as inversions and deletions through recombination between homologous elements in yeast (Wilke *et al.*, 1992; Dunham *et al.*, 2002).

In addition to the acquisition of new phenotypes and the reproducibility at the endpoint of evolution, a great deal of interest centres on determination of the mechanistic changes which occur during the evolutionary process (Sniegowski, 1999; Fong *et al.*, 2005). Several evolution studies have used gene expression micorarrays to study evolution (Cooper, 2001; Ferea *et al.*, 1999; Riehle *et al.*, 2003) but were only able to draw conclusions on a small sub-set of genes due

to limitations presented by statistical data analysis (Hess *et al.*, 2001). However recent improvements in gene expression arrays and data analysis allow for improved statistical significance of gene sets (Storey and Tibshirani, 2003; Venkatasubbarao, 2004).

At the commencement of this study no attempts to adaptively evolve commercial strains of *S. cerevisiae* yeast, whether used for winemaking, brewing or baking, had been reported. It has however been suggested that some of the existing commercial strains, identified using traditional isolation and evaluation methods may in fact be the result of adaptive evolution (Querol *et al.*, 2003).

1.6. Conclusions

It remains highly unlikely that a single yeast can be isolated or developed that possesses an ideal combination of all or most oenological characteristics. At any rate, rapidly changing trends in the beverage processes and consumer demands translate to equally rapid changes in the performance criteria expected of fermentation yeasts (Pretorius and van der Westhuizen, 1991; Rainieri and Pretorius, 2000). The continued development of improved or tailored wine yeast strains is therefore essential.

The targets of strain development largely relate to the improved wine quality and the economics of production, where optimisation of fermentation efficiency plays an important role (Pretorius and Bauer, 2002). Specifically, strain improvement could be directed at enhancing biological stress resilience, control of microbial spoilage and the affect on sensory quality (Barre *et al.*, 1993; Ostergaard *et al.*, 2000; Dequin, 2001). Oxygen, nitrogen and stress tolerance play an integral role in fermentation performance, thus optimisation of processes surrounding these nutrients would create yeast strains with higher efficiency through an ability to adapt quickly to the changing environment, as well as minimising the risk of stuck or sluggish fermentations (Ivorra *et al.*, 1999).

There are numerous techniques, which can be used in directed strain development programmes, but adaptive evolution provides a resourceful non-recombinant genetic approach. Changes that arise through this method are based on genetic adaptations, which may range from simple alterations of DNA-sequences, such as point mutations, to dynamic events in the genome structure (Zelder and Hauer, 2000). The latter are expected to offer advantages in terms of the stability of the evolved phenotype(s). Consequently, adaptive evolution offers a resource for directed strain improvement with distinct advantages over other techniques described so far. For this reason adaptive evolution was explored through the present study as a means of generating derivatives of commercial wine yeast strains which are better suited to the challenges of completing oenological fermentations. Strains generated in this way may also provide a useful window into processes of molecular evolution (Schulte, 2001).

Chapter 2 Materials and methods

2.1 Yeast strains and maintenance

The strains used in this study were *Saccharomyces cerevisiae*, as listed in Table 2.1. Strains were cultured in YEPD (yeast extract, 10 g l^{-1}, bacteriological peptone, 20 g l^{-1}, D-glucose, 20 g l^{-1}) at 30°C with shaking at 160 rpm and maintained at 4°C on YEPD-agar (with the addition of 20 g l^{-1} bacteriological agar), or at -80°C as glycerol stocks (culture grown overnight in YEPD and then supplemented with glycerol (final concentration 15 % v/v)).

2.2 Bacterial strains and maintenance

Escherichia coli was utilised in this study for the amplification of plasmid DNA. *E. coli* was cultured in Luria-Bertani (LB) broth (tryptone, 10 g l^{-1}, yeast extract, 5 g l^{-1}, NaCl, 10 g l^{-1}) at 37°C with shaking at 160 rpm, and maintained on LB-agar (LB with the addition of 20 g l^{-1} bacteriological agar). To maintain plasmids during growth (where required) 100 μg l^{-1} of ampicillin was added to LB.

2.3 Culture media

2.3.1 Media for yeast cultures

S. cerevisiae strains were routinely grown in YEPD.

2.3.2 Chemically defined grape juice media

A chemically defined grape juice media (CDGJM) was used to resemble oenological conditions (Henschke and Jiranek, 1993), with known concentrations of all components (Appendix I).

Table 2.1 Strains of *Saccharomyces cerevisiae* used in this study.

Yeast Strain	Genotype	Source
L-2056	Industrial wine yeast	Lallemand Pty Ltd
C911D	L-2056 *hoΔMAT*a	(Walker *et al.*, 2003)
FM5-M	Adaptively evolved mutant of C911D (~250 generations, mixed culture)	This study
FM16-M	Adaptively evolved mutant of L-2056 (~350 generations, mixed culture)	This study
FM5-1 → FM5-10	Clonal cultures isolated from FM5-M	This study
FM16-1 → FM16-10	Clonal cultures isolated from FM16-M	This study
1922	BY4741 *hxt1Δ::kanMX4*	(Winzeler *et al.*, 1999)
586	BY4741 *hxt2Δ::kanMX4*	(Winzeler *et al.*, 1999)
4182	BY4741 *hxt3Δ::kanMX4*	(Winzeler *et al.*, 1999)
3913	BY4741 *gdh2Δ::kanMX4*	(Winzeler *et al.*, 1999)
6388	BY4741 *glc3Δ::kanMX4*	(Winzeler *et al.*, 1999)
2767	BY4741 *ald6Δ::kanMX4*	(Winzeler *et al.*, 1999)
1272	BY4741 *ino1Δ::kanMX4*	(Winzeler *et al.*, 1999)
4959	BY4741 *hap4Δ::kanMX4*	(Winzeler *et al.*, 1999)
5575	BY4741 *gph1Δ::kanMX4*	(Winzeler *et al.*, 1999)
4525	BY4741 *rck1Δ::kanMX4*	(Winzeler *et al.*, 1999)
C911D *hxt1*	L-2056 *hoΔ hxt1Δ::kanMX4 MAT*a	This study
C911D *hxt2*	L-2056 *hoΔ hxt2Δ::kanMX4 MAT*a	This study
C911D *hxt3*	L-2056 *hoΔ hxt3Δ::kanMX4 MAT*a	This study
C911D *gdh2*	L-2056 *hoΔ gdh2Δ::kanMX4 MAT*a	This study
C911D *glc3*	L-2056 *hoΔ glc3Δ::kanMX4 MAT*a	This study
C911D *ald6*	L-2056 *hoΔ ald6Δ::kanMX4 MAT*a	This study
C911D *ino1*	L-2056 *hoΔ ino1Δ::kanMX4 MAT*a	This study
C911D *hap4*	L-2056 *hoΔ hap4Δ::kanMX4 MAT*a	This study
L-2056 *ald6*	L-2056 *ald6Δ::kanMX4/ald6Δ::kanMX4*	This study
L-2056 *gph1*	L-2056 *gph1Δ::kanMX4/gph1Δ::kanMX4*	This study
L-2056 *rck1*	L-2056 *rck1Δ::kanMX4/rck1Δ::kanMX4*	This study
C911D adh2^{UP}	C911D integrant [pCMTDH3BADH2^{UP}]	This study
C911D adr1^{UP}	C911D integrant [pCMTDH3AADR1^{UP}]	This study
C911D vid24^{UP}	C911D integrant [pCMTDH3EVID24^{UP}]	This study

2.4 Growth and fermentation

2.4.1 Laboratory scale anaerobic fermentations

Fermentations were carried out in triplicate (unless otherwise indicated) and were inoculated (to 1 x 10^5 cells ml^{-1}) from overnight YEPD cultures of the appropriate yeast strain in 100 ml batches of CDGJM in modified 250 ml Erlenmeyer flasks. An anaerobic environment was achieved by fitting flasks with a fermentation lock (to allow the release of CO_2) and sparging for 3 minutes with nitrogen at an approximate flow rate of 500 ml min^{-1}. Flasks were incubated at 30°C with shaking at 160 rpm and samples were regularly collected via a port fitted with a rubber septum (suba seal®) using a needle and syringe. Optical density at 600 nm of appropriately diluted samples was used as an estimate of yeast cell growth. Samples were then clarified by centrifugation and stored at -20°C.

2.4.2 Dry cell weight determination

Millipore filters (0.22 μm, GSWG, 47 mm diameter) were pre-prepared by drying in a microwave oven on medium power setting for 10 minutes, followed by cooling in a desiccator and weighing. Culture samples (2.5 - 10 ml, depending on cell density) were collected on filters under vacuum, washed with 2 x volume of double distilled water (Elga® purified water), re-microwaved (medium power setting for 10 minutes) and cooled in a dessicator before re-weighing to determine dry cell weight.

2.4.3 Determination of glucose and fructose

An estimation of glucose concentration, and thus the progress of fermentation was obtained during the initial stages of fermentation with a hand held refractometer (Atago), and during the final stages of fermentation using clinitest indicator tablets (Bayer). Accurate determination of glucose and fructose concentration was performed with a Roche spectrophotometric enzymatic analysis kit (Arrow Scientific #0139106), usually performed after

the completion of the experiment on samples collected at regimental time intervals.

Spectrophotometric enzymatic assays, were performed as described by the manufacturer, with some modifications. Briefly, 300 µl of working solution (0.25 M triethanolamine, 3.3 mM $MgSO_4$, 0.37 mM NADP, 2.67 mM ATP and 13.2 µg ml^{-1} of hexokinase) was placed in a well of a 48 well micro-titre plate (corrected to a 1 cm light path) and OD_{340} (A_1) was measured with a µQuant micro-titre plate spectrophotometer (BioTek Instruments). 10 µl of sample was added with mixing and the plates were then incubated at room temperature for 15 minutes. The OD_{340} (A_2) was measured again, 2 µl of phosphoglucose isomerase was added with mixing and the OD_{340} (A_3) measured after 15 minutes at room temperature. The concentration of glucose and fructose present in the sample was then determined using the following formula:

$$\Delta A_{x1} = A_2 - A_1$$

$$\Delta A_{x2} = A_3 - A_2$$

$$C_{glucose} = (\Delta A_{B1} - \Delta A_{S1}) \times 0.89514 \times Df$$

$$C_{fructose} = (\Delta A_{B2} - \Delta A_{S2}) \times 0.86460 \times Df$$

Where *x* denotes either the sample (S) or the blank (B), of which the blank is an assay performed with water. Further to this, C is the concentration of the metabolite (g l^{-1}). Df is the dilution factor.

2.4.4 Determination of key metabolites by HPLC

Ethanol, glycerol, acetic acid, succinic acid and acetaldehyde were analysed from terminal fermentation samples by HPLC. Samples were filtered through 0.45 µm PVDF syringe filters (Millipore). HPLC analysis was performed on undiluted and diluted (1:20) samples on an Aminex HPX-87H column (300 mm x 7.8 mm) (BioRad). Elution was performed at 60°C with 2.5 mM H_2SO_4 at a flow rate of 0.5 ml min^{-1}. Detection was performed by means of a RID-10A refractive index detector and a SPD-10A UV – visible detector at 210 nm

(Shimadzu). Quantitation was achieved by comparison with prepared standards in CDGJM using Class VP integration software (Shimadzu).

2.4.5 Sterol determination

Total intracellular sterols were extracted as reported by Breivik and Owades (1957) with slight modification. Strains were grown in 50 ml of YEPD at 30°C for 18 h with shaking at 160 rpm. Cells were then harvested by centrifugation at 5,000 x *g* for 5 min and washed once with sterile distilled water. The net wet weight of the cell pellet was determined. Three millilitres of 25 % alcoholic potassium hydroxide solution (25 g of KOH and 36 ml of sterile distilled water, brought to 100 ml with 100 % ethanol) was added to each pellet and mixed by vortex for 1 min. Cell suspensions were then incubated at 80°C for 1 h. Tubes were allowed to cool to room temperature and sterols were extracted by addition of a mixture of 1 ml of sterile distilled water and 3 ml of *n*-heptane and vigorous vortex mixing for 3 min. The heptane layer was transferred to a clean borosilicate glass screw-cap tube and stored at -20°C for up to 24 h. A 2 ml aliquot of sterol extract was diluted fivefold in 100 % ethanol and scanned spectrophotometrically between 200 and 300 nm. The presence of ergosterol and the late sterol intermediate 24(28) dehydroergosterol (DHE) in the extracted sample resulted in a characteristic four-peaked spectrum (Arthington-Skaggs *et al.*, 1999). The absence of detectable ergosterol in extracts was indicated by a flat line.

Ergosterol content was calculated as a percentage of the wet weight of the cells by the following equations:

percent ergosterol + percent 24(28) DHE = [(A281.5/290) × F]/pellet weight

percent 24(28) DHE = [(A230/518) × F]/pellet weight

percent ergosterol = [percent ergosterol + percent 24(28) DHE] - percent 24(28) DHE.

where F is the factor for dilution in ethanol and 290 and 518 are the E values (in percent per centimeter) determined for crystalline ergosterol and 24(28) DHE, respectively (Arthington-Skaggs *et al.*, 1999).

2.5 Yeast classical genetics

Protocols used here were based on those reported by Guthrie and Fink (1991).

2.5.1 Yeast mating

A loop full of fresh overnight culture from YEPD agar was mixed thoroughly with a yeast strain of the opposite mating type on a thin YEPD plate and incubated for 2 hours at 30°C. Mating cells were then identified by their characteristic barbell shape and separated from the mixed population using a micromanipulator (Olympus). Isolated diploid strains were then incubated for a further 48 hours at 30°C.

2.5.2 Yeast sporulation

Cells were plated onto solid potassium acetate sporulation medium (1 % potassium acetate, 0.1 % yeast extract, 0.05 % glucose, 2 % bacto-agar) and incubated for up to 10 days at 30°C. The formation of asci was monitored by microscopic examination of a wet mount.

Spores were prepared for spore-cell mating experiments by digestion of the ascus wall with β-glucuronidase, as below, suspensions were vortexed for 1 minute and incubated for 8-12 hours at 30°C. The suspension was then vortexed for 2 minutes, centrifuged for 2 minutes at 2,000 x *g*, washed in 1 ml of TE buffer (Appendix I), and finally resuspended in 50 µl of TE buffer.

2.5.3 Ascus microdissection

To disrupt the ascus wall, a loop full of culture from a sporulation plate was resuspended in 1 ml of 100 U ml^{-1} β-glucuronidase (Sigma G-7770) and incubated for 15 – 60 minutes at 30°C. The spore suspension was then streaked

onto a thin YEPD plate, and what appeared as intact tetrads were isolated and dissected using a micromanipulator (Olympus).

2.6 Nucleic acid isolation

2.6.1 Isolation of genomic DNA from S. cerevisiae

Approximately 2 x 10^9 cells from a 10 ml YEPD stationary phase culture were collected by centrifugation at 20,000 x *g* and washed in 1.0 ml of sterile reagent grade water. Cell pellets were resuspended in 0.2 ml of cell lysis solution (2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris-Cl (pH 8.0) and 1 mM Na_2EDTA) and 0.2 ml of phenol:chloroform (5:1) and 0.3 g of acid washed glass beads were added. Samples were vortexed for 4 minutes, 0.2 ml of TE was added and then samples were centrifuged at 20,000 x *g* for 2 minutes. The aqueous layer was collected in a fresh tube to which 1 ml of 100 % ethanol was added and samples were centrifuged as before and the supernatant discarded. The pellet was resuspended in 0.4 ml of TE, containing 3 µl of RNase cocktail (1.5 U of RNase A and 60 U of RNase T1) and samples were incubated for 30 minutes at 30°C. Genomic DNA was precipitated with 10 µl of 4 M ammonium acetate and 1 ml of 100 % ethanol, and then collected by centrifugation at 20,000 x *g* for 2 minutes. The pellet was air-dried and resuspended in reagent grade water.

2.6.2 RNA preparations from yeast

Approximately 5 x 10^8 yeast cells were harvested by centrifugation at 12,800 x *g* for 5 minutes. The pellet was washed in 1 ml PBS (Appendix 1), followed by resuspension in 0.5 ml of TRIZOL® reagent (Invitrogen, Cat# 15596-026), in a 2 ml screw capped centrifuge tube. Samples were plunged into liquid nitrogen for 20 seconds, and then allowed to thaw on ice. 0.3 g of acid washed glass beads were then added and samples were vortexed for 2 minutes. Following this, samples were incubated at 65°C for 3 minutes, and then after a 100 µl addition of chloroform, were shaken vigorously by hand for 15 seconds. After 5 minutes at room temperature cell debris was pelleted by centrifugation at

12,800 x *g*, for 10 minutes at 4°C. The top, clear phase was collected in a new tube, 250 µl of isopropanol was added, the tube was gently mixed by inversion 6 times and then incubated at room temperature for 10 minutes. RNA was collected by centrifugation (12,800 x *g*, 10 min, 4°C). Pellets were washed in 75 % ethanol (vortexed and re-centrifuged for 5 minutes at 12,800 x *g* at 4°C). The washed pellet was air-dried for 10 minutes or until all ethanol was removed. Finally RNA was dissolved in 100 µl of RNase free water (reagent grade water treated overnight at room temperature with 0.05 % diethyl pyrocarbonate (DEPC) and autoclaved) and incubated at 65°C for 5 minutes, with occasional mixing to resuspend the pellet. RNA samples were stored at -80°C.

To ensure the complete removal of contaminating DNA and protein, RNA preparations used were cleaned using RNeasy columns (Qiagen, Cat # 74104), as per manufacturers instructions, immediately before use.

2.6.3 Determination of DNA or RNA concentration

The concentration of DNA and RNA preparations was determined by the absorbance of a suitably diluted sample at 260 nm, given that the OD_{260} of a solution of double stranded DNA at 50 µg ml^{-1} or RNA at 40 µg ml^{-1} is approximately 1. Purity of nucleic acids (from protein) was also estimated by determination of the absorbance at 280 nm, and comparison of the ratio of OD_{260}:OD_{280}, with a ratio of 1.8 - 2.0 being deemed adequate.

2.6.4 Plasmid preparation from E. coli

Plasmids were routinely isolated by alkaline lysis, similar to the method described in Sambrook (2001). Thus *E. coli* cultures were grown overnight in selective media from a single colony and from 1.5 ml of culture, cells were harvested by centrifugation. Cells were then resuspended in 100 µl of Solution 1 (50 mM D-glucose, 25 mM Tris-HCl [pH 8.0] and 10 mM EDTA [pH 8.0]), 200 µl of Solution 2 (0.2 M NaOH and 1 % SDS) were added and the tube

inverted 4 times. Following this, 150 µl of Solution 3 (3 M potassium acetate and 11.5 % [v/v] glacial acetic acid) were added and the tube was vortexed in an inverted position for 10 seconds and incubated on ice for 15 - 30 minutes. Each sample was then centrifuged (1,800 x *g*, 10 min), the supernatant transferred to a fresh tube with 1 µl of RNase cocktail (0.5 U of RNase A and 20 U of RNase T1) and incubated for 30 - 60 minutes at 37°C. Following this, samples were extracted with an equal volume of phenol/chloroform (5:1), the upper phase was collected and precipitated with 250 µl of 7.5 M ammonium acetate and 0.7 volumes of isopropanol. Samples were incubated for 15 minutes on ice before centrifugation at 18,000 x *g* for 15 minutes. The pellets were washed with ice-cold 70% ethanol and air-dried. Plasmid DNA was resuspended in 50 µl of reagent grade water or TE buffer and stored at –20°C.

High purity plasmid DNA preparations were prepared using MoBio plasmid miniprep kit (MoBio, Cat# 12300-250).

2.7 Molecular cloning techniques

2.7.1 Restriction endonuclease digestion of DNA

1-10 µg of DNA was incubated with 5-50 U of restriction enzyme with the manufacturer recommended buffer, in a final volume of 50 µl at 37°C either for 2 hours or overnight, If desired, digested DNA was cleaned for further use using the Qiagen QiaQuick PCR purification kit (Qiagen, Cat# 28106), as per manufacturer's instructions.

2.7.2 Dephosphorylation of vector DNA

Approximately 2 µg of restriction digested plasmid DNA was agarose gel purified, with the use of the Qiagen QiaQuick gel extraction kit (Qiagen, Cat# 28704), and then de-phosphorylated with typically 0.1-0.5 U of Shrimp Alkaline Phosphatase (SAP) per mole of DNA ends and 2 µl of 10 x SAP buffer in a total volume of 20 µl. The reaction was incubated at 37°C for 60 minutes, and then was heat inactivated by incubation at 65°C for 10 minutes.

DNA was cleaned using the QiaQuick PCR purification kit (Qiagen), according to the manufactures instruction. The pellet was briefly dried under vacuum and resuspended in 10 µl of reagent grade water.

2.7.3 Ligation of DNA into plasmid

Ligation of restriction digested DNA into appropriately digested and alkaline phosphatase treated vector DNA was performed with T4 DNA ligase (0.1 - 50 Weiss Units) and the associated buffer as directed by the manufacturer (New England Biolabs) in a maximum total volume of 15 µl. The molar ratio of insert to vector ends was prepared at 5:1 whenever possible. In some instances, polyethylene glycol, $MgCl_2$ and ATP were also added, typically in the concentrations of 9.3 % (v/v), 3.33 mM and 0.33 mM, respectively.

2.7.4 Preparation and use of competent **E. coli** *cells for transformation*

An overnight culture of *E. coli* DH5α cells was diluted 1:10 in LB and grown to an OD_{580} of 0.4. The cells were chilled on ice for 10 minutes and then pelleted at 4°C at 1,300 x *g* for 7 minutes. The pellet was gently resuspended in cold $CaCl_2$ solution (60 mM $CaCl_2$, 15% [v/v] glycerol, 10 mM PIPES pH 7.0) and left on ice for 30 minutes. Cells were then washed twice by pelleting at 1,300 x *g* for 5 minutes at 4°C and resuspended in 10 ml of cold $CaCl_2$ solution before finally resuspending cells in $CaCl_2$ solution and storing in 200 µl aliquots at -80°C.

Previously prepared competent cells were thawed at room temperature, gently mixed and added to no more than 15 µl of ligation mix before incubation on ice for 30 minutes. Cells were heat shocked at 42°C for 60 seconds and then returned to the ice bath for 5 minutes. 2 ml of LB was added and the samples were incubated at 37°C for 45 minutes on a rotating wheel. Transformed cells were spread onto LB plates containing the appropriate antibiotic, and incubated overnight at 37°C.

2.7.5 Self cloning over-expression strains

Integrative expression vectors were constructed according to Hirosawa *et al.* (2004). In brief, the constitutive *TDH3* promoter was amplified using primers listed in Table 2.2. The amplified fragments were then digested with enzymes listed in Table 2.3 and inserted into the same sites of pGG119 to form pCMTDH3*x* (vectors also listed in Table 2.3). Genes of interest were amplified and digested using primers and restriction enzymes listed in Table 2.3 and inserted into the same sites in the appropriate pCMTDH3*x* vector also listed in Table 2.3.

2.7.6 High efficiency transformation of S. cerevisiae *using lithium acetate*

An overnight culture (YEPD) was inoculated from a stationary phase culture, previously inoculated from a single colony, and grown to a density of 2 x 10^7 cells ml^{-1} (OD_{600} of approximately 0.4). Cells from 50 ml of culture were then harvested at 2,700 x *g* for 5 min and resuspended in 25 ml of reagent grade water. Cells were again harvested and resuspended in 1 ml freshly prepared buffered lithium solution (100 mM LiAc, 100 mM Tri-Cl (pH 8.0), 10 mM EDTA). 200 µl of the cell suspension was mixed with 200 µg of carrier DNA (Sigma D-1626) (prepared by boiling a 2 mg ml^{-1} stock in TE buffer for 10 minutes, and then cooling on ice for 5 minutes), 2-5 µg of transforming DNA (in a volume of less than 20 µl), and 1.2 ml of PEG solution (40 % polyethylene glycol in buffered lithium solution). Each transformation was then incubated at 30°C for 30 minutes followed by a heat shock at 42°C for 45 minutes. Cells were harvested at 2,700 x *g* for 5 minutes and resuspended in 200 µl of TE before plating on selective media. Where geneticin was used as a selection, heat shocked cells were cultured without selection for 1-2 hours before plating on media with 400 mg l^{-1} of geneticin.

Table 2.2 PCR primers for the construction of over-expression plasmids used in this study.

Primer	Sequence (5' → 3')*
TDH3-1	GGGCGGCCGGACGTCTTTATCATTATCAATACTGCCATTTC
TDH3-2	GGGCGGCCGCCTAGGTTTATCATTATCAATACTGCCATTTC
TDH3-3	GGGACTAGTGACGTCTTTATCATTATCAATACTGCCATTTCA
TDH3-4	GCATCTAGACCATGGTTGTTTGTTTATGTGTGTTTATTCGA
TDH3-5	GCACCCGGGCCATGGTTGTTTGTTTATGTGTGTTTATTCG
ADH2-NCO	GGGCCATGGATGTCTATTCCAGAAACTCAAAAAG
ADH2-XBA	GGGTCTAGAAATTTCTGGTAAACTGGATAAGC
ADH2-UPEAG	GAGCGGCCGTCTTTATCAACGAAGGGC
ADH2-UCAVR	GGGCCTAGGACCAAGAAGAAACAAGAAGTGA
ADR1-NCO	GGGCCATGGATGGCTAACGTAGAAAAACCAAAC
ADR1-XMA	GGGCCCGGGATCGACGAAATCAGGTTGGT
ADR1-UPEAG	ATACGGCCGGGGGAAGGTTATATCTTTG
ADR1-UCAAT	GGGGACGTCGTAGCAGAATATTTTTCTGAAGTG
VID24-NCO	GGGCCATGGATGATCAATAATCCTAAGGTAGACA
VID24-XMA	GGGCCCGGGATTCTTGATATGTTACCAG
VID24-UPSPE	GGGACTAGTATAATGAAACGGGGATGG
VID24-UCAAT	TTTGACGTCCTTGCGATATGTGTTGG

*Underlined text denotes restriction site.

Table 2.3 Plasmids, primers and insertion sites for construction of integrative over-expression cassettes.

Plasmid	Promoter Foward primer	Promoter Reverse primer	5' Promoter restriction sites	Insertion of gene	3' Promoter restriction sites	Insertion of upstream sequence	Linearisation restriction site for transformation
pCMTDH3A	TDH3-1	TDH3-4	*Nco*I *Xba*I	pCMTDH3AADR1	*Eag*I *Aat*II	pCMTDH3AADR1^{UP}	*Sph*I
pCMTDH3B	TDH3-2	TDH3-4	*Nco*I *Xba*I	pCMTDH3BADH2	*Eag*I *Avr*II	pCMTDH3BADH2^{UP}	*Aoc*I
pCMTDH3E	TDH3-3	TDH3-5	*Nco*I *Xba*I	pCMTDH3EVID24	*Spe*I *Aat*II	pCMTDH3EVID24^{UP}	*Afl*II

2.8 Nucleic acid amplification procedures

2.8.1 Polymerase Chain Reaction (PCR)

PCR amplification was performed in 12.5 - 50 µl reactions typically containing 1U DyNAzyme EXT DNA polymerase (Finnzymes F-505S), 1 x DyNAzyme EXT PCR buffer, 100 pmol primer, 50 ng plasmid DNA or 200 ng genomic DNA, 200 - 400 µM dNTPs. $MgCl_2$ concentrations and cycling parameters were optimised for each PCR reaction. Primers used for PCR amplification are listed in Table 2.4. Cycling reactions were conducted using an Eppendorf *Mastercycler gradient* thermocycler.

2.8.2 Sequencing reactions

Sequencing cycle reactions were set up as recommended by Applied Biosystems (ABI): a 20 µl volume containing 3.2 pmol of primer, 50 - 200 ng of template DNA and 4 µl of ABI Big Dye sequencing reagent. PCR reactions were as follows: 25 cycles of 96°C, 10 seconds; 50°C, 5 seconds and 60°C, 4 minutes. Sequencing products were precipitated with 80 µl of 75% (v/v) isopropanol, where reactions were vortexed and incubated for 30 minutes at room temperature, before centrifugation for 20 minutes at 20,000 x *g*. Supernatants were carefully aspirated and pellets were washed with 250 µl of 75 % (v/v) isopropanol. Air-dried pellets were sent to the IMVS Molecular Pathology sequencing service for separation and analysis using an ABI automated sequencer.

Primers used for sequencing the *GCR1* promoter are listed in Table 2.5.

2.8.3 PCR labelling of probes for Southern blot analysis

DNA probes were labelled with Digoxigenin-11-dUTP (DIG), by incorporation into PCR products using DyNAzyme EXT DNA polymerase. The PCR DIG Probe Synthesis Kit (Roche Cat# 1 636 090) was used as per manufacturers instructions.

Table 2.4 PCR primers for construction of deletion strains used in this study.

Primer	Sequence (5' → 3')
G418F	AAAAGACTCACGTTTCGAGGC
G418R	CGAGCATCAAATGAAACTGC
HXT1A	TGAGAAAAAGAATATTCATCAAGTGC
HXT1D	GAACTATGTATAGCGCCTATGTGGT
HXT2A	CGACAACAATAAGTCGCCCATTTTTTAT
HXT2D	TCAATACCTCGAAGCAGCGTTTCA
HXT3A	GGAGAAAGAAACAACTCAATAACGA
HXT3D	GTTTTCTAACTCTACTGCGCACTT
GDH2A	GCAATTGGGGTAAAAATACACATTA
GDH2D	AGTTTTTATTGATTTTCGATCGTTG
GLC3A	CTGATGAGCTTTCACTATCTCTTCC
GLC3D	AACCAATGCTTCGTTTTAGACATAC
ALD6A	CATCCAGCTTCTATATCGCTTTAAC
ALD6D	AATATGATTTTGTGTGGGATGTTTT
INO1A	CTTTTTCTTCGTTCCTTTTGTTCTT
INO1D	TTGTGTTTTTCAGCTTCTTCTTTCT
HAP4A	TTAATTCCTTCACCTCTCTAAACCC
HAP4D	AACGGATATGTGAAAATGCTCTTAG
GPH1A	GAGGAAATAAGTAAGGGGTGGATAA
GPH1D	TTCGAGTAAGTGATAATAAGGACCG
RCK1A	GATTTTCAGTTATATGGAGTGGGTG
RCK1D	CAAGTTTTTCCGTGTTTCTAGGATA

Table 2.5 PCR primers for the sequencing of the *GCR1* promoter region.

Primer	Sequence (5' → 3')
GCR1AF	CATGGGATGCAAGAACACCAA
GCR1AR	TAGACGCTGAAACGCCTGTTT
GCR1BF	CCGCTCCTAATTTAAAGTCACTTG
GCR1BR	GGATGCTTTTAATTCGTGAAACAAG
GCR1CF	CCCAACTCATCTTCCTAATGCTG
GCR1CR	CAAGTAGGTCGGAAAGGTTTGTG

2.9 Microarray analysis

2.9.1 Labelling and hybridisation

Labelled cDNA probes were prepared using the SuperScript Indirect cDNA labelling system (Invitrogen) as per the manufacturers instructions. In brief, 20 µg of template RNA was denatured with the addition of 5 µg anchored oligo $(dT)_{20}$. The reaction was incubated at 70°C for five minutes and quenched on ice for five minutes. cDNA was synthesised with 1x first strand buffer, 0.3 mM DTT, dG, dA and dC nucleotides added at 0.5 mM, dTTP at 0.16 mM and amino allyl modified dUTP at 0.34 mM. 800 U of Superscript III (Invitrogen) was then added before incubation at 46°C for three hours. Following this, RNA was hydrolysed by the addition of 0.3 N NaOH. First strand cDNA was then purified with QiaQuick PCR purification columns, before ethanol precipitation of cDNA. The pelleted cDNA was resuspended in 5 µl of 50 mM sodium bicarbonate (pH 9.0). 5 µl of resuspended fluorescent dyes were then added to cDNA, either Cy3 (Amersham Biosciences, #PA 23001) or Cy5 (Amersham Biosciences, #PA 25001) to reciprocal samples, before mixing and incubation at room temperature in the dark for one hour. The fluorescently labelled cDNA was purifed through the QiaQuick PCR purification column, and dried in a rotary vacuum.

Yeast oligonucleotide slides (Ramaciotti Centre, UNSW), which are duplicate spotted with 40-mer elements/gene, were used for all microarray experiments. Slides were blocked prior to hybridisation according to manufactuers instruction (Schott Nexterion). Probes were resuspended in 12.5 µl of DIG Easy Hyb buffer (Roche) before compliment dye samples were combined and loaded onto a 110 mm x 75 mm coverslip and placed onto the printed slide. Slides were hybridised overnight at 37°C, washed with 0.5 x SSC buffer (3 M NaCl, 0.3 M tri-sodium citrate) and 0.01% SDS to dislodge the coverslip, followed by 5 minutes in each of 0.5 x SSC, 0.01% and 0.5 x SSC. A 3 minute wash in 0.2 x SSC was followed by drying in a centrifuge at 750 RPM for 5 minutes, and storage in the dark prior to scanning.

Hybridised slides were scanned using an Axon 4100, with GenePix 4.0 software (Axon). The images were then saved as 16 bit TIFF single image files, which were scanned outputs of the Cy3 (red) and Cy5 (green) channels.

2.9.2 Microarray data analysis

Statistical analysis of microarrays was conducted using SPOT (CSIRO) and R packages on *Bioconductor* (www.bioconductor.org). Raw intensity data from microarray images were generated in SPOT cDNA Microarray Image Analysis software in accordance with the users manual. In brief, batches of files were established, as a list which combines the separate wavelength TIFF images/slide of replicated and reverse dyed slides that were to identify differential gene expression. A template was then created which outlined the grids of spots before using a seeded region growing (SRG) algorithm to calculate spot intensities, where each pixel value in a scanned image represented a level of hybridisation at a specific location on the slide. Finally, a non-linear filter called morphological opening was applied to adjust for background correction. The software package used for data analysis and normalisation of this raw data was *limma* (Linear Modelling for Microarray Analysis) as per the users guide (Wettenhall and Smyth, 2004).

2.10 Southern blot analysis of genomic DNA

Appropriately digested yeast genomic DNA was heat treated at 65°C for 20 minutes, and was then combined with gel loading buffer (30% [v/v] glycerol, 0.25% [w/v] bromophenol blue, and 0.25% [v/v] xylene cyanol) before separation on a 1–2 % TAE (Appendix I) agarose gel. DIG-labelled molecular weight markers were also included (Roche DNA Molecular Weight III, Cat#1 218 603). After separation of the DNA the gel was depurinated by incubation in depurination buffer (250 mM HCl) for 10 minutes. The gel was then rinsed with reagent grade water before denaturation of the DNA by twice incubating in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 15 minutes. Again the gel was rinsed with reagent grade water and then neutralised by submersion in

neutralisation solution (1.5 M Tris-Cl, 1.5 M NaCl, pH 7.5) twice for 15 minutes.

DNA was transferred from the gel to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech) by capillary transfer using 20 x SSC. After transfer, DNA was cross-linked to the nylon membrane using an Amersham Life Sciences UV cross-linker (254 nm @ 70,000 $\mu J/cm^2$).

DIG Easy Hyb buffer (Roche) was used for pre-hybridisation and hybridisation. Nylon membranes were placed in glass hybridisation tubes with 30 ml of DIG Easy Hyb buffer and pre-hybridised for 2 hours at 42°C. Probes (PCR labelled with DIG) were boiled for 10 minutes followed by rapid cooling in an ice bath, and the denatured probe diluted in DIG Easy Hyb buffer was hybridised to the membrane bound DNA overnight at 42°C. Following this the membrane was washed twice in 2 x SSC, 0.1 % SDS at room temperature for 15 minutes, followed by two more 15 minute washes in 0.5 x SSC, 0.1% SDS at 68°C.

The membrane was then equilibrated in maleic acid buffer (1 M maleic acid, 1.5 M NaCl, pH 7.5) for 1 minute. All of the following washes were performed at room temperature. The membrane was blocked by incubation in 1 x blocking solution (Roche) dissolved in maleic acid buffer. Anti-digoxigenin-AP antisera (Roche) was diluted 1:10,000 in fresh blocking solution and the membrane incubated in this solution for 30 minutes with gentle agitation. The membrane was then washed twice for 15 minutes in maleic acid wash buffer (maleic acid buffer with 0.3 % Tween-20) before equilibration in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5). The membrane was sealed in a plastic bag containing 0.5 ml of ECF substrate/100 cm^2 (Amersham Life Sciences, Cat# 1067873) and incubated in the dark for 16 hours. Fluorescent bands were detected using a Storm® phosphoimager with a blue chemifluorescent filter.

Chapter 3 Generation of novel yeast strains using adaptive evolution

3.1 Introduction

To alleviate some of the stresses experienced by wine yeast strains during fermentation, strain optimisation programs have been an ongoing measure by researchers. Non-recombinant strategies to improve stress resistance in industrial yeast have included extensive selection and breeding programs, as well as the transient acquisition of resistance via physiological adaptation (Brown and Oliver, 1982; Attfield, 1997). More recently, the use of chemostats to provide a highly controlled environment, so as to produce fitter genetic variants of laboratory yeasts with respect to biomass yield, glucose consumption and ethanol production (Ferea *et al.*, 1999) or the partial introduction of anaerobic xylose utilisation has proven quite successful (Sonderegger and Sauer, 2003). Additional to this the derivation of yeast resistant to multiple stresses using adaptive evolution has also been studied. Work by Cakar *et al.* (2005) utilised a diverse (ethyl methane sulfonate mutagenised) population of a laboratory strain of *S. cerevisiae* employing both batch and continuous cultivation, under the selective conditions of oxidative and heat stresses to develop and isolate stress resistant strains.

Adaptive evolution is defined by a set of random mutations that are beneficial under a selective pressure (Foster, 1999). Adaptation by natural selection occurs through the spread and substitution of mutations that improve the organism and its reproductive success in a particular environment (Elena and Lenski, 2003). While applied to laboratory yeasts, this approach has yet to be widely utilised in programs aimed at the improvement of industrial yeasts, particularly wine yeasts.

The purpose of this initial work was to apply the mechanism of adaptive evolution to derive new wine yeast strains, which were highly suited to the stresses of fermentation. Due to the complex nature of an industrial grape juice fermentation, we chose to utilise a system where a specific subset of the

stresses prevalent under such conditions were enforced, reflecting typical New World winemaking procedures. To do so we used a sequential batch fermentation system with each batch inoculated at a low density before an initial oxidative propagation step, which was followed by extended anaerobic fermentation. Furthermore, the medium used was of low pH (3.5), high osmolarity (200 g l^{-1} sugar) and devoid of sterols and unsaturated fatty acids.

Aerobic cultivation of the starter culture allows for the production of sterols and unsaturated fatty acids which are essential for growth, especially under anaerobic conditions (Andreasen and Stier, 1953; Andreasen and Stier, 1954). Without this step, cell division results in the dilution of these compounds amongst daughter cells to concentrations at which membrane integrity and cellular function are compromised. Thus fermentations which become anaerobic would represent a significant stress for the inoculated yeast. Furthermore, it was hypothesised that a low inoculum density (1×10^5 cells ml^{-1} as opposed to the current industry standard of 5×10^6 cells ml^{-1}) would exacerbate the situation by encouraging further cell division and dilution of the essential sterols and fatty acids (Arnezeder and Hampel, 1991; Ferreira *et al.*, 2004). Given the importance of these compounds it was speculated that superior strains might include those whose advantage comes from a mutation(s) which allows them to over-accumulate sterols and fatty acids during aerobic propagation. Other bases for enhancement of strains might, of course, also be possible.

Apart from resembling the way in which starter cultures are propagated in industry, exposure of the culture to oxidative stress offered the possibility that tolerance would develop not only to this stress, but perhaps other stresses (Lewis *et al.*, 1997) such as the damage caused by ethanol and heat, both of which are prevalent under oenological conditions (Collinson and Dawes, 1992; Costa *et al.*, 1993). Further stresses (low pH and high osmolarity) were also included in the experimental design, as it was hypothesised that this multi-stress approach would result in the adaptation of the yeast culture to one or

more of these parameters thereby yielding derivatives that were better able to grow and ferment under one or more of the imposed stresses.

Further to this, two populations of different ploidy were used to investigate the effect of gene copy number on the rate of adaptation. *S. cerevisiae* is known to proliferate through a mitotic cell cycle usually in a diploid state. However under certain environmental conditions, diploids can undergo sporulation, which consists of both meiosis and spore formation, to form haploids, also capable of mitotic proliferation. It is also possible for haploid cells to switch between the a and α mating types due to their homothallic nature (as reviewed by Herskowitz, 1988), thus allowing for mating, by cell fusion, within a population from the same progenitor to form diploid cells. Consequently, we used both the haploid and diploid forms to compare the evolutionary response both phenotypically and genotypically, in strains that were highly homologous.

L-2056 was chosen as the foundation population for these evolution experiments, as a haploid derivative (C911D) had already been constructed in this laboratory (Walker *et al.*, 2003), thereby allowing both phenotypic and genotypic comparison of strains of similar genetic background with respect to ploidy. L-2056 was originally isolated from vineyards in the Côtes du Rhône and belongs to *S. cerevisiae*. This strain is reported to have a moderate fermentation rate, a relatively high nutrient requirement, good alcohol tolerance as well as low sulfur dioxide and volatile acidity production (Lallemand Pty Ltd). L-2056 is also widely used throughout the New World wine industry (A. Markides, pers. comm.). The construction of C911D involved the deletion of the *HO* endonuclease gene, normally allowing mating type switching and thus a homothallic lifecycle. Following this, the selection marker (*KanMX*) used to delete the open reading frame of *HO* was removed, thus the genome of C911D contains minimal foreign gene sequences.

3.2 Results

Fermentations conducted throughout the course of this experimentation were performed in a chemically defined grape juice media (CDGJM (Appendix I)).

This contained 200 g l^{-1} sugar in equimolar amounts of glucose and fructose, and assimilable nitrogen supplied as ammonia at 600 mg (FAN) l^{-1}. The media was adjusted to a pH of 3.5 before filter sterilisation prior to use. Fermentations were conducted in a 30°C temperature controlled room.

3.2.1 Chemostat selection and analysis of candidate mutants

In order to isolate yeast strains that display phenotypes of enhanced fermentation performance under stressful wine-like conditions, strains L-2056 and C911D were subjected to adaptive evolution through sequential batch fermentation. Within each cycle, pre-conditioning via oxidative propagation was carried out by sparging the culture with compressed air at a rate of 2 ml min^{-1} for 4 hours. Fermentation was then allowed to complete at 25°C (residual sugar was measured by Clinitest®, with < 2.5 g l^{-1} deemed "dry"). Following this the vessel was drained and replenished with fresh medium. The residual cells remaining in the vessel inoculated the new batch to the order of 1 x 10^5 cells ml^{-1}. The culture was then pre-conditioned with compressed air again.

Each cycle consisted of approximately 11 generations (population doublings) and every 50 generations (occurring each 6 to 7 weeks) aliquots were taken from each biostat and analysed further. Specifically, comparative small-scale batch fermentations by the culture samples (assumed to be a mixed culture) were conducted. These comparative fermentations were performed under similar conditions to those of the selection process, that is by using a low inoculation rate and a chemically defined grape juice medium of the same formulation. The large number of fermentations that had to be undertaken, and the limited availability of 25°C space at the time necessitated the conduct of these fermentations at 30°C. Fermentation duration by each culture collected at 50-generation intervals was seen to match that of the parent up until a point where an adaptive mutation(s) is proposed to have occurred. That is, after 250 generations under selective pressure for C911D and 350 generations for L-2056. The distinctiveness of cultures derived from C911D (FM5-M) and L-2056 (FM16-M) (M denotes the mixed culture) was initially evident in terms of fermentation duration.

3.2.2 Comparison of fermentation durations of C911D and isolates FM1-M through to FM5-M

As mentioned previously isolates (FM1-M through to FM4-M) collected up to 200 generations of the sequential batch culture initially inoculated with C911D, shared similar fermentation characteristics to those of the parent strain (Table 3.1). However, FM5-M, isolated after 250 generations had passed, displayed improved fermentation properties. Included was an ability to finish fermentation in 50.3 % of the time required for the parent strain (i.e. 79 hrs versus 159 hrs).

3.2.3 Comparison of fermentation durations of L-2056 and isolates FM10-M through to FM16-M

The differences observed between the evolved isolate FM16-M and the parent were analogous to those described above for the haploid (Table 3.1). It was found that the phenotypic characteristics demonstrated by the 50 generation isolates, up to and including FM15-M were the same as those of the parent strain, L-2056. However, FM16-M, selected after 350 generations, was found in small-scale fermentations to catabolise all available sugar after 97 hours, whereas all previous isolates and L-2056 were unable to complete fermentation.

3.3 Conclusions

a) A sequential batch fermentation of a wine-like medium appears suitable for the adaptive evolution of an industrial *Saccharomyces cerevisiae* strain to a population with improved properties.

b) Isolate FM5-M collected after 250 generations of adaptive evolution displayed improved fermentation performance compared to the parental haploid wine yeast derivative, C911D.

c) Isolate FM16-M collected after 350 generations of adaptive evolution displayed improved fermentation performance compared to parental wine yeast strain L-2056.

Table 3.1 The fermentation duration of mixed culture isolates taken from the culture originally inoculated with parent strains C911D and L-2056 subjected to the selective conditions of adaptive evolution.

Yeast Strain	Fermentation Duration[1]	
	(hours)	% of parent
C911D *(0)*	159.0 ± 4.0	100.0 ± 4.0
FM1-M *(50)*	159.0 ± 4.0	100.0 ± 4.0
FM2-M *(100)*	159.0 ± 4.0	100.0 ± 4.0
FM3-M *(150)*	159.0 ± 4.0	100.0 ± 4.0
FM4-M *(200)*	159.0 ± 4.0	100.0 ± 4.0
FM5-M *(250)*	79.0 ± 4.0	50.3 ± 4.0
L-2056 *(0)*	DNC	N/A
FM10-M *(50)*	DNC	N/A
FM11-M *(100)*	DNC	N/A
FM12-M *(150)*	DNC	N/A
FM13-M *(200)*	DNC	N/A
FM14-M *(250)*	DNC	N/A
FM15-M *(300)*	DNC	N/A
FM16-M *(350)*	97.0 ± 4.0	N/A

Fermentations were performed in CDGJM. Data is the mean of triplicate fermentations ± standard deviation (sampling intervals). Italicised data in brackets indicates the approximate number of generations that had elapsed since initiation of the experiment.[1] DNC: did not complete fermentation after 200 hours; N/A: Not applicable.

3.4 Discussion

Adaptive evolution has been described as the result of post-mutational sorting by the process of natural selection (Metzgar and Wills, 2000). The work of Metzgar and Wills (2000) was based on the premise that environment dependent sorting, leading to selection of adaptive mutations under starvation conditions would result in a response to cope with the stressful environment imposed (Rosenberg *et al.*, 1998), thus yielding isolates better suited to the constraints of fermentation. It has been implied that the presence of numerous stresses as typically imposed under oenological conditions may have contributed to the unintentional adaptive evolution of contemporary strains from less suited progenitors (Querol *et al.*, 2003). We therefore hypothesised that deliberate application of a selective pressure through a laboratory-based adaptive evolution experiment could be used to yield yeast more adept at completing the fermentation of high concentrations of glucose and fructose during periods of prolonged anaerobiosis. A wine-like medium with limited oxygen supplementation and the absence of oxygen substitutes (sterols and unsaturated fatty acids) was applied to adaptively evolve the wine yeast strain L-2056 and its haploid derivative, C911D.

The fermentation duration for generational isolates up to and including FM4-M (200 generations) and FM15-M (300 generations), was similar to that of the parent strain in each case. However, the haploid isolate adapted to the selective pressure over a period of 250 generations, and the commercial isolate in approximately 350 generations, whereby they were able to complete fermentation sooner than their parent (Table 3.1). Consequently, it is proposed that a defining mutation or mutations had occurred which allowed for such properties to be displayed after the time where FM4-M and FM15-M had been collected.

A shortened fermentation duration was chosen as a simple screen for the selection of strains likely to be adaptively evolved to possess improved characteristics. While in itself a rapid fermentation may be considered undesirable when occurring under optimal conditions, where conditions are

sub-optimal, such as when an oxygen deficiency results in a protracted fermentation, this improvement in yeast performance would be highly prized by winemakers. Furthermore, it is important to reiterate that the enhanced sugar catabolism of the evolved isolates compared to the inability of L-2056 to complete fermentation or the protracted fermentation by C911D, would be of great interest to the wine industry (Pretorius, 2000). This is the case, because failure to achieve dryness in the production of dry table wines leaves the final product out of specification, with poor palate structure and possible off-flavours, thereby severely reducing the commercial value of the wine (Iland and Gago, 2002).

It is not immediately obvious how the evolved populations differ genotypically in order to display improved fermentation phenotypes. It is likely that the flux through glycolysis has been altered so as to allow the evolved populations to finish fermentation *per se* and, in the case of FM5-M, in a shorter time than the parent. Glucose transport, hexose phosphorylation, and phosophofructokinase and pyruvate kinase activity have all been proposed to have a central role in glycolysis (Michnick *et al.*, 1997; Diderich *et al.*, 1999b; Reijenga *et al.*, 2001). The correlation between the capacity of these biological activities and glycolytic flux has been difficult to substantiate (Nissen *et al.*, 1997; van Hoek *et al.*, 2000), however glucose transport has been postulated as the most important and potentially rate-limiting step in glycolysis (Gancedo and Serrano, 1989; Diderich *et al.*, 1999b; Elbing *et al.*, 2004). As a consequence, there are multiple mechanisms which could result in the decreased fermentation duration observed in the FM5-M and FM16-M populations.

S. cerevisiae possess 20 different hexose transporters that transport glucose and fructose (the primary grape sugars) as well as mannose by facilitated diffusion (see reviews by Lagunas, 1993; Boles and Hollenberg, 1997; Ozcan and Johnston, 1999). The net rate of hexose transport is determined by both the timing and degree to which the transporters are expressed (i.e. the number of transporters in the plasma membrane) and their relative affinity for the various hexoses (Luyten *et al.*, 2002). Given that FM5-M and FM16-M have sustained

sugar catabolic rates from the mid stationary phase to the end of fermentation, it could be rationalised that the influence of the hexose transporter thought to be pre-eminent at this time, Hxt3p, is increased in evolved isolates. This low affinity transporter is the only carrier to be expressed throughout fermentation, being expressed maximally at growth arrest and declining in abundance over the course of stationary phase (Perez *et al.*, 2005). Furthermore, when the relevant structural gene *HXT3* is expressed alone in a *Hxt* null mutant, the cells still have the capacity to undergo rapid fermentation during stationary phase under starvation conditions (Luyten *et al.*, 2002).

The induction of *HXT6* and *HXT7* may also be of importance in evolved isolates, these high affinity carriers are induced at the end of the growth phase. Further to this, glucose induction of the *HXT* genes is mediated by a glucose repression mechanism involving a zinc-finger-containing protein Rtg1p. In the absence of glucose, Rgt1p binds to *HXT* promoters and represses expression, whereas glucose inactivates Rgt1p repressor function, leading to derepression of *HXT* genes (Marshall-Carlson *et al.*, 1991; Erickson and Johnston, 1994; Ozcan and Johnston, 1995). The glucose signal that triggers inhibition of Rtg1p function appears to be mediated via both Rtg2p and Snf3p which are thought to serve as sensors of glucose (Ozcan *et al.*, 1996a; Ozcan *et al.*, 1998). As such, a mutation in *SNF3* and/or *RTG2* could result in an unregulated repression of *HXT* genes (Marshall-Carlson *et al.*, 1990; Ozcan *et al.*, 1996a).

The pre-conditioning of the culture to an oxidative stress may have resulted in high levels of reactive oxygen intermediates (ROI), through the reduction of oxygen to water, perturbing cell redox status and leading to damage to lipids, protein and DNA and eventually cell death (Godon *et al.*, 1998). Yeast can adapt to oxidative stress by induction of two pathways. Firstly, the generation of proteins directly related to cellular antioxidant defence such as Trx2p, Sod1p, Sod2p, and Cup1p (van Loon *et al.*, 1986; Gralla and Valentine, 1991; Chae *et al.*, 1994; Liu and Thiele, 1996). Secondly, the enzymatic redirection of metabolic fluxes slowing pathways like glycolysis through the synthesis of NADPH (e.g. Pgm1p and Tdh2p) (Collinson and Dawes, 1992; Godon *et al.*,

1998). The differential regulation of such genes associated with these pathways could be responsible for an adaptation to the oxidative environment. Furthermore, the response system expressed due to the oxidative stress may have activated stress response mechanisms against the other imposed environmental stresses, consequently enabling isolates FM5-M and FM16-M to finish fermentation within a shorter duration due to cross-protection.

It was assumed that the decreased fermentation duration of the evolved mutant isolates was not linked to nitrogen efficiency, given the ready availability of a utilisable form (ammonia, 600 mg N l^{-1}) during the adaptive evolution fermentation (Jiranek *et al.*, 1995a; Gardner *et al.*, 2002; Gardner *et al.*, 2005). Any alteration in glycolytic flux, as proposed as a basis for the improved fermentative abilities of the evolved isolates is therefore unlikely to be associated with a reduced inactivation of sugar transporters which ordinarily occurs promptly upon nitrogen starvation (Busturia and Lagunas, 1986; Riballo and Lagunas, 1994; Krampe and Boles, 2002).

Adaptive evolution of the haploid strain C911D to yield a derivative better suited to the applied stress occurred some 100 generations sooner than it did for the commercial strain (presumed to be a diploid). Despite this work relating to a singular experiment it is tempting to speculate that ploidy may be important to this outcome (Orr and Otto, 1994; Zeyl *et al.*, 2003). By definition, the occurrence of a new allele requires both a molecular mutation event as well as a fixation process, often requiring many generations (Zeyl, 2004), whereby the new allele replaces all others in the population. While higher ploidy may reduce the waiting period for an adaptive mutation to occur by offering more alleles from which to arise, the fixing time for non-dominant mutations may be increased, because they confer smaller selective advantages on heterozygotes compared with haploids (Paquin and Adams, 1983; Zeyl *et al.*, 2003). Which effect is most important is determined by population size. In a larger population, like those in this experiment, it is the fixing time not the waiting time for the mutation which takes precedence, consequently haploids are expected to evolve faster (Orr and Otto, 1994), as occurred here.

Numerous groups have isolated adapted mutants using strictly continuous culture with defined working volumes and specified dilution rates (Ferea *et al.*, 1999; Sonderegger and Sauer, 2003). Here, the employment of a sequential batch cultivation system ensured that any mutants selected would be suited to fermentation under oenological conditions wherein media composition changes throughout the fermentation. In this way selection of mutations that are disadvantageous to general fermentation performance has been minimised. This system, although useful in this application, extends the duration of the selection process considerably. In a similar study investigating the adaptation of a haploid recombinant strain to anaerobic xylose utilisation, Sonderegger and Sauer (2003) isolated an adaptive mutant after 460 generations in a period of 266 days, whereas the isolation of FM5 after approximately 250 generations required 198 days. Given the low inoculation rate, each fermentation was expected to yield approximately 11 generations. The difference in total duration between our batch fed continuous fermentation and a strictly continuous culture is mainly due to the prevalence of stationary phase under normal fermentation conditions. In a strictly continuous fermentation this phase can be minimised by dilution of the system with fresh medium to ensure that cells remain in a logarithmic phase.

Adaptive evolution has therefore proven successful for the isolation from a commercial wine yeast strain and a haploid derivative, a population that has improved fermentation performance compared to the respective parent strain. The screen for decreased fermentation duration allowed for the simple monitoring of cultures over the period of the experiment, where isolates FM5-M and FM16-M displayed significantly reduced fermentation duration compared to preceding isolates, but also their respective parents. Their observed phenotype is suggested to be associated with a sustained sugar catabolism arising from an increased glycolytic flux.

Chapter 4 Preliminary phenotypic and genotypic characterisation of adaptively evolved cultures FM5-M and FM16-M

4.1 Introduction

In Chapter 3, the isolation of adaptively evolved cultures FM16-M and FM5-M from the wine yeast L-2056 and its haploid derivative, C911D, was described. These mixed cultures were able to complete a model oenological fermentation more effectively than their parent strain. Having been selected under the pressure of multiple stresses, typical of the winemaking fermentation, a more detailed characterisation of these cultures was sought in order to determine the range and magnitude of the phenotypic alterations, particularly those of oenological relevance. For instance, given the inclusion of an aerobic propagation phase at the commencement of each batch fermentation, it was of interest to determine whether sterol and unsaturated fatty acid levels were altered in evolved cultures. In addition, fermentation trials were examined more extensively so as to confirm fermentation kinetics and determine the yield of key metabolites. An alteration in the latter might suggest the metabolic basis for the enhancement of the evolved cultures. Given the important contribution that yeast make to wine flavour and quality (Fleet, 1990; Fleet and Heard, 1993; Lema *et al.*, 1996; Lambrechts and Pretorius, 2000), the yield of certain metabolites, such as ethanol and glycerol, was of particular interest.

To better understand how the observed phenotypic changes of the adaptively evolved mutants were being conferred, an analysis of the transcriptome was undertaken by cDNA microarray. At the level of transcription, cDNA microarrays allow accurate, genome-wide mapping of regulatory responses (Horak and Snyder, 2002; Boer *et al.*, 2003). Given the nature of an adaptive evolution project, it is assumed that under conditions of continued stress and nutrient limitation, a series of adaptive mutations have occurred within the population (Ferea *et al.*, 1999). Consequently, with the whole sequence of the *S. cerevisiae* genome known (Goffeau *et al.*, 1996), the use of cDNA microarrays for the systematic investigation of gene expression was employed

as a tool to compare these cultures transcriptional profiles at several points during fermentation (DeRisi *et al.*, 1997; Spellman *et al.*, 1998), thus providing clues as to the genotypic differences between parent and evolved culture.

There are very few sequences of wine yeast that have been determined (either published or databased), however the homology between wine yeast and S288c has been theorised to be greater than 99 % (Masneuf *et al.*, 1998). A recent study found high homology to S288c across four wine strains at the DNA level (Dunn *et al.*, 2005). This supports the validity of the use of sequences of S288c and products designed around them, such as S288c microarray chips for wine yeast analysis (Puig *et al.*, 1998; Puig and Perez-Ortin, 2000).

There have been limited studies monitoring global transcript levels in wine fermentations/oenological conditions (Backhus *et al.*, 2001; Rossignol *et al.*, 2003; Zuzuarregui *et al.*, 2006). Of greatest note Backhus *et al.* (2001) grew the wine yeast UCD 2100 in a minimal must media (Giudici and Kunkee, 1994), under conditions similar to that of an oenological fermentation, and compared expression between early and mid/late fermentation in both low and high assimilable nitrogen. Rossignol *et al.* (2003) grew the wine yeast EC1118 (Lallemand) in a synthetic grape must (Bely *et al.*, 1990a), comparing transcriptional response at five different time points during fermentation to a reference of early exponential phase. Zuzuarregui *et al.* (2006) compared the transcriptional activity of ICV 16 (Fermicru primeur; DSM) and ICV 27 (UCLM S-377; Springer Oenologie) in a synthetic must fermentation. Thus utilising such techniques provided ample scope to add to this knowledge database.

In this study we sought to characterise the evolved populations by comparing their gene expression profile with that of their parents via microarray analysis using the chips developed for the laboratory yeast S288c.

4.2 Results

To determine the effect of the continued propagation of the strains C911D and L-2056, which theoretically resulted in adaptively evolved progeny, single batch fermentations and metabolite analysis of these fermentations were conducted. The conditions used were similar to the small-scale batch fermentations highlighted in Chapter 3, with monitoring of not only fermentation duration but also glucose and fructose catabolism. The major metabolites produced, that is, succinic, acetic and lactic acids, as well as acetaldehyde, glycerol and ethanol were measured from terminal samples by HPLC. Measures of ergosterol and fatty acids were also conducted on a selection of fermentations.

4.2.1 Characteristics of FM5-M and C911D during fermentation

During the course of the fermentation of a CDGJM containing 200 g l^{-1} of glucose and fructose, FM5-M was found to be able to complete fermentation in approximately half the time required for C911D (79 h *vs.* 159 h). It was also found to have an increased dry cell weight (6.4 mg ml^{-1} versus 5.7 mg ml^{-1} for C911D) representing an increase of 12.0 % (Table 4.1). Of particular industrial importance, glycerol and ethanol showed altered production, with an increase in glycerol from 6.2 g l^{-1} to 7.1 g l^{-1} (an increase of *ca.* 0.9 g l^{-1} or 15.4 %). A concurrent decrease in ethanol was found for FM5-M of some 13.8 % (i.e. 88.0 g l^{-1} *vs* 102.0 g l^{-1}). Increases were noted in the production of acetic acid and acetaldehyde by FM5-M (16.6 % and 6.8 % respectively). Figures 4.1A, B and C, depict the profiles of sugar catabolised and glycerol and ethanol produced during fermentation. It is evident that at a point between 20 and 40 hours after inoculation, FM5-M shows elevated catabolism of sugars and synthesis of glycerol, with a concurrent, albeit mild, increase in ethanol production compared to C911D. The time point where there is a change in sugar catabolism and metabolite production is referred to in this study as the reference window of divergence (RWD).

Table 4.1 Biomass, fermentation duration and metabolite yields of adaptively evolved isolate FM5-M derived from C911D.

Parameter[a]	C911D	FM5-M
Dry weight (mg ml^{-1})	5.70 ± 0.09	112.0 ± 0.3
Fermentation duration (h)	159.0 ± 2.0	50.3 ± 2.0
Succinic acid (g l^{-1})	0.28 ± 0.01	130.6 ± 0.1
Lactic acid (g l^{-1})	0.23 ± 0.02	143.7 ± 0.1
Glycerol (g l^{-1})	6.19 ± 0.12	115.4 ± 0.1
Acetic acid (g l^{-1})	1.70 ± 0.04	116.6 ± 0.1
Acetaldehyde (g l^{-1})	0.03 ± 0.01	106.8 ± 0.1
Ethanol (g l^{-1})	102.00 ± 0.80	86.2 ± 0.2

[a] Biomass and metabolite yields determined in terminal fermentation samples. Absolute values ± standard deviation are shown for C911D. Data for FM5-M is expressed as a percentage of C911D.

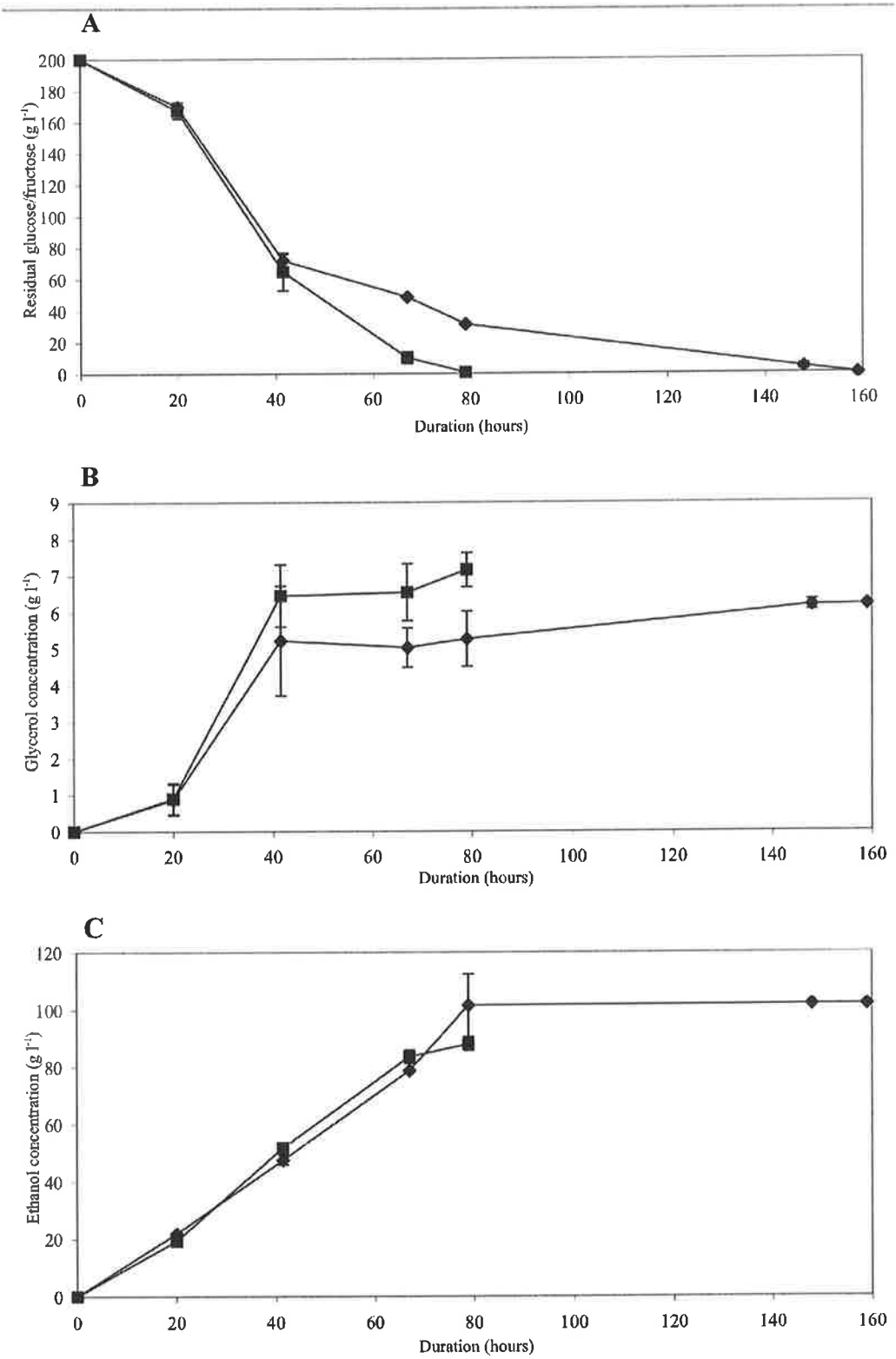


Figure 4.1 Catabolism of total monosaccharides (A), production of glycerol (B) and production of ethanol (C) by the mixed culture isolate FM5-M (■) and the parent strain C911D (◆). Fermentations were performed in CDGJM containing 200 g l^{-1} glucose/fructose. Data points are the mean of triplicate fermentations ± standard deviation.

Mixed culture isolates FM1 through FM4 were also screened for major differences in fermentation kinetics and metabolite yield in comparison to C911D, however no significant differences were found (data not shown).

4.2.2 Lipid quantitation of C911D and FM5-M

Fatty acids and ergosterol are essential for plasma membrane integrity, therefore enabling the cell to complete an oenological fermentation with greater viability and fermentative capacity (Arnezeder and Hampel, 1991; Mauricio, 1991; Valero *et al.*, 2001). The cellular fatty acid and ergosterol content was therefore determined because it was hypothesised that these substituents may be present in the evolved mutant in altered amounts, thereby accounting for the enhanced properties of this culture. Surprisingly there was a reduction in the ergosterol content of FM5-M compared to its parent C911D, in both the YEPD-grown inoculum (stationary phase cells) and at the end of fermentation (Table 4.2). Thus the YEPD pre-culture of FM5-M contained 3.3 % ergosterol by cell wet weight, which equated to only 85.8 % of the content in the C911D culture. A larger difference was found in cells collected at the end of fermentation, where FM5-M produced only 62.8 % of the ergosterol content of the parent strain (*ca.* 2.3 % opposed to 3.6 % for FM5-M and C911D, respectively).

Determination of major fatty acids using GC-MS were unreliable with the equipment available and thus these values, which suggested no significant differences existed between mixed cultures and their parents, are not reported here.

4.2.3 Characteristics of FM16-M and L-2056 during fermentation

The evolved mixed culture FM16-M was able to catabolise available sugar within a period of 97 hours, as opposed to L-2056, which was unable to finish fermentation and was deemed to have stuck after 200 hours (Table 4.3). Unlike FM5-M *vs* C911D, there was no significant difference between FM16-M and L-2056 in terms of biomass formation measured as dry cell weight from

Table 4.2 Ergosterol content as a percentage of culture dry weight for adaptively evolved isolate FM5-M and parent C911D.

Sample[a]	C911D	FM5-M
Starter culture	3.84 ± 0.35	3.29 ± 0.17 (85.8%)
End of fermentation	3.58 ± 0.30	2.25 ± 0.22 (62.8%)

[a] Analyses were performed on stationary phase cultures at the end of the starter propagation (18 hrs) in YPD and at the end of the fermentation of the experimental cultures. Absolute values ± standard deviation are shown for C911D and FM5-M. Values are the mean of three determinations ± standard deviation. FM5-M data expressed as a percentage of C911D is shown in brackets.

Table 4.3 Biomass, fermentation duration and metabolite yields of adaptively evolved isolate FM16-M derived from L-2056.

Parameter[a]	L-2056	FM16-M
Dry weight (mg ml^{-1})	4.70 ± 0.08	102.0 ± 0.3
Fermentation duration (h)	did not complete	(97 hrs)
Succinic acid (g l^{-1})	0.16 ± 0.08	127.6 ± 0.1
Lactic acid (g l^{-1})	0.24 ± 0.05	148.9 ± 0.1
Glycerol (g l^{-1})	7.98 ± 0.22	114.2 ± 0.1
Acetic acid (g l^{-1})	1.54 ± 0.03	119.7 ± 0.1
Acetaldehyde (g l^{-1})	0.07 ± 0.01	100.3 ± 0.1
Ethanol (g l^{-1})	97.96 ± 1.49	99.1 ± 5.9

[a] Biomass and metabolite yields determined in terminal fermentation samples. Absolute values ± standard deviation are shown for L-2056. Data for FM16-M is expressed as a percentage of L-2056.

terminal samples. There was also little deviation between FM16-M and L-2056 in the production of ethanol. As seen for the evolved haploid mixed culture, glycerol production also increased (14 %) in FM16-M (i.e. 9.1 g l^{-1} versus 8.0 g l^{-1}; Table 4.3). A time course for the production of ethanol and glycerol was also determined (Figure 4.2). As for the FM5-M *vs* C911D comparison, there is an increased rate of sugar catabolism in FM16-M, with elevated glycerol production between the 20 hour and 40 hour time points. Increases in succinic, acetic and lactic acid production were also observed in FM16-M compared to L-2056 (Table 4.3).

Culture growth, estimated by change in absorbance during exponential growth and microscopic cell counts at the end of fermentation, was also examined and found not to differ between evolved cultures and their respective parent strain (data not shown). Due to L-2056 being unable to complete fermentation, comparisons of total metabolite yields were not possible as the carbon not catabolised by the latter must be taken into account. When considered in this way, it can be seen that the maximum specific rate of glycerol and ethanol production by FM16-M exceeds that of the parental strain (see Figure 4.3, 30 hr time point). This corresponds to the change in fermentation kinetics of sugar consumed and glycerol produced (Figure 4.2A and B).

A reference window of divergence (RWD) was identified between 20 and 40 hours in both the FM5-M *vs* C911D and FM16-M *vs* L-2056 fermentation comparisons. This time point highlights where the evolved mixed cultures began to demonstrate an ability to maintain a higher rate of sugar catabolism than their respective parents. Concurrent increases in glycerol production also occurred (Figures 4.1B and 4.2B). The RWD is further highlighted in Figures 4.3A and B, where metabolite produced as a function of carbon catabolised is at a peak at a time which approximates the RWD for the L-2056, FM16-M comparison. This time point was therefore considered an important reference point for subsequent characterisation of the evolved cultures relative to their parents.

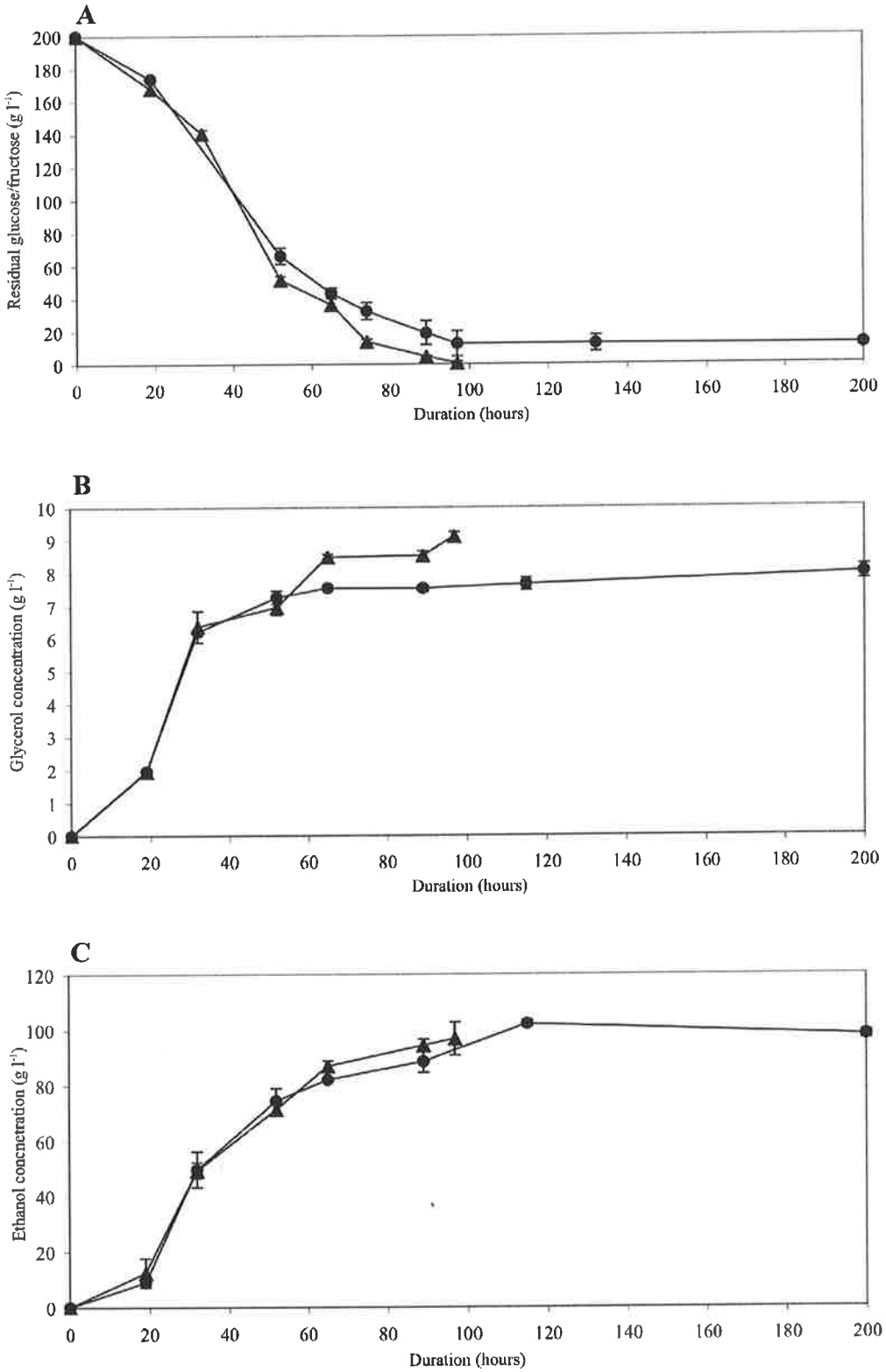


Figure 4.2 Catabolism of total monosaccharides (A), production of glycerol (B) and production of ethanol (C) by the mixed culture isolate FM16-M (▲) and the parent strain L-2056 (●). Fermentations were performed in CDGJM containing 200 g l^{-1} glucose/fructose. Data points are the mean of triplicate fermentations ± standard deviation.

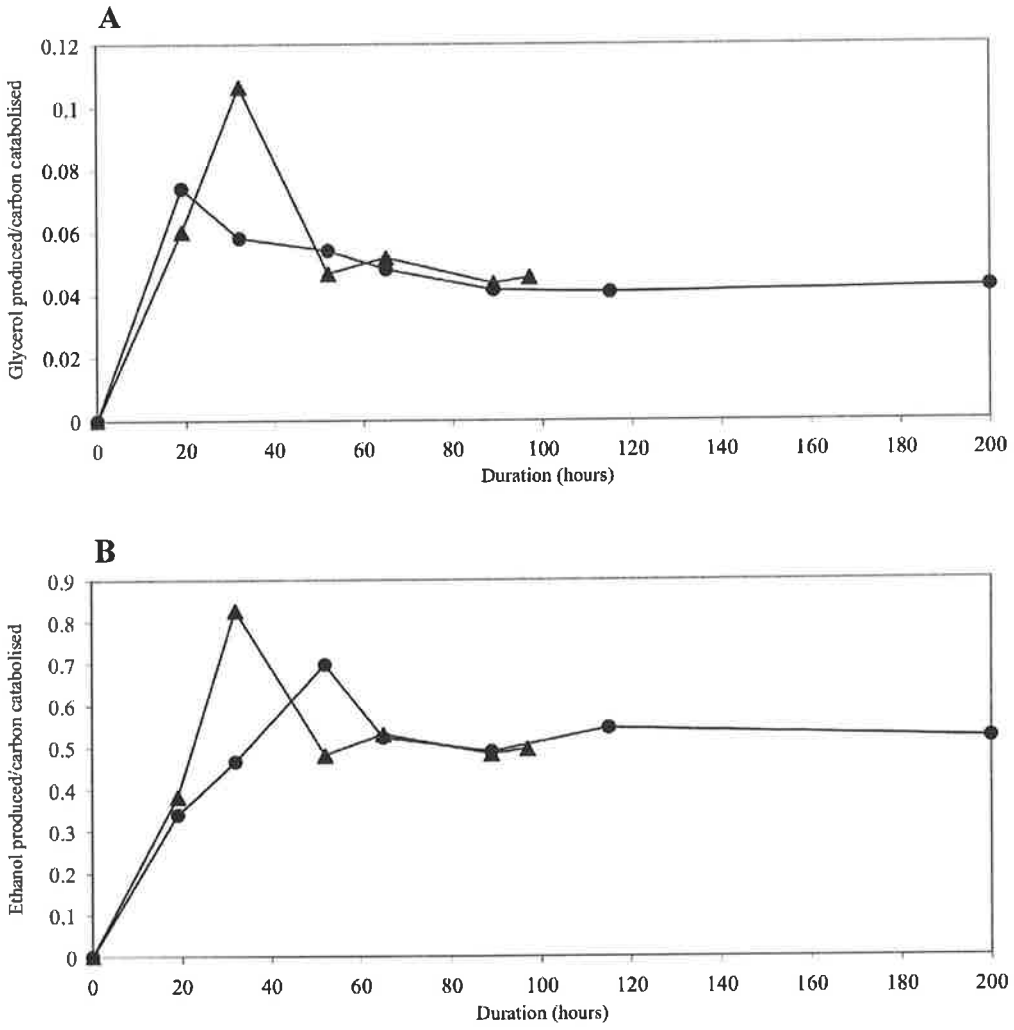


Figure 4.3 Glycerol (A) and ethanol (B) production as a function of total monosaccharides catabolised during the course of fermentation, by the mutant isolate FM16-M (▲) and the parent strain L-2056 (●). Fermentations were performed in CDGJM containing 200 g l^{-1} glucose/fructose.

As for the comparison of metabolite production in strains C911D and FM1 - FM4, mixed culture isolates FM10 through to FM15 displayed metabolite profiles very similar to that of the parent L-2056 (data not shown). Unfortunately, due to availability of instrumentation, ergosterol and fatty acid analysis for FM16-M and L-2056 could not be completed.

4.2.4 Transcriptome analysis

At this point of the study, populations had been generated whose collective fermentative abilities and/or metabolite yields differed from those of the corresponding parental strain. It was recognised that 1) the makeup of the population of the evolved cultures could range from simple (i.e. a single clone) to complex (i.e. multiple clones with differing properties, the sum of which yielded the observed behaviour for the population) and 2) that in either case, the differences seen between parents and evolved populations might not necessarily be attributable to alterations in the expression of one or a small number of genes, but rather could be due to a complex profile of novel gene expression patterns (Ferea *et al.*, 1999). Accordingly, a broad transcriptional profiling approach was used essentially as a means of screening for target genes which could subsequently be pursued through further or more detailed investigation. Therefore microarray analysis was used.

It was reasoned that in some cases where a novel attribute was in evidence, a detectable change in transcript of related genes would exist whether arising from a single clone or a mixed population of clones. One possible exception being rare transcripts of some regulatory genes.

S. cerevisiae oligonucleotide microarrays were obtained from the Ramaciotti Centre for Gene Function Analysis (Sydney, Australia) and consisted of 40-mer oligonucleotide probes for 6,250 yeast open reading frames (S288c background (MWG Biotec)) printed in duplicate on epoxy-coated glass microarray slides (Schott). At the time of these experiments, these were the only slides printed in Australia with a *S. cerevisiae* background. The use of an Affymetrix platform was ruled out, due to the high cost and scarce availability

of scanners associated with such arrays at this time. The implementation of protocols within the group for both labelling of probes and hybridisation to slides, while scanning slides at a local facility was also a priority.

Fermentation is a dynamic process, where transcriptional responses have been shown to be highly varied throughout (Rossignol *et al.*, 2003). Consequently cultures were examined at time points where the greatest phenotypic differences between the evolved mixed culture (FM5-M and FM16-M) and its parent (C911D and L-2056) were expected. Also cultures grown from an initial population of $< 5 \times 10^5$ cells ml^{-1} are commonly sampled at a density of 1×10^7 cells ml^{-1} since separate cultures are considered physiologically similar and in a state of rapid growth at this point (DeRisi *et al.*, 1997). Therefore, RNA was isolated at three sample points during fermentation, these were at a cell density of 1×10^7 cells ml^{-1} and time points approximating a change in carbon catabolism. These were, *ca.* 1 hr before and 2 hrs after this noted change, within the RWD, under conditions which mimicked that of the selection process. The assumption was that at the collection of the pre-RWD sample, both the evolved and parent cultures would display similar fermentation rates and would be exposed to similar concentrations of glucose and fructose as well as glycerol and ethanol. By the post-RWD sample point, however, evolved cultures had exhibited distinct rates and degree of sugar catabolism and yields of associated metabolites for at least 2 hours.

Microarrays were conducted in triplicate reverse dye experiments, where triplicate fermentations were conducted for evolved and parent strains. RNA was isolated as described by Higgins *et al.* (2003), and then partnered with a reciprocal biological replicate, reverse transcribed and labelled with either the fluorophore Cy3 or Cy5, and hybridised to the glass microarray slide (DeRisi, 2003). Experiments for each partnered subset of RNA were labelled with the reverse dye set. Evolved mixed cultures were analysed with respect to the parent for each time point to monitor the differences between evolved culture and parent as a function of physiological response. Dye partner set

configurations were standardised for all comparisons and an example is given in Table 4.4.

Although cDNA microarrays have been successfully employed for studies of lab strains, adaptation of the protocols to industrial strains in an oenological ferment environment posed several challenges, specifically relating to sensitivity (Zhou and Thompson, 2002). While high total RNA concentrations were easily isolated, at later sample points during fermentation when cells were in stationary phase such preparations provided low concentrations of mRNA (Riou *et al.*, 1997). As a result, it was consistently difficult to attain high quality hybridisations and, consequently, spot intensities.

For statistical analysis of the cDNA microarray data the *limma* (Linear Modelling for Microarray Analysis) software package (Smyth and Speed, 2003; Smyth, 2004; Smyth *et al.*, 2005) from Bioconductor (http://www.bioconductor.org) was used. Appropriate filtering and normalization preprocessing of the data were performed prior to the final analyses according to the help files of *limma* and the *limma* User's Guide included with the software (Wettenhall and Smyth, 2004). This software has been reported as one of the most commonly used packages for normalization and statistical analysis of cDNA microarrays (Xia *et al.*, 2005), implementing an empirical Bayes linear modeling approach (Smyth, 2004). The same statistical tests and criteria were applied to all studies, where genes with associated p-value ≤ 0.01 were considered as differentially expressed.

4.2.4.1 Transcriptome analysis of FM5-M relative to C911D under oenological conditions

Major metabolites were quantified from samples from which cDNA microarrays were also performed. At mid exponential phase, cell numbers for the cultures were $1.23 \pm 0.05 \times 10^7$ cells ml^{-1} (FM5-M) and $1.1 \pm 0.2 \times 10^7$ cells ml^{-1} (C911D), and there was little difference between the cultures in terms of the amounts of sugar catabolised or metabolites produced (Table 4.5). The latter also applied to the following time point. Post-RWD the expected trend in

Table 4.4 An example of dye partner set configurations used for cDNA microarrays analysis.

Slide Number	**RNA from, labelled with**	
	Cy3	**Cy5**
1	Parent – BR # 1	Evolved – BR # 1
2	Evolved – BR # 1	Parent – BR # 1
3	Parent – BR # 2	Evolved – BR # 2
4	Evolved – BR # 2	Parent – BR # 2
5	Parent – BR # 3	Evolved – BR # 3
6	Evolved – BR # 3	Parent – BR # 3

BR denotes biological replication, that is, RNA extracted from fermentation replicate prior to reverse transcription and labelling, and prior to hybridisation to a microarray slide.

Table 4.5 Key metabolites of samples from RNA isolation during the FM5-M, C911D microarray analysis fermentations.

Metabolite ($g\ l^{-1}$)	Mid Exponential		Pre- RWD		Post- RWD	
	C911D	**FM5-M**	**C911D**	**FM5-M**	**C911D**	**FM5-M**
Glucose/fructose	187.3 ± 0.1	184.3 ± 0.1	106.4 ± 5.6	98.1 ± 6.8	99.4 ± 5.3	88.3 ± 5.6
Glycerol	1.6 ± 0.1	1.7 ± 0.1	6.6 ± 0.3	6.9 ± 0.3	6.8 ± 0.2	7.5 ± 0.1
Ethanol	7.9 ± 0.3	8.1 ± 0.4	53.6 ± 4.7	59.3 ± 3.9	58.8 ± 1.6	63.9 ± 3.9

Values are the mean of 2 determinations ex 3 replicates ± standard deviation.

the metabolite data was observed, that is more sugar (11.2 %) has been catabolised by the evolved culture (i.e. 9.8 g catabolised over the 3 hours from pre-RWD in FM5-M as opposed to 7.0 g for C911D). Concurrently, a 10.3 % increase in glycerol was observed along with an 8.7 % increase in ethanol accumulation in the FM5-M culture. It is noteworthy however that the rate of ethanol accumulation in the FM5-M (1.5 g h^{-1}) culture was less than that of the C911D (1.7 g h^{-1}) culture, calculated as a function of the pre- and post-RWD sample collections.

Those genes whose transcripts differed upon comparison of FM5-M and C911D at the mid exponential phase (*ca.* 1 x 10^7 cells ml^{-1}) are listed in Table 4.6 in functional groups. The most striking observation was the general decrease in transcript in FM5-M. Thus out of 80 genes identified, 59 showed reduced transcript. Examples of this were genes involved with amino acid biosynthesis, lipid biosynthesis, carbohydrate metabolism, stress response and transport. Interestingly there was also a down-regulation of seven genes associated with methionine metabolism and five genes associated with glucose transport at this time point. Up-regulation was observed for two genes which encode heat shock proteins (*HSP10* and *HSP150*) as well as two genes associated with oxidative stress (*TRX2* and *SOD2*). Additionally, a decrease in the transcript of *GLC3*, the product which determines the extent of branching in glycogen particles (Rowen *et al.*, 1992; Thon *et al.*, 1992) was observed.

Before the RWD, up-regulation of genes involved with carbohydrate metabolism as well as DNA and RNA metabolism is also evident (Table 4.7). An increase in transcript of the copper transport genes, *CUP1-1*, *CUP1-2* and the hexose transporter, *HXT5*, amongst others, is also evident.

Only two gene transcripts were found to have modified regulation post-RWD, these were *VID24*, a negative regulator of gluconeogenesis and *HXT4*, a hexose transporter, both being increased in FM5-M (Table 4.8).

Table 4.6 Differentially expressed FM5-M genes during fermentation at mid exponential phase (1 x 10^7 cells ml^{-1})

Function	ORF (gene name)	Fold induction or repression	Function	ORF (gene name)	Fold induction or repression
Amino acid and derivative metabolism	YJR137c (*ECM17*)	-2.3	Transport	YGL187c (*COX4*)	1.5
	YCL018w (*LEU2*)	1.3		YLR380w (*CSR1*)	-1.4
	YDR234w (*LYS4*)	-1.8		YEL093c (*CYC7*)	-1.5
	YNR050c (*LYS9*)	-1.9		YHR094c (*HXT1*)	-2.2
	YIL094c (*LYS12*)	-1.6		YMR011w (*HXT2*)	-1.7
	YJR010w (*MET3*)	-2.5		YDR345c (*HXT3*)	-1.6
	YER091c (*MET6*)	-2.7		YDR343c (*HXT6*)	-4.7
	YGL125w (*MET13*)	-1.7		YDR342c (*HXT7*)	-2.7
	YKL001c (*MET14*)	-4.1		YBL005w (*PDR3*)	3.5
	YPR167c (*MET16*)	-1.6		YEL017c-a (*PMP2*)	2.0
	YLR303w (*MET17*)	-2.5		YNL055c (*POR1*)	1.6
	YIL128w (*MET18*)	-2.4		YGR009c (SEC9)	-1.6
	YLR180w (*SAM1*)	-2.0		YBR294w (*SUL1*)	-2.3
				YNL044w (*YIP3*)	-1.6

Table 4.6 (Cont.)

Function	ORF (gene name)	Fold induction or repression
Carbohydrate metabolism	YCL050c (*ARA1*)	1.6
	YEL011w (*GLC3*)	-1.9
	YKL109w (*HAP4*)	-1.6
	YJL153c (*INO1*)	-1.7
	YOR142w (*LSC1*)	3.7
	YGL209c (*MIG2*)	-1.6
	YJL052w (*TDH1*)	2.0
	YML100w (*TSL1*)	1.6
Cell wall organization and biogenesis	YBR149w (*CIS3*)	-1.4
	YDR055w (*PST1*)	1.9
Lipid biosynthesis	YGR175c (*ERG1*)	-1.6
	YLR056w (*ERG3*)	-1.6
	YPL038w (*ERG10*)	-1.3
Other	YPL061w (*ALD6*)	-3.2
	YCL050c (*APA1*)	1.6
	YLR216c (*CPR6*)	2.2
	YJL172w (*CPS1*)	-1.7
	YDL215c (*GDH2*)	-1.5
	YML004c (*GLO1*)	-1.5
	YMR217w (*GUA1*)	-1.8
	YGR249w (*MGA1*)	-1.7
	YLR058c (*SHM2*)	-1.7
Unknown	YJL200c (*ACO2*)	-2.1
	YBR302c (*COS2*)	-1.4
	YPL095c (*EEB1*)	2.1
	YHL021c (*FMP12*)	-1.9

Table 4.6 (Cont.)

Function	ORF (gene name)	Fold induction or repression	Function	ORF (gene name)	Fold induction or repression
Lipid biosynthesis	YGR060w (*ERG25*)	-1.8	Unknown	YGR243w (*FMP43*)	-2.4
	YBL039c (*URA7*)	-1.7		YGR052w (*FMP48*)	-2.2
Organelle Organisation and biogensis	YHR089c (*GAR1*)	-1.4		YML125c (PGA3)	1.5
	YOR310c (*NOP58*)	-1.6		YOL084w (PHM7)	-2.3
	YGL039w	-1.6		YLD375w (*STP3*)	-1.4
Protein biosynthesis	YOR133w (*EFT1*)	-1.5		YLR327c (*TMA10*)	-3.8
	YDR385w (*EFT2*)	-1.5		YBBR111c (*YSA1*)	1.4
	YGR285c (*ZUO1*)	-1.5		YER067w	-3.0
Response to stress	YOR020c (*HSP10*)	1.8		YDR340w	3.2
	YJL159w (*HSP150*)	2.1		YOR385w	-1.5
	YNL056w (*NCE103*)	-1.8		YKR075c	-2.2
	YLR350w (*ORM2*)	-1.8		YDL121c	-1.6
	YLL039w(*UBI4*)	1.9			
	YHR008c (*SOD2*)	1.5			
	YGR209c (*TRX2*)	1.7			

Fermentations were performed in CDGJM containing 200 g l^{-1} glucose/ fructose. Data shown as a function of expression relative to parent C911D.

Table 4.7 Differentially expressed FM5-M genes during fermentation pre-RWD.

Function	ORF (gene name)	Fold induction or repression	Function	ORF (gene name)	Fold induction or repression
DNA metabolism	YMR046c	2.6	RNA metabolism	YFL001w (*DEG1*)	2.5
	YMR051c	2.6		YHR088w (*RPF1*)	1.8
	YMR045c	2.1	Carbohydrate	YDR216w (*ADR1*)	2.4
	YNL054w-b	3.2	metabolism	YBR018c (*GAL7*)	2.5
	YLR410w-b	3.4		YNL037c (*IDH1*)	1.7
	YGR161w-a	3.4		YIL162w (*SUC2*)	1.6
	YDR261w-a	3.6	Cell wall organization and biogenesis	YKL096w-a (*CWP2*)	-4.1
	YDR261w-b	3.3	Lipid metabolism	YDR072w (*IPT1*)	-1.5
	YDR210w-c	2.6	Transport	YHR096c (*HXT5*)	1.8
	YBL101w-b	2.3		YPL036w (*PMA2*)	-2.3
	YBL101w-a	3.4		YPL092w (*SSU1*)	-4.2
	YGR161w-b	2.5	Other	YOL165c (*AAD15*)	1.8
				YHR055C (*CUP1-2*)	1.8
	YGR161c-c	2.5		YHR053c (*CUP1-1*)	2.1

Fermentations were performed in CDGJM containing 200 g l^{-1} glucose/fructose. Data shown as a function of expression relative to parent C911D.

Table 4.8 Differentially expressed FM5-M genes during fermentation post-RWD.

Function	**ORF (gene name)**	**Fold induction or repression**
Carbohydrate metabolism	YBR105c (*VID24*)	1.5
Transport	YHR092c (*HXT4*)	1.7

Fermentations were performed in CDGJM containing 200 g l^{-1} glucose/ fructose. Data shown as a function of expression relative to parent C911D.

4.2.4.2 Transcriptome analysis of FM16-M relative to L-2056 under oenological conditions

Major parameters of fermentation media were determined from samples used for cDNA microarray analysis at time points similar to that of the FM5-M *vs* C911D comparison (Table 4.9). Mid exponential phase cell numbers were 1.0 ± 0.05 x 10^7 cells ml^{-1} for FM16-M and 1.1 ± 0.11 x 10^7 cells ml^{-1} for L-2056. Concentrations of sugars, glycerol and ethanol at mid exponential phase were also similar in the FM16-M and L-2056 cultures. Before RWD the glucose/fructose and glycerol concentrations of FM16-M were similar to that of L-2056, however the ethanol concentrations were marginally higher. Post-RWD, FM16-M had catabolised 8 g l^{-1} more sugar and produced 0.4 g l^{-1} more glycerol.

There were no significant transcriptional differences between FM16-M and L-2056 detected at the mid-exponential phase, however there was a small list of genes representing differences in transcripts either side of the RWD sample point (Table 4.10 and 4.11).

Genes that appeared up-regulated pre-RWD included a number involved with stress response, specifically two that encode heat shock proteins (*HSP26* and *HSP30*). There was a decrease in transcript of genes associated with organelle organization and biogenesis and transport, specifically four hexose transporters *HXT2, HXT4, HXT6* and *HXT7* (Table 4.10). However two genes associated with carbohydrate transport showed an increase in transcript in the mutant culture FM16-M (*TPO2* and *PIC2*). Also of note is an increase in *ADH2*, whose gene product is required for the reduction of acetaldehyde to ethanol.

After the RWD, seven genes were found to have differential transcript regulation, all being down-regulated in FM16-M (Table 4.11). Thus there was a decrease in the transcript of *GPH1* encoding a nonessential glycogen phosphorylase regulated by stress response elements. A decrease in *RCK1*, a gene thought to be associated with oxidative stress, was also noted. There was

Table 4.9 Key metabolites of samples from RNA isolation during the FM16-M, L-2056 microarray analysis fermentations.

Metabolite ($g\ l^{-1}$)	Mid Exponential		Pre- RWD		Post- RWD	
	L-2056	**FM16-M**	**L-2056**	**FM16-M**	**L-2056**	**FM16-M**
Glucose/fructose	173.6 ± 0.1	167.7 ± 0.1	66.8 ± 4.2	65.3 ± 5.1	66.0 ± 3.0	58.0 ± 0.6
Glycerol	1.9 ± 0.1	1.9 ± 0.1	7.3 ± 0.5	7.4 ± 0.2	7.4 ± 0.2	7.8 ± 0.1
Ethanol	8.9 ± 0.4	9.3 ± 0.2	66.5 ± 1.5	71.6 ± 2.4	73.5 ± 1.8	76.2 ± 3.0

Values are the mean of 2 determinations ex 3 replicates ± standard deviation.

Table 4.10 Differentially expressed FM16-M genes during fermentation pre-RWD.

Function	**ORF (gene name)**	**Fold induction or repression**
Amino Acid and Derivative metabolism	YPR035w (*GLN1*)	1.6
	YNR050c (*LYS9*)	-1.5
Carbohydrate Metabolism	YFR053c (*HXK1*)	-1.5
	YBR299w (*MAL32*)	-1.7
	YDR043c (*NRG1*)	1.5
Cell wall organization and biogenesis	YDR955w (*PST1*)	1.8
	YBR067c (*TIP1*)	1.6
Conjugation	YDL039w (*PRM7*)	-1.8
Generation of precursor metabolites and energy	YMR303c (*ADH2*)	1.7
Homeostasis	YLR109w (*AHP1*)	2.0
Lipid metabolism	YDR492w (*IZH1*)	1.5
Organelle organization and biogenesis	YMR033w (*ARP9*)	-1.8
	YMR053c (*STB2*)	-1.8
	YYIR012w (*SQT1*)	-1.8

Table 4.10 (Cont.)

Function	ORF (gene name)	Fold induction or repression
Stress response	YBR072w (*HSP26*)	3.0
	YCR021c (*HSP30*)	2.3
	YMR175w (*SIP18*)	2.5
	YOR010c (*TIR2*)	-2.5
	YNL060w (*YGP1*)	1.5
Transport	YMR011w (*HXT2*)	-1.7
	YHR092c (*HXT4*)	-1.8
	YDR343c (*HXT6*)	-1.8
	YDR342c (*HXT7*)	-1.9
	YPR032w (*SRO7*)	-1.9
	YER053c (*PIC2*)	2.0
	YGR138c (*TPO2*)	1.7
Unknown	YER053c-a	1.6
	YBR085c-a	1.5
	YPR170c	-1.7
	YER067w	-1.5
	YMR034c	-1.6

Fermentations were performed in CDGJM containing 200 g l^{-1} glucose/ fructose. Data shown as a function of expression relative to parent L-2056.

Table 4.11 Differentially expressed FM16-M genes during fermentation post-RWD.

Function	**ORF (gene name)**	**Fold induction or repression**
Carbohydrate metabolism	YPR160w (*GPH1*)	-2.0
Cell cycle	YGL158w (*RCK1*)	-2.0
Generation of precursor metabolites	YGL256w (*ADH4*)	-1.7
Transcription	YGL162w (*SUT1*)	-2.0
Unknown	YGR043c	-1.6
	YLR392c	-1.9
	YOL161c	-2.0

Fermentations were performed in CDGJM containing 200 g l^{-1} glucose/ fructose. Data shown as a function of expression relative to parent L-2056.

also a decrease in transcript for *ADH4*, an alcohol dehydrogenase, along with three genes of unknown function.

4.3 Conclusions

a) FM5-M and FM16-M have increased sugar catabolism, reflected in their shorter fermentation durations.
b) FM5-M and FM16-M demonstrate desirable metabolite production (of oenological significance), in particular decreased ethanol and increased glycerol yields.
c) Transcriptional profiles of FM5-M and FM16-M are different to the respective parent, and offer an insight to the basis for the phenotypes observed in the evolved cultures.

4.4 Discussion

4.4.1 Metabolite biomass and lipid yields

FM5-M and FM16-M were selected from an adaptively evolving culture due to their shortened fermentation duration (Chapter 3). We were interested to determine the extent of adaptation by the evolved cultures and their resultant desirable (and potentially undesirable) fermentative properties. Consequently, other characteristics which might be considered to contribute to the overall desirability of wine strain performance were analysed and are reported here. These include dry cell weight, the major organic acids: lactic, acetic and succinic, as well as acetaldehyde, glycerol and ethanol yields.

Aside from reduced fermentation duration (the benefits of which have been discussed in Chapter 3), the evolved culture FM5-M also demonstrated a desirable metabolite production profile. A 15.4 % (0.9 g l^{-1}) increase in glycerol and a decrease in ethanol (14.1 g l^{-1}, *ca.* 86.2 % of C911D) formation were noted and recognised as being of particular interest in an industrial context. Increases in acetic and succinic acids and acetaldehyde were also evident, and possibly occur at the expense of ethanol (Nevoigt and Stahl, 1996; Michnick *et al.*, 1997; Remize *et al.*, 1999; Dequin, 2001; Nevoigt *et al.*,

2002). FM16-M had an increased production of succinic, lactic and acetic acids as well as potentially favourable increases (14.2 % or 1.1 g l^{-1}) in the concentration of glycerol in terminal fermentation samples. Further to this, it is noteworthy that although the majority of the sugar is catabolised in the L-2056 fermentation by approximately 90 hours, there is still *ca.* 12 g l^{-1} residual sugar; a concentration which in an industrial context would deem the wine unsuitable for dry wine production and microbiologically unstable (Iland and Gago, 2002).

Glycerol is the third major metabolite formed during fermentation after ethanol and carbon dioxide (Remize *et al.*, 1999) and is considered important to wine composition. Although aromatically neutral, glycerol can be perceived on the palate as sweetness (Noble and Bursick, 1984), highlighting the potential for enhanced mouthfeel (Nieuwoudt *et al.*, 2002). Reported concentrations of glycerol in wine range from 4.2 to 10.4 g l^{-1} (Radler and Schutz, 1982), with the threshold reported as 5.4 g l^{-1} (Noble and Bursick, 1984). Consequently, it could be assumed that the concentrations produced by the mutants during fermentation could significantly affect the structure of a wine.

Recombinant strains that over-produce glycerol have been shown to yield elevated amounts of acetic acid as well (Michnick *et al.*, 1997; Remize *et al.*, 1999; de Barros Lopes *et al.*, 2000; Prior *et al.*, 2000; Eglinton *et al.*, 2002). This was also the case for both FM5-M (*ca.* 2.0 g l^{-1} *vs* 1.7 g l^{-1} for C911D) and FM16-M (*ca.* 1.8 g l^{-1} *vs* 1.5 g l^{-1} for L-2056). It has been previously published that acetic acid is highly undesirable in wine above 0.8 g l^{-1} often leading to an overpowering vinegar-like aroma (Fleet and Heard, 1993). However, such thresholds for desirability are dependent on the wine style. At elevated concentrations, acetic acid in wine can also lead to enhanced sweetness on the nose and greater complexity on the palate (unpublished data; N Bourke, McLaren Vale Vintners, pers comm.; Iland and Gago, 2002)

With the increase observed in both glycerol and acetic acid produced by the evolved mixed cultures, it is assumed that there is a re-routing of the flux

through glycolysis. In the instance of FM5-M the increase of these metabolites appears to be at the expense of ethanol. Reduced ethanol production could have arisen through the sequestering of carbon away from ethanol with the need to regenerate NADH instead occurring by oxidation of acetaldehyde to acetic acid (Eglinton *et al.*, 2002), or formation of glycerol from dihydroxyacetone phosphate. For the diploid culture, FM16-M, no reduction in ethanol was apparent, but as with FM5-M there was an increase in glycerol and acetic acid. A detailed fluxome analysis (Gombert *et al.*, 2001; Christensen *et al.*, 2002) would be useful in determining the metabolic origin of these compounds in each evolved culture. Similarly the possibility that an increased succinic acid production occurs as an attempt by the cell at maintenance of redox balance might also be explored in this way.

In a winemaking context, a reduced ethanol yield can be a very positive outcome. Ethanol increases the perception of 'heat' on the back palate and masks many flavour and aroma compounds of wine (Iland and Gago, 2002). Thus the avoidance of higher ethanol concentrations, particularly those resulting from the use of over-ripened fruit can be most beneficial. There is currently much interest in so-called 'low-ethanol' yeasts (Iland and Gago, 2002) particularly from New World wine producing countries such as Australia and USA where flavour-ripe grapes are readily achieved and the resulting wine favoured by consumers. A reduced ethanol yield would presumably also benefit the yeast itself by reducing the toxic effects of this metabolite during fermentation (Virginie *et al.*, 2001). In fact the superior performance of FM5-M is likely to, at least in part, be a result of the lower ethanol concentration of these fermentations.

The increase in biomass production (dry cell weight) by FM5-M may be related to the reduced production of ethanol. Similar findings were described by Aranda and del Olmo (2003). For instance, given the reduction in ethanol production by FM5-M, it could be argued that the effects of ethanol on the cell membrane would be lessened compared to the parent C911D. Consequently, reserve compounds, such as trehalose and glycogen, which are known to

enhance cell viability by conferring stress tolerance may be produced in lesser quantities by the adaptively evolved haploid thus enhancing biomass accumulation instead (Plourde-Owobi *et al.*, 2000).

It was theorised that due to the extended anaerobic fermentation, coupled with the initial oxidative stress, an adaptively evolved strain may be equipped to produce greater quantities of sterols and/or unsaturated fatty acids in turn offering greater cellular protection, and thus longevity and osmotolerance (Higgins *et al.*, 2003). Quantitation of ergosterol was used as an indicator for total cellular sterols, as it has been identified to account for up to 90 % of the total cellular sterol content (Arnezeder and Hampel, 1991). However lipid analysis of FM5-M yielded results contrary to what was initially hypothesised, where a reduction in the cellular ergosterol content was seen in FM5-M, in both pre-culture and terminal fermentation samples. Zitomer and Lowry (1992) found that high levels of oxygen caused by over-aeration can decrease the expression of *ERG11* (encoding the enzyme which catalyses the C-14 demethylation of lanasterol in the ergosterol biosynthesis pathway) and *OLE1* (encoding for the fatty acid desaturase required for monosaturated fatty acid synthesis) to low levels and subsequently reduced production of these compounds. Given that the conditions used here included a post-inoculation aeration step, these findings offer one putative mechanism by which the reduced quantity of ergosterol in FM5-M could have arisen.

Intracellular lipid particles are nearly entirely comprised of triacylglycerols and steryl esters and are suggested to constitute a reserve of lipid material (Leber *et al.*, 1994). Under conditions of fatty acid deficiency as might occur during an anaerobic oenological fermentation (Wakil *et al.*, 1983; Belviso *et al.*, 2004), both triacylglycerols and steryl esters are mobilised and their fatty acids are used for the synthesis of phospholipids (Daum and Paltauf, 1980). Thus it is possible that FM5-M addresses a reduction in ergosterol content by virtue of increased lipid reserves.

The phenotypes observed for cultures FM5-M and FM16-M show great promise in an industrial context. Notably the decreased fermentation duration, implies that these cultures are better able to complete fermentation under imposing stressful conditions, encountered during the winemaking process. In this way, losses of commercial product through incomplete fermentations, which is currently a significant problem worldwide, might be reduced. Additionally, the elevated concentration of glycerol noted in both mixed cultures is a highly desirable phenotype, especially given the direction in New World winemaking for the production of wines with enhanced mouthfeel.

4.4.2 Transcriptome analysis

Whole transcriptome analysis was undertaken to aid in the elucidation of what genotypic changes had occurred to confer the superior performance of evolved cultures during fermentation (Streelman and Kocher, 2000). It was theorised that by comparing transcripts at the data points of mid exponential phase as well as pre- and post-RWD we would be better able to elucidate genotypic alterations which led to the observed phenotypes in the adaptively evolved cultures. Thus cellular machinery associated with the observed phenotypes of FM5-M and FM16-M was expected to be differentially expressed around the time where differences in performance and metabolite yield between parent and evolved cultures were observed during fermentation.

It was our intention to minimise noise in this analysis by use of triplicated biological replicates, as well as replicated technical samples coupled with dye swaps to provide greater basis for testing differences within the mutant and parent groups and slides (Churchill, 2002). However, microarray analysis can only offer an insight into the transcriptional alterations associated with the observed phenotypic traits of the evolved mutants. Further substantiation is required by independent validation of the array data, for example by means of quantitative real time PCR or Northern analysis. Moreover it would be important to monitor expression of protein products (Chuaqul *et al.*, 2002).

These caveats aside, the analysis highlighted 107 (in the haploid comparison) and 38 (in the diploid comparison) genes and putative ORFs whose transcripts was altered. These genes can be functionally grouped according to their reported involvement in key processes, including: transcription, transport (sugars, sulphate amongst others), metabolism (carbon, DNA, RNA, lipid, amino acids and derivatives), generation of precursor metabolites and energy, cell cycle, stress response, cell wall and organelle organization and biogenesis, conjugation and homeostasis. The identification of genes belonging to such groups concurs with the range of functions required of a wine yeast and the stresses it is exposed to during fermentation. Surprisingly the list of transcripts which were differentially expressed between adaptively evolved cultures and their parent was small. This fact is in contrast to the reports of others (Ferea *et al.*, 1999; Sonderegger *et al.*, 2004), whose selective pressures were arguably simpler than the multiple stresses and growth phases experienced by the cultures in this study. This may suggest that only a small number of modifications had occurred during the evolutionary process, or many changes in individual clones were hidden.

Comparison of transcripts in FM5-M to that of C911D at mid exponential phase yields data that shows a general trend of down regulation (*ca.* 73 % of transcripts found to be differentially expressed were shown to be down regulated in FM5-M). mRNA production is linked to cell cycle and obviously decreased during stationary phase cells (Sogin and Saunders, 1980). Consequently the general decrease in transcripts as a whole seen early in fermentation could confer an advantage to mutants by expending less energy on non-essential pathways, thus enabling FM5-M to finish fermentation within a shorter duration than C911D.

The elucidation of total functional changes through transcriptional differences generated in microarray analysis is difficult, consequently a subset only of genes with differential expression thought to be important are discussed below. Of the down-regulated genes, in the FM5-M *vs.* C911D comparison (mid exponential phase), a decrease in transcript of five hexose transporter genes,

HXT1, HXT2, HXT3, HXT6 and *HXT7* was observed. Despite such reductions however, sugar catabolism in FM5-M at this time point (*ca.* 15 hours, Figure 4.1A) was greater compared to the parent. It is known that in a wild-type yeast, these *HXT* genes are regulated with respect to glucose concentration, though not necessarily co-ordinately (Ozcan and Johnston, 1995). *HXT* expression is also influenced by osmotic pressure (Hirayama *et al.*, 1995; Rep *et al.*, 2000) and nutrient starvation (Diderich *et al.*, 1999a). Hxt2p, Hxt6p and Hxt7p are carriers with high or moderate affinity and are induced by low glucose concentrations and repressed by high glucose concentrations (Ozcan and Johnston, 1995; Reifenberger *et al.*, 1997). As a consequence, Hxt2p, Hxt6p and Hxt7p are thought to bear little significance on the rate of sugar catabolism early in fermentation, when sugar concentrations are high. Down-regulation of low affinity transporters, Hxt1p and Hxt3p, may also have limited effect at such time in fermentation. For instance, Hxt1p is induced by high sugar yet has been shown to be non-essential under oenological conditions (Luyten *et al.*, 2002), mainly due to the redundancy in this family of transporters. The impact of a down-regulation of *HXT3* in FM5-M is more difficult to elucidate, although it is thought that this gene is only weakly regulated by glucose concentration (Perez *et al.*, 2005). It is therefore suggested that the reduced expression of the five highlighted *HXT* genes is indicative of their functionality not being essential to the performance of the evolved cultures. Instead it is their reduced expression that is beneficial to the culture, perhaps through the lessened metabolic burden associated with their synthesis. Alternatively, down-regulation of these *HXT* genes may merely be an indirect outcome of other changes, perhaps to the functioning of a common regulator of sugar transport and catabolic processes.

In *S. cerevisiae*, the regulation of metabolism of glycogen (a major storage carbohydrate) is correlated with growth phase of the cell. This polysaccharide is virtually absent from actively growing cultures yet accumulates in large amounts (< 2.6 % dry cell weight) (Rothman-Denes and Cabib, 1970; Gunja-Smith *et al.*, 1979), in cells which are undergoing metabolic transitions such as starvation, diauxic growth lags, or approach to stationary phase (Lillie and

Pringle, 1980). The observed reduction in *GLC3* transcript in FM5-M may reflect a decrease in the production of glycogen. Such an outcome perhaps provides surplus energy and/or biosynthetic precursors for biomass formation, which in turn benefits fermentative performance of the evolved culture, FM5-M.

A decrease in transcript in four genes associated with ergosterol biosynthesis (*ERG1, ERG3, ERG10* and *ERG25*) is in agreement with the reduced ergosterol concentration determined for the mutant FM5-M. These four genes participate in the ergosterol biosynthetic pathway at different points. *ERG1* (encoding squalene epoxidase) when over-expressed leads to the accumulation of ergosterol (Veen *et al.*, 2003), thus a decrease in transcript theoretically will yield reduced end product.

The down-regulation of seven genes associated with methionine biosynthesis, at the mid exponential phase sample would suggest a decrease in the production of sulfur containing amino acids. L-methionine is as essential amino acid required for protein biosynthesis and participates together with ATP, in the formation of *S*-adenosylmethionine (AdoMet) (Mizunuma *et al.*, 2004). The expression of the *MET* genes is coordinately regulated in response to an increase in the intracellular concentration of AdoMet (Thomas *et al.*, 1989). AdoMet is a sulfonium compound that is used in a large number biological methylation reactions (Thomas and Surdin-Kerjan, 1997; Lu, 2000). Down-regulation of *MET* genes could impact on the fermentative capacity of FM5-M in several ways. For example, reduced formation of AdoMet might make more ATP available to cells of the evolved culture for use in glycolysis. Alternatively, a reduction in AdoMet-dependent methyltransferases (which act on a wide variety of target molecules, including DNA, RNA, proteins, amongst other molecules; Cheng and Roberts, 2001), may lead to increased transcriptional regulation genes important during fermentation (specifically glycolytic genes) through decreased methylation of DNA.

Hap4p is thought to be the major regulatory protein of the Hap2/3/4/5p complex required for transcriptional induction of respiratory components (Blom *et al.*, 2000). *HAP4* is repressed by glucose and induced by diauxic shift (DeRisi *et al.*, 1997). The reduced expression of *HAP4* noted in FM5-M may simply be further repression of respiratory genes. *HAP4* has been found to be strongly induced during the shift from fermentative to respiratory conditions. Such induction of *HAP4* might only be possible in this study during the early stages of fermentation (up to ~ 8 hr) before available oxygen is presumed to be utilised. Otherwise, the down-regulation of *HAP4* early in fermentation may in fact be relevant to the increased expression of *HXT5* before the RWD. Putative regulatory elements in the promoter of *HXT5* include two Hap2/3/4/5p complex binding sites (Verwaal *et al.*, 2004). In contrast to the other hexose transporters, expression of *HXT5* is determined by the growth rate of cells and not by the extracellular glucose concentration (Verwaal *et al.*, 2004). Furthermore it is expressed when growth rate decreases and maximally induced under glucose depletion (Verwaal *et al.*, 2002). Therefore the shortened fermentation duration of FM5-M might be due to the higher rate of sugar uptake mediated by Hxt5p which in turn produces greater availability of sugars for glycolysis.

The increase in transcripts of *TRX2* and *SOD2* mid exponential phase and *CUP1-1* and *CUP1-2* pre RWD offers a proposed role in protecting the cell as gene products function as antioxidants (Liu and Thiele, 1996; Perez-Torrado *et al.*, 2005).

The up-regulation of *ADR1* is also of interest, as it may contribute to the reduced ethanol production by FM5-M. *ADR1* was initially characterised as a positive regulator of the glucose repressed, alcohol dehydrogenase *ADH2* (Ciriacy, 1975; Ciriacy, 1979). The derepression of *ADR1* mRNA translation appears to also be affected by glucose (Verwaal *et al.*, 2004), and there are reports that glucose may also regulate at the post-translational level. Since Adr1p is a substrate for cAMP-dependent protein kinases *in vitro* and since increased kinase activity *in vivo* inhibits *ADH2* expression, it was suggested

that an increase in the cAMP level during growth on glucose would cause the phosphorylation of Adr1p (Cherry *et al.*, 1989). The phosphorylated Adr1p would still bind to the *ADH2* promoter but would not interact with the transcription machinery (Taylor and Young, 1990). In this case the oxidation of ethanol to acetaldehyde by the isoenzyme *ADH2* would still be possible.

After the RWD the increased transcript of *HXT4* offers a mechanism for the sustained sugar catabolism in FM5-M. Transcription of *HXT4*, which encodes a moderate affinity hexose transporter, is regulated by Gcr1p and Gcr2p (Turkel and Bisson, 1999). This in fact is a commonality of several of the groups of genes observed to be differentially expressed in these arrays, where genes with differential transcript regulation in the ergosterol pathway also have putative *GCR1* binding sites in their promoters (searches were conducted using the Cold Spring Harbour Promoter database, see Zho and Zhang, 1999). Further to this, all of the genes found to be differentially expressed at the mid exponential phase which are involved in amino acid biosynthesis or are hexose transporters all have putative *GCR1* transcriptional regulation binding sites in their promoters. Therefore a mutation in the *GCR1* gene may be responsible for some of the observed transcriptional events, thus the sequencing of this gene may prove informative.

Comparing transcripts of FM16-M and L-2056 from the mid exponential phase sample point revealed no genes to be differentially expressed, implying that genomic changes to FM16-M do not affect the transcriptome of cells in this physiological state. Pre RWD, of the FM16-M and L-2056 comparison, an increase in transcript of *GLN1*, which encodes for glutamine synthetase (Mitchell and Magasanik, 1983), is observed. It is assumed that increased glutamine synthetase (as the product of increased transcript of *GLN1*) in FM16-M may stimulate glycolysis and replace depleted ATP, and perhaps NADH (Flores-Samaniego *et al.*, 1993; Larsson *et al.*, 1997). There is also a striking increase in transcript of genes associated with stress responses. Notably Hahn and Thiele (2004) found a negative role of Snf1p on expression of *HSP26* and *HSP30* by inhibition of Msn2p/Msn4p under abrupt glucose

depletion conditions. Accordingly, the gene *SIP18*, also found up-regulated here, has been proposed to be regulated by the Msn2p/Msn4p transcriptional factor (Treger *et al.*, 1998). The observed increase in transcript in genes associated with the stress response is likely due to adaptation of the cell to the stressful conditions prevalent under oenological conditions, post diauxic shift (Riou *et al.*, 1997; Brosnan *et al.*, 2000).

There is a down regulation, also at the pre-RWD comparison (FM16-M *vs* L-2056), of *HXT2*, *HXT4*, *HXT6* and *HXT7*. A similar phenomena was noted at mid-exponential sampling in the haploid comparison, however the reason for this transcriptional change is difficult to elucidate without further investigation.

Post-RWD there is a decrease in the transcript of *GPH1*, encoding glycogen phosphorylase, which has three putative Stress Response Elements (STREs) in the upstream region (Sunnarborg *et al.*, 2001). Given that the accumulation of glycogen often occurs in response to nutrient limitation (De Winde *et al.*, 1997), it could be inferred that the environmental constraints were of less effect in the FM16-M compared to L-2056 cultures. This could possibly be due to the up-regulation of genes which encode stress response proteins, therefore enabling the cell to continue functioning. Furthermore the decreased transcript in glycogen metabolism would theoretically provide more glucose 6-phosphate for glycolysis.

Surprisingly there is a transcript increase in *ADH2* before the RWD and a decrease in *ADH4* after the RWD. These two genes encode for alcohol dehydrogenases, and the alteration in their expression could lead to a change in the redox state of the cell (Michnick *et al.*, 1997), since these enzymes produce NAD^+ (Bakker *et al.*, 2000). More specifically the decrease in transcript observed in *ADH4* could result in the greater accumulation of ethanol, as strains with low *ADH* activity have been proven to do (Ciriacy, 1975; Wills and Phelps, 1975; Johansson and Sjostrom, 1984).

Due to the dynamic nature of fermentation and hence the changing composition of the fermentation medium, it is difficult to attain transcriptional data comparing two phenotypically different strains from truly equivalent conditions. That is, the transcriptional differences that were observed during fermentation, specifically after the RWD, between these two sets of isolated mixed cultures, may in fact be a reflection of differing media compositions. Confirmation of this could be achieved by comparison of a set of concentrations of sugar both pre- and post-RWD. Consequently, it is worthwhile re-iterating that the selection of the pre- and post-RWD sample points, was based on the assumption that the increased catabolism of sugars by evolved mutant cultures would in fact arise out of alterations in abundance of selected transcripts during the within the period up to and including the RWD.

Although the gene lists generated from cDNA microarray analysis are relatively concise, it is still difficult to elucidate the genotypic mechanisms responsible for the observed phenotype in the mutant cultures. A few possible mechanisms have been put forward, however further characterisation is clearly required. In addition to specifically investigating the roles of selected highlighted genes through gene deletion or over-expression studies (as appropriate), determination of the degree of heterogeneity of the evolved culture and a transcriptional profiling of individual clones is proposed.

Chapter 5 Phenotypic and transcriptional characterisation of clones isolated from FM5-M and FM16-M

5.1 Introduction

It was reasoned that sequential cultures maintained for extended periods as occurs in long term evolution experiments, would be heterogenous (Rosenzweig *et al.*, 1994; Sauer, 2001). The validity of this was tested for the FM5-M and FM16-M populations, by determining the phenotypic characters and transcript profile of clonal cultures or 'single colony isolates' from the mixed cultures.

It is clear that in most circumstances populations of mixed cultures will demonstrate a degree of phenotypic heterogeneity. Typically yeast in industrial oenological conditions are of a mixed culture, particularly in the early stages of fermentation (Fleet and Heard, 1993; Fleet, 2003). In fact, even New World wine producers that have typically exploited pure culture technology, are increasingly trialling mixed or uninoculated cultures to achieve enhanced flavour diversity and thus complexity in finished wines (Lambrechts and Pretorius, 2000; Fleet, 2003; Howell *et al.*, 2006). However, the characterisation of single isolates in this study was deemed important to reveal the cross section of strains responsible for the overall fermentation attributes observed in the mixed culture.

5.2 Results

Fermentations were conducted as previously described, with triplicate experiments of parent and mixed evolved cultures as well as single cultures of each of the ten clonal isolates from FM5-M (FM5-1 → FM5-10) and FM16-M (FM16-1 → FM16-10).

As reported in Chapters 3 and 4, adaptive evolution experiments yielded the isolate FM5-M, after 250 generations, thought to be adaptively evolved. This was initially based on the premise that this strain was able to conduct fermentation under the imposed stressful conditions, within a shorter duration

(Figure 4.1A). Further phenotypic studies revealed that the mutant FM5-M did not retain this ability, a fact which may be indicative of a reversion of the causal mutation, possibly due to having insufficient fixing time (Galitski and Roth, 1996).

5.2.1 Comparison between FM5- cultures – biomass formation, fermentation duration and major metabolites

Ten clones were randomly isolated from single colonies derived from the FM5-M culture (FM5-1 through FM5-10), and their fermentation characteristics determined (Table 5.1). Of the ten single isolates analysed, only one was found inferior to FM5-M (FM5-8) because of its inability to finish fermentation. This finding also demonstrates the heterogeneity of the mixed culture. The data from FM5-8 was excluded from further discussion as the reduced sugar catabolism by this strain prevented meaningful comparisons of biomass and metabolite yields between the strains.

In these experiments the mixed culture, FM5-M, was found to produce succinic and lactic acids at similar concentrations to the parent, however differences are observed in the mean production of these by the clonal cultures. An increase in succinic acid (9.4 % in the clonal cultures, as opposed to a reduction of 2.6 % in FM5-M) and a 13.4 % decrease in lactic acid (no change was found in FM5-M) were noted. The mean data generated from the clonal cultures for glycerol, acetic acid, acetaldehyde and ethanol also largely reflected that of the mixed culture. Specifically, all clones produced more glycerol than the parent, with a range of 7.2 g l^{-1} to 8.2 g l^{-1}, an increase of 2.2 % to 15.2 % respectively, whereas FM5-M produced 10.3 % more glycerol than C911D (Figure 5.1). One clonal culture (FM5-1) produced marginally less acetic acid, the remainder however produced between 1.5 g l^{-1} to 1.7 g l^{-1}, (5.8 % to 27.6 % more than the parent C911D), similar to the 9.1 % increase observed in FM5-M.

Acetaldehyde concentrations produced by the clonal cultures also demonstrated variation, ranging from 90.0 % to 150.0 % of the production noted in the parent C911D, where FM5-M produced 120 % more than that of C911D. The

Table 5.1 Dry weight, fermentation duration and major metabolites of evolved isolate FM5-M, clonal isolates thereof and the parental population of C911D.

Parameter[a]	C911D	FM5-M	Mean of clones (Range)
Dry weight (mg ml^{-1})	6.24 ± 0.24	108.6	105.4 (92.5-155.6)
Fermentation duration (h)	62.0 ± 1.0	100	100 (100)
Succinic acid (g l^{-1})	0.22 ± 0.01	97.4	109.4 (89.7-125.1)
Lactic acid (g l^{-1})	0.34 ± 0.03	100.0	88.6 (83.5-123.9)
Glycerol (g l^{-1})	7.09 ± 0.07	110.3	108.2 (102.2-115.2)
Acetic acid (g l^{-1})	1.39 ± 0.03	109.1	113.9 (97.8-127.6)
Acetaldehyde (g l^{-1})	0.03 ± 0.00	120.0	110.0 (90.0-150.0)
Ethanol (g l^{-1})	102.36 ± 1.17	86.5	89.3 (87.1-100.3)

[a] Biomass and metabolite yields determined in terminal fermentation samples. Absolute values ± standard deviation are shown for C911D. Data for FM5-M (mixed culture) and the mean of the 9 single isolate clones are expressed as a percentage of C911D.

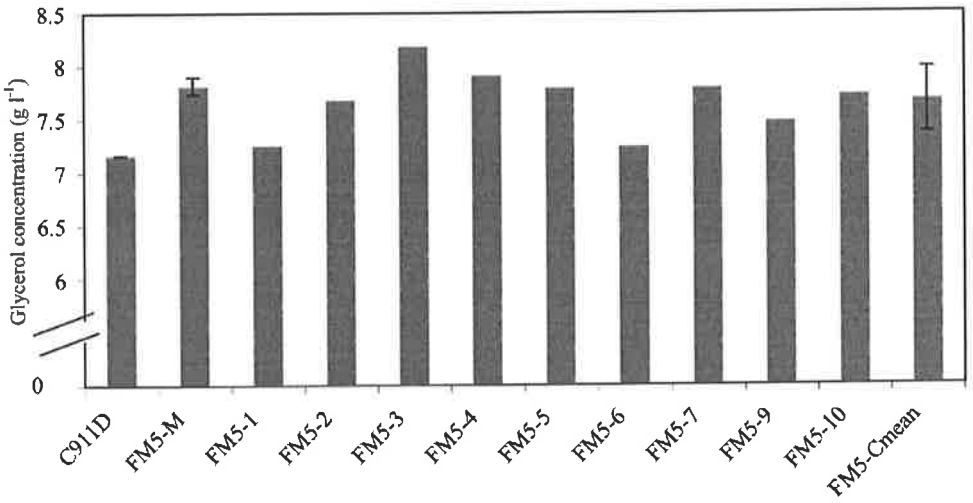


Figure 5.1 The concentration of glycerol produced by C911D, the mixed evolved culture (FM5-M) and clonal isolates thereof (FM5-1 to FM5-10). Fermentations were performed in CDGJM. Values for C911D and FM5-M are the mean of triplicate fermentations, Cmean is the mean of clone fermentations ± standard deviation. Data for isolate FM5-8 has been excluded as this culture failed to complete fermentation.

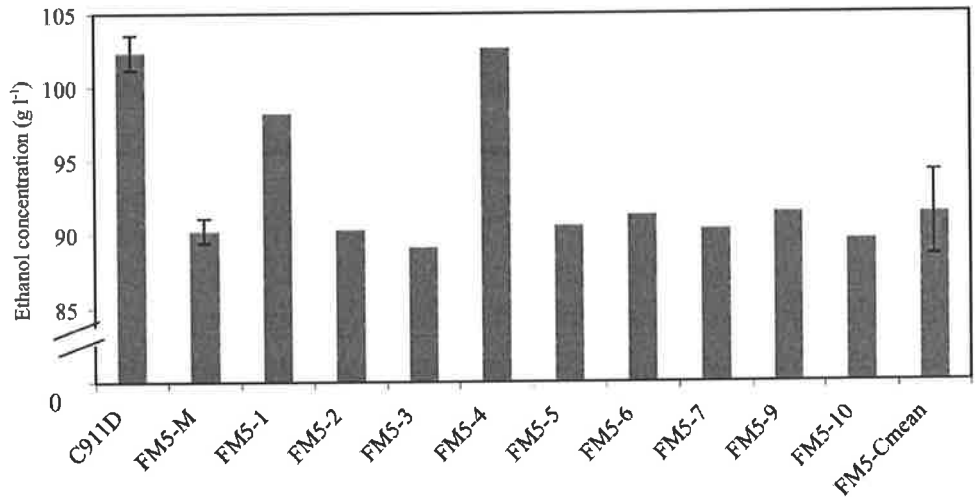


Figure 5.2 The concentration of ethanol produced by C911D, the mixed evolved culture (FM5-M) and clonal isolates thereof (FM5-1 to FM5-10). Fermentations were performed in CDGJM. Values for C911D and FM5-M are the mean of triplicate fermentations, Cmean is the mean of clone fermentations ± standard deviation. Data for isolate FM5-8 has been excluded as this culture failed to complete fermentation.

concentration of ethanol was found to be on average somewhat reduced from that of the parental population, being 89.3 % and 86.5 % for the clonal cultures and the mixed culture, respectively. Isolates FM5-1 and FM5-4 produced similar concentrations of ethanol to C911D (98.2 g l^{-1} and 102.6 g l^{-1} *vs* 102.4 g l^{-1} respectively) where all other clonal cultures produced between 89.1 g l^{-1} and 91.5 g l^{-1} (87.1 % - 89.2 % of that produced by C911D (Figure 5.2)). Again this mean is similar to the level of production seen for FM5-M (*ca.* 90.2 g l^{-1}).

Akin to the mixed culture, the clonal cultures demonstrated on average an ability to produce more biomass than the parent (i.e. 105.4 % as dry weight), except in the case of FM5-1 and FM5-2, which produced less biomass (95.7 % and 92.5 % of the C911D yield).

5.2.2 Comparison between FM16- cultures – biomass formation, fermentation duration and major metabolites

As with the analysis of the clonal cultures derived from the haploid adaptively evolved mutant FM5-M, one strain was found unable to complete fermentation (FM16-4), again highlighting the heterogeneity of the mixed culture. This strain was not included in the mean calculations. The mean fermentation durations and dry cellular weights for the remaining clones closely resembled that of FM16-M (Table 5.2), with all nine clonal cultures completing fermentation sooner (i.e. 77.5 hr to 119 hr) than the parent L-2056 (140 hr; Figure 5.3). Glycerol production, was found to be similar to, or greater than the parent (Figure 5.4). The most striking result was that of clonal culture FM16-7, which produced some 18 % more glycerol than its parent strain. In general, the concentration of succinic acid was found to be marginally less in the isolates compared to the mixed culture, although there was a large variation in the accumulation of this metabolite (50.4 % to 109.6 %).

The acetic acid produced by clonal cultures was similar to that of FM16-M, ranging from 1.6 g l^{-1} to 1.8 g l^{-1}, with the mean for the parent being 1.8 g l^{-1}. The dramatic increase found in lactic acid produced by FM16-M is reflected in only two of the clonal cultures, the remainder showing a minimal increase. On

Table 5.2 Dry weight, fermentation duration and major metabolites of evolved isolate FM16-M, clonal isolates thereof and the parental population of L-2056.

Parameter[a]	L-2056	FM16-M	Mean of clones (Range)
Dry weight (mg ml^{-1})	4.67 ± 0.07	98.7	95.7 (85.1-101.6)
Fermentation duration (h)	140.0 ± 1.5	68.9	70.3 (55.4-85.0)
Succinic acid (g l^{-1})	0.35 ± 0.05	92.9	79.0 (50.4-109.6)
Lactic acid (g l^{-1})	0.16 ± 0.04	150.4	119.2 (58.5-218.6)
Glycerol (g l^{-1})	7.54 ± 0.11	112.0	109.2 (98.9-131.2)
Acetic acid (g l^{-1})	1.67 ± 0.02	105.8	98.2 (92.3-105.3)
Acetaldehyde (g l^{-1})	0.07 ± 0.00	90.5	106.4 (85.7-128.6)
Ethanol (g l^{-1})	106.28 ± 1.21	96.5	98.8 (94.4-101.1)

[a] Biomass and metabolite yields determined in terminal fermentation samples. Absolute values ± standard deviation are shown for L-2056. Data for FM16-M (mixed culture) and the mean of the 9 single isolate clones are expressed as a percentage of L-2056.

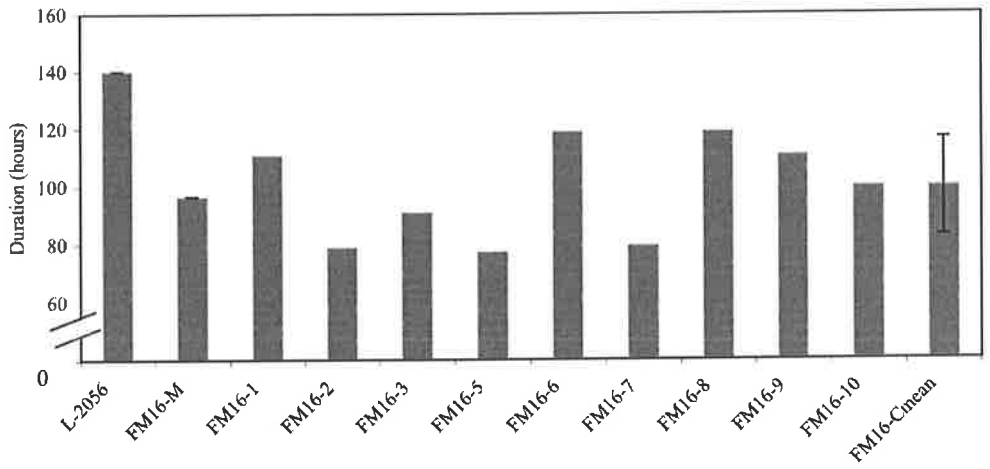


Figure 5.3 Fermentation duration of L-2056, the mixed evolved culture (FM16-M) and clonal isolates thereof (FM16-1 to FM16-10). Fermentations were performed in CDGJM. Values for L-2056 and FM16-M are the mean of triplicate fermentations, Cmean is the mean of the clone fermentations ± standard deviation. Data for isolate FM16-4 has been excluded as this culture failed to complete fermentation.

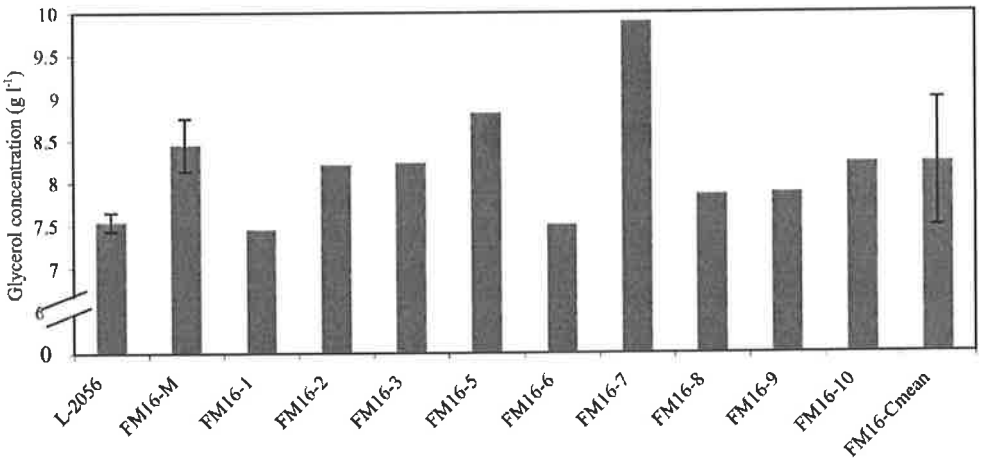


Figure 5.4 The concentration of glycerol produced by L-2056, the mixed evolved culture (FM16-M) and clonal isolates thereof (FM16-1 to FM16-10). Fermentations were performed in CDGJM. Values for L-2056 and FM16-M are the mean of triplicate fermentations, Cmean is the mean of the clone fermentations ± standard deviation. Data for isolate FM16-4 has been excluded as this culture failed to complete fermentation.

average the nine clones produce approximately 19.2 % more lactic acid than the parent strain L-2056. The mean acetaldehyde concentration produced by the clonal cultures is 106.4 % of that produced by L-2056, which contrasts to the production by FM16-M, being 90.5 % of that produced by the parent. Negligible differences were noted in the formation of biomass.

5.2.3 Further characterisation of FM16-5 and FM16-7

Further characterisation of improved isolates was undertaken through the conduct of triplicate fermentations comparing FM16-5 and FM16-7 to L-2056. These two clonal cultures were chosen based on their reduced fermentation duration and increased glycerol production. Small-scale fermentations performed with FM16-5 and FM16-7 were undertaken as previously described (Chapter 3). It was confirmed that both the clonal cultures were able to complete fermentation within 87.5 (FM16-5) and 70 (FM16-7) hours as opposed to the parent, which was unable to finish fermentation after 140 hours and left a residual sugar concentration of 42.1 g l^{-1}. Contrasting to that observed in the mixed culture, greater growth (measured as OD_{600}; Figure 5.5) was indicated for FM16-5 and FM16-7 after 40 hours compared to L-2056. Interestingly there was little difference in cell numbers or dry cell weight between the two isolates and the parent (Table 5.3). A comparison between evolved and parent cultures of metabolites produced is difficult to make due to the high residual sugar concentration in the parent fermentations (*ca.* 21% of total sugar remaining in the L-2056 culture). For this reason, measured metabolites were greatly elevated in terminal fermentation samples from the evolved cultures (Table 5.3). However a divergence in metabolite yield is already clearly seen at the RWD between 25 and 40 hours as rates of sugar catabolised decline in the parental strain but remain high in the clones (Figures, 5.6A - C).

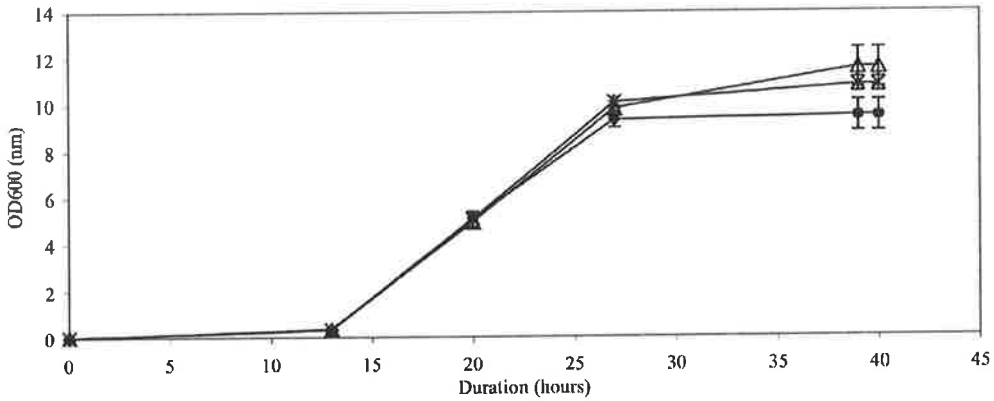


Figure 5.5 Biomass accumulation (as a function of OD 600) by the mutant clonal isolates FM16-5 (✳) and FM16-7 (△) as well as the parent strain L-2056 (●). Fermentations were performed in CDGJM containing 200 g l^{-1} glucose / fructose. Data points are the mean of triplicate fermentations ± standard deviation.

Table 5.3 Cell enumeration, dry weight, fermentation duration and major metabolites of evolved clonal isolates FM16-5 and FM16-7 and the parental population of L-2056.

Parameter[a]	L-2056	FM16-5	FM16-7
Cell number (x 10^8 cells / ml)	2.27 ± 0.31	2.39 ± 0.19	2.16 ± 0.12
Dry weight (mg ml^{-1})	5.70 ± 0.09	5.83 ± 0.13	5.88 ± 0.22
Fermentation duration (h)	DNC	87.5 ± 3.0	70.0 ± 2.0
Succinic acid (g l^{-1})	0.18 ± 0.01	0.21 ± 0.02	0.21 ± 0.02
Lactic acid (g l^{-1})	0.24 ± 0.02	0.56 ± 0.04	0.54 ± 0.05
Glycerol (g l^{-1})	7.96 ± 0.25	10.08 ± 0.05	10.14 ± 0.27
Acetic acid (g l^{-1})	1.54 ± 0.07	1.61 ± 0.03	1.65 ± 0.16
Acetaldehyde (g l^{-1})	0.03 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Ethanol (g l^{-1})	87.5 ± 5.5	109.1 ± 0.1	108.5 ± 2.5

[a] Biomass and metabolite yields determined in terminal fermentation samples. Absolute values ± standard deviation are shown. DNC; did not complete.

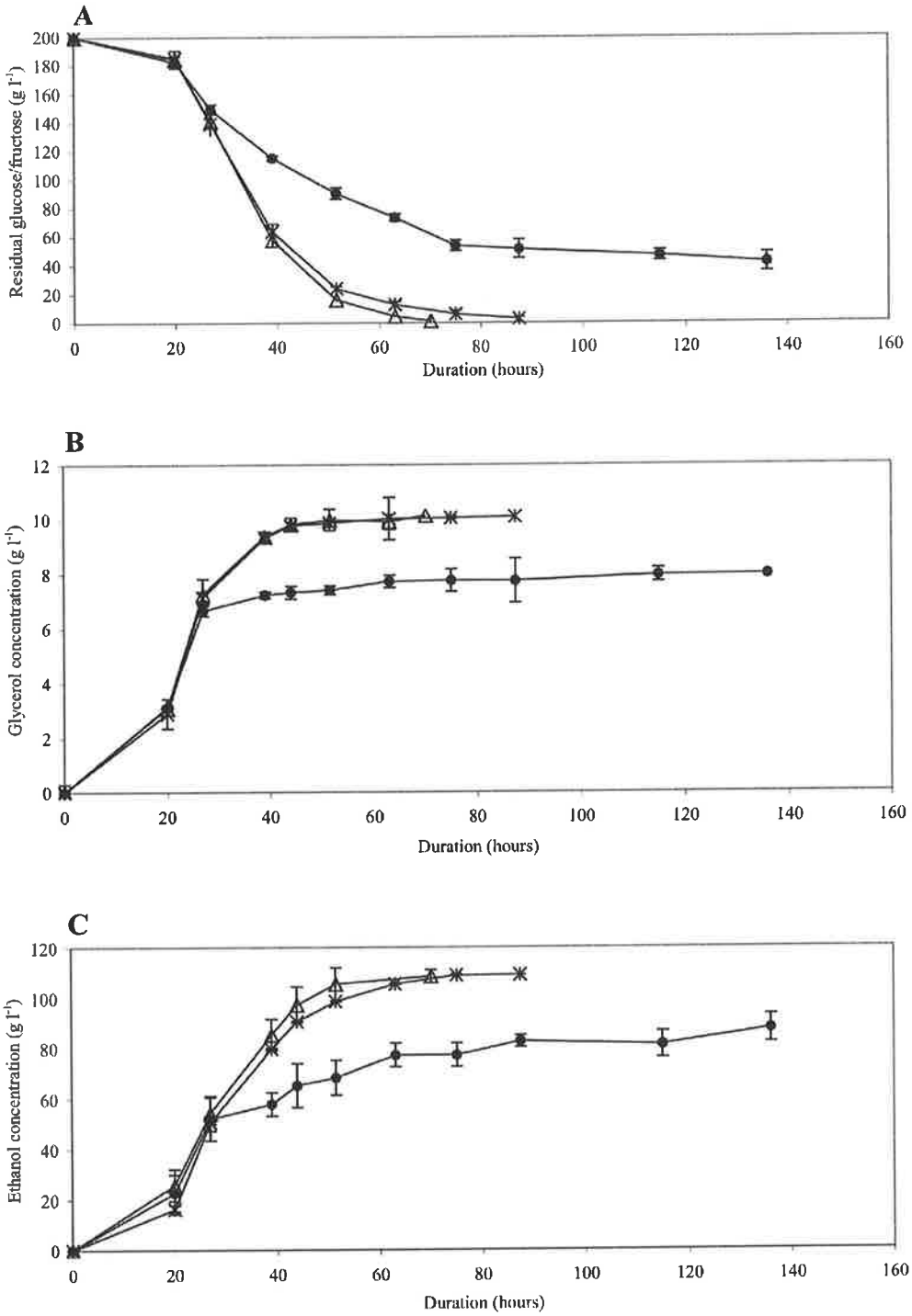


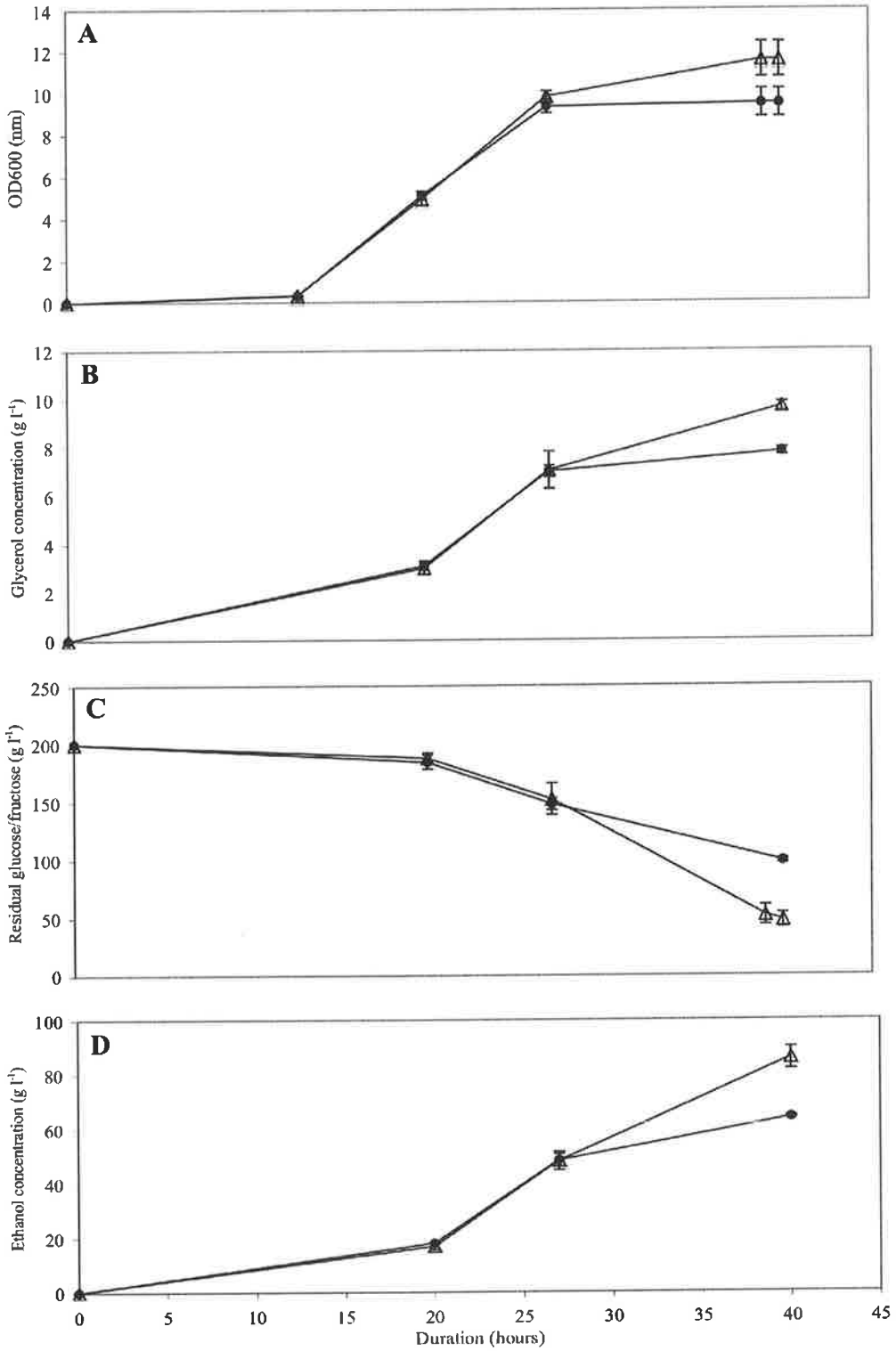
Figure 5.6 Catabolism of total monosaccharides (A), production of glycerol (B) and production of ethanol (C), by the mutant clonal isolates FM16-5 (✳) and FM16-7 (△) as well as the parent strain L-2056 (●). Fermentations were performed in CDGJM containing 200 g l^{-1} glucose / fructose. Data points are the mean of triplicate fermentations ± standard deviation.

5.2.4 Transcriptional responses in FM16-7 relative to L-2056 under oenological conditions.

To further elucidate the extent of heterogeneity of the mixed culture and thus the transcriptional activities measured in Chapter 4, we analysed the transcript abundance of FM16-7, compared to L-2056 (labelling and hybridisation conducted by Mark van der Hoek, Adelaide Microarray Consortium). It was thought that the observed phenotype of shorter fermentation duration and increased glycerol concentration would be best monitored post-RWD, where the differences in fermentation performance are prevalent. This was based on the assumption that cells sensing carbon and nutrient depletion coupled with increasing ethanol toxicity prevalent post-RWD would result in changes in gene expression, synthesis of protective molecules, and/or the modulation of protein activity by posttranslational modifications or subcellular localisation (Estruch, 2000). Such responses are proposed to contribute to the associated phenotype of heightened sugar catabolism and glycerol metabolism post-RWD. Consequently, it was thought beneficial to extend the sampling time beyond 2 hours post-RWD, as had been used previously.

Fermentation was monitored to confirm the phenotype of FM16-7. The profiles (biomass accumulation, sugar catabolism, glycerol and ethanol metabolism) of these triplicate microarray cultures (Figure 5.7A - D) replicated the fermentations discussed above (Figure 5.5 and 5.6A - C). Thus the catabolism of sugars by FM16-7 and L-2056 was similar for the early part of fermentation, until a point between 25 and 30 hours after inoculation where the RWD occurred. The measured metabolites from samples used for RNA isolation and subsequent microarrays (Table 5.4) revealed that the mutant had catabolised 76.1 % of available sugar, as opposed to 50.7 % by the parent at the point of sampling (40 hours). Similar trends are observed in the production of ethanol and glycerol.

Where differences in transcript abundance were detected between FM16-7 and L-2056, the tendency was for abundance to be reduced (Table 5.5). Notably, there was a decrease in transcript of *GCR1,* a glycolysis transcriptional



Figures 5.7 Biomass accumulation (A), catabolism of total monosaccharides (B), and production of glycerol (C) and ethanol (D) by the mutant clonal isolate FM16-7 (△) and the parent strain L-2056 (●). Fermentations for microarray analysis were performed in CDGJM containing 200 g l^{-1} glucose / fructose. Data points are the mean of triplicate fermentations ± standard deviation.

Table 5.4 Key metabolites from RNA isolation during the FM16-7, L-2056 microarray analysis fermentations.

Metabolite (g l^{-1})	**L-2056**	**FM16-7**
Sugar (g l^{-1})	98.7 ± 4.2	47.9 ± 6.0
Glycerol (g l^{-1})	7.7 ± 0.2	9.6 ± 0.2
Ethanol (g l^{-1})	64.0 ± 0.4	85.9 ± 4.1

Values are the mean of 2 determinations ex 3 replicates ± standard deviation.

Table 5.5 Differentially expressed genes in FM16-7 during fermentation post-RWD.

Function	ORF (gene name)	Fold induction or repression	Gene or protein description
DNA metabolism	YFL001w (*DEG1*)*	-1.5	
	YBL003c (*HTA2*)	-1.6	
	YBL002w (*HTB2*)*	-1.7	
	YER104w (*RTT105*)*	-1.6	
	YPL283c (*YRF1-7*)*	-1.6	
	YDR316w-a*	-1.5	
	YGR161w-b*	-1.4	
	YER137c-a*	-1.4	
RNA metabolism	YLR129w (*DIP2*)*	1.6	
	YGL120c (*PRP43*)*	1.4	
	YLR222c (*UTP13*)*	1.8	
	YHR087w*	-1.5	
Carbohydrate metabolism	YPL075w (*GCR1*)*	-1.9	Glycolysis transcriptional activater
	YGL253w (*HXK2*)*	-1.6	Hexokinase isoenzyme
	YKL035w (*UGP1*)*	-1.4	UDP glucose phosphorylase
	YHR046c (*IMN1*)*	-1.8	Inositol monophosphatase
Generation of precursor metabolites	YMR083w (*ADH3*)	-1.7	Mitochondrial alcohol dehydrogenase
	YGL256w (*ADH4*)	-2.2	Mitochondrial alcohol dehydrogenase
	YCR005c (*CIT2*)*	-1.6	Citrate synthase
	YNL052w (*COX5A*)*	1.6	Subunit of cytochrome oxidase c
	YCL039w (*GID7*)*	-1.6	
	YJR009c (*TDH2*)*	-1.6	Glyceraldehyde 3-phosohate dehydrogenase
Lipid metabolism	YOL002c (*IZH2*)*	1.6	Membrane protein involved in zinc metabolism
	YBR183w (*YPC1*)*	-1.6	Alkaline ceramidase
Response to stress	YLL026w (*HSP104*)*	-2.6	Heat shock protein
	YMR175w (*SIP18*)*	1.8	Protein of unknown function induced by osmotic stress
	YER103w (*SSA4*)*	-2.2	Heat shock protein
	YGR211w (*ZPR1*)*	-1.9	Essential protein with 2 zinc fingers
Signal transduction	YNL255c (*GIS2*)*	1.6	Protein proposed to be involved with RAS / cAMP signalling

Table 5.5 (cont.)

Function	ORF (gene name)	Fold induction or repression	Gene or protein description
Protein biosynthesis	YBR199w (*KTR4*)	-1.4	Putative mannosyltransferase
	YEL054c (*RPL12A*)*	1.4	
	YPL220w (*RPL1A*)*	1.5	
	YJR094w-a (*RPL43B*)*	1.6	
	YIL069c (*RPS24B*)*	1.4	
	YNL178w (*RPS3*)*	1.5	
	YNL007c (*SIS1*)*	-1.7	
	YBR101c (*FES1*)*	-1.8	
Transport	YGR142w (*BTN2*)*	-1.8	Protein that modulates arginine uptake
	YMR255w (*GFD1*)*	-1.4	
	YHR092c (*HXT4*)*	1.3	Hexose transporter
	YNL142w (*MEP2*)	-1.5	Ammonium permease
	YNR053c (*NOG2*)*	1.5	Putative GTPase
	YOR273c (*TPO4*)*	-1.9	Polyamine transport protein
	YKR104w*	-1.5	
Other	YML022w (*APT1*)*	1.8	Adenine phosphoribosyltransferase
	YMR217w (*GUA1*)*	1.4	GMP synthase
	YHL032c (*GUT1*)*	1.5	Glycerol kinase
Unknown	YEL070w (*DSF1*)*	1.8	
	YNL036w (*MTQ1*)*	-1.4	
	YML056c (*IMD4*)	1.4	
	YHR179w (*OYE2*)*	-1.4	
	YER037w (*PHM8*)*	-1.6	
	YDL048c (*STP4*)*	-1.8	
	YLR162w*	-8.5	
	YMR253c*	-1.5	
	YNL024c*	-1.6	
	YER130c	-1.6	
	YBR116c*	1.5	

* denotes those genes with a Gcr1p promoter binding element.

activator, as well as a decrease in two of the alcohol dehydrogenases (*ADH3* and *ADH4*). A decrease was also noted in carbohydrate metabolism and DNA metabolism, as well as two genes associated with stress response. However the *GID7* transcript increased. A general trend of increased transcript in genes involved with protein biosynthesis was seen. In addition, interestingly, two genes encoding heat shock proteins and *MEP2* an ammonium permease were down regulated.

The expression data generated for the isolate FM16-7 shares several commonalities with the mixed culture FM16-M, compared to the parent, that is the decrease in transcript associated with *ADH4* is also seen in the mixed culture post-RWD. The up-regulation of *SIP18* and *IZH2*, and the down-regulation of the gene encoding hexokinase, *HXK2*, pre-RWD. Contrasting this, an increase in the transcript associated with *HXT4* is noted in the clone, however in the mixed culture, a decrease was observed pre-RWD. It is important to reiterate that these sample points were at different times during fermentation, consequently it is possible that a different list of genes has been generated, since the medium composition is likely to have differed. The commonality of *HXT* genes across the microarrays conducted suggests the importance of this group of genes to the phenotype of the adaptively evolved mutants.

5.2.5 Sequencing of the GCR1 promoter

Searches were again conducted using the Saccharomyces Promoter Database (Zho and Zhang, 1999), yielding a high prevalence of Gcr1p transcriptional activator sequences in the promoter region of genes found differentially expressed, sequencing of the *GCR1* promoter was undertaken to further explore the possible involvement of a mutation in the observed phenotypes (with the assistance of Dr. Jennie Gardner). Four base pair changes were observed, and G → A (-55), C → T (-275), G → A (-336) and A → G (-829) (L-2056 → FM16-7) (Figure 5.8). Notably none of these mutations changed any currently recognisable transcription factor binding sites.

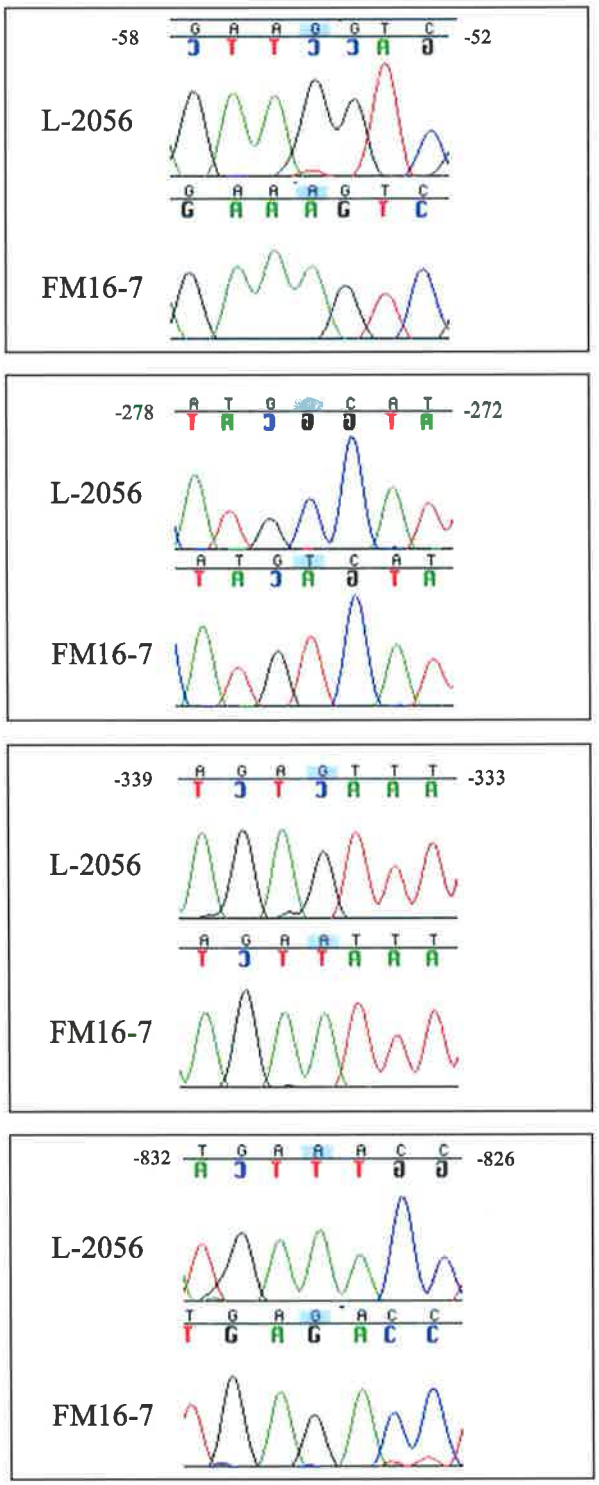


Figure 5.8 Sequencing chromatograms of the *GCR1* promoter show the four base pair mutations. Change in sequence denoted by highlighted base.

5.2.6 Phenotype microarray analysis of FM16-7 and L-2056

To gain further insight into the phenotypic changes of FM16-7, a phenotype microarray was conducted. Phenotype microarrays (PMs) are an assay of approximately 2000 culture traits, including tests for the main catabolic pathways for carbon, nitrogen and phosphorus utilisation. There are also assessments of the sensitivity of the cells to various osmolytes and pH values. The remaining assays test the sensitivity of cells to a wide range of chemicals, including antibiotics, antimetabolites, membrane-active agents, respiratory inhibitors and toxic metals. Each individual reaction is monitored by the reduction of a tetrazolium dye, due to the occurrence of cellular respiration (Bochner, 1989).

The PMs were conducted by Dr Michael Ziman at Biolog Inc, California, USA. Cell suspensions of either strain were inoculated into PM plates, before incubation for 48 hours (30°C) in the OmniLog — an instrument that functions as an incubator and a spectrophotometer. The OmniLog cycles each PM plate in front of an imaging head every 15 minutes, measuring and recording the reduction of tetrazolium. Kinetic plots are generated for each well and the reference and experimental populations are plotted in red and green respectively. The differential phenotypes are highlighted by overlaying the graphs, and as an example where no change is apparent between the cell types, graphs are coloured in yellow.

No differences were found in carbon and nitrogen metabolism, nutrient stimulation, osmotic sensitivity and pH growth and control. FM16-7 was however found to respire less when in the presence of adenosine-2',3' cyclic monophosphate, dithiophosphate, cytidine-2',3'-cyclic monophosphate and thymidine 3',5'-cyclic monophosphate. FM16-7 was also found to have increased respiration in comparison to L-2056 when in the presence of potassium chromate.

5.3 Conclusions

a) FM5-M and FM16-M are heterogenous cultures.
b) 9 out of 10 clonal cultures of FM5-M and FM16-M demonstrate similar phenotypes to the originating mixed cultures.
c) Strains FM16-5 and FM16-7 demonstrated superior fermentation attributes to both L-2056 and FM16-M, with regard to fermentation duration and glycerol production.
d) The *HXT* gene set may be of importance to the phenotype of FM16- cultures.
e) A transcriptional activator of glycolysis (*GCR1*) is down-regulated in FM16-7.
f) Sequencing of the *GCR1* promoter revealed 4 base pair changes in FM16-7 compared to parent L-2056.
g) Phenotype arrays yielded differences between FM16-7 and L-2056 in phosphate metabolism and chromate sensitivity.

5.4 Discussion

Comparison of the evolved clonal and mixed cultures yielded fermentation data similar to previously reported findings, with one exception. Over time and numerous replications of experiments it became evident that FM5-M was no longer able to complete fermentation within a shorter duration than the parent C911D. This is suggested to be due to reversion of the evolved trait, perhaps because the purported causal genetic change had not become fixed in the population (Galitski and Roth, 1996). If the putative mutation conferring the decreased fermentation duration was one that was highly transcribed, this in itself might have contributed to reversion. Studies of induced DNA damage and repair have indicated that the transcribed strand of an active gene is targeted for preferential repair (Drapkin *et al.*, 1994; Hanawalt *et al.*, 1994; Datta and Jinks-Robertson, 1995).

A comparison of metabolite data collected from mixed and clonal cultures revealed general similarity to that of previously reported findings (Chapters 3 and 4). FM5 mixed and clonal cultures demonstrated elevated glycerol

production, decreased ethanol production and an increased biomass. FM16 mixed and clonal cultures also had elevated glycerol and decreased fermentation duration compared to the parent L-2056. Both sets of mutants also produced elevated concentrations of acetic acid. The range of concentrations evident in the clonal cultures clearly highlights the heterogeneity in both of the mixed evolved cultures. Further iterated by the detection of 1 of 10 clonal cultures from each of the mixed cultures that was unable to finish fermentation. Their exclusion from the tabulated data was essentially to allow comparison of data from completed fermentations only. It is however still possible that in the context of the mixed culture, these poorly-fermenting clones still make a marked contribution to the overall phenotype.

Confirmation of the retention of desirable phenotypes from the initial clonal analysis was sought. Thus two strains, FM16-5 and FM16-7, were subsequently chosen and analysed in replicated experiments. Both of these strains had phenotypes similar to, or improved upon, that of the mixed culture. Importantly clonal cultures were able to complete fermentation under the imposed conditions whereas L-2056 was unable. Further to this, these strains produced increased concentrations of glycerol, *ca.* 4.1 % more than previously reported for the mixed culture and 33 % more than the parent (completed fermentation).

Glycerol, as mentioned previously, is an important metabolite produced during fermentation for numerous reasons. It is also of special interest from an oenological point of view because of its associated effects upon mouthfeel and perceivable sweetness. Metabolically, glycerol production serves as a redox valve to dispose of excess reduced equivalents (NADH) (Lagunas and Gancedo, 1973; van Dijken and Scheffers, 1986), in brief, to maintain cytosolic redox equilibrium conducive to sustaining glycolytic catabolism (van Dijken *et al.*, 1986; Albers *et al.*, 1996). Glycerol is formed by the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate concomitant with NADH oxidation and NAD^+-dependent glycerol 3-dehydrogenases (Albertyn *et al.*, 1992; Cronwright *et al.*, 2002). The NAD^+-dependent glycerol

3-dehydrogenase is encoded for by two isogenes *GPD1* and *GPD2*, of which *GPD2* has found to be subject to anaerobic induction (Albertyn *et al.*, 1994; Ericksson *et al.*, 1995). Expression of *GPD1* increases in cells exposed to raised external osmolarity, which in turn causes an increased production and intracellular accumulation of glycerol (Andre *et al.*, 1991; Albertyn *et al.*, 1994; Ericksson *et al.*, 1995). This osmoregulatory response serves to counter the dehydration and loss of cell volume that is experienced by cells subject to increased osmotic stress. The osmotic control of *GPD1* expression is possibly executed via a osmosensing signal transduction cascade, called the HOG (High Osmolarity Glycerol) pathway, involving homologues of mitogen-activated protein (MAP) kinases and of a bacterial phosphorelay system (Albertyn *et al.*, 1994; Maeda *et al.*, 1994; Posas *et al.*, 1996). Gpp1p and Gpp2p (glycerol 3-phosphatases) are subsequently responsible for the dephosphorylation of glycerol 3-phospate to glycerol. These isoenzymes work in conjunction with Gpd1p and Gpd2p, where they have different physiological roles: the Gpd1p-Gpp2p combination is mainly involved with glycerol production during osmotic stress, whereas the Gpd2p-Gpp1p combination is primarily involved in adjusting the NADH:NAD redox balance under anaerobic conditions (Albertyn *et al.*, 1994; Ansell *et al.*, 1997; Pahlman *et al.*, 2001). The over-expression of either the *GPD1* or *GPD2* gene in *S. cerevisiae* has been proven by many groups to increase the production of glycerol (Michnick *et al.*, 1997; Remize *et al.*, 1999; de Barros Lopes *et al.*, 2000). No significant differences in the expression of these genes were found in the studies conducted here, however it would be worthwhile to further investigate the expression of these genes throughout the course of fermentation.

Under anaerobic conditions, alike to oenological conditions, the increase in glycerol concentration in FM5 and FM16 mixed and clonal cultures must reflect a change in the redox status of these cultures, and potentially a greater excess of reduced equivalents like NADH. This could be explained by an up-regulation of the gene *GPD2*, conversely a down-regulation in *GPD1* due to greater osmotic tolerance, with a subsequent balance in transcription resultant in *GPD2*, due to coordinate regulation and given the isoforms have similar

affinity for their substrates (Ansell *et al.*, 1997). Further to this, it is feasible that the maintenance of elevated rates of sugar catabolism during stationary phase evident in FM16-M, is due to the elevated concentration of glycerol compared to parent strains and its role as a compatible solute (Remize *et al.*, 1999).

The increased acetic acid concentrations have been discussed with regard to the industrial perspective. Metabolically, yeast produce acetate as an intermediate of the pyruvate dehydrogenase (PDH) bypass, which converts pyruvate into acetyl-CoA in a series of reactions catalysed by pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ACDH) and acetyl-CoA synthase (ACS) (Saint-Prix *et al.*, 2004). It has been theorised that a physiological role of acetate may be the regeneration of reducing equivalents (NADH and NADPH) for maintaining the redox balance (Remize *et al.*, 2000). It could be assumed that cytosolic Ald6p, or the mitochondrial Ald5p ACDH isoform, (Navarro-Avino *et al.*, 1999), could be responsible for the elevated production of acetic acid in the evolved strains, as it is proposed that these enzymes contribute to acetate formation during fermentation (Wang *et al.*, 1998). Further to this, disruption of *ALD6*, could result in the over-expression of *ALD4*, and consequently elevated acetic acid concentrations (Saint-Prix *et al.*, 2004). It could be suggested that most acetaldehyde produced is being shuttled to ethanol production via alcohol dehydrogenase (Lutstorf and Megnet, 1968; Beier *et al.*, 1985) or acetic acid production via acetaldehyde dehydrogenases. However there appears to be limited variation in the acetaldehyde produced by these strains, and thus this route for formation might be considered negligible, especially given the concentrations of acetaldehyde reported in wine range between 4 and 500 mg l^{-1} (Liu and Pilone, 2000). An increase in transcripts of *ALD4* and *ALD6* were noted in FM16-7, however associated p values were *ca.* 0.1 and thus fell outside of our stringent cut off for significance.

The disparity in succinic acid concentrations is difficult to explain, due to the large variation observed over the multitude of fermentations. However, during fermentation, the TCA pathway operates in a branched fashion and it is thought

that most succinic acid is formed by the reductive branch of the TCA pathway (Camarasa *et al.*, 2003). An alternative route of succinate production arises when glutamate is present in high concentrations. In this case, glutamate is catabolised to 2-oxoglutarate, which in turn is oxidatively decarboxylated to succinic acid via acetyl-CoA (Albers *et al.*, 1998). However in these experiments, ammonia was the sole nitrogen source, thus glutamate is actually synthesised with the aid of products of the retrograde genes (The retrograde response) together with early parts of the oxidative branch of the TCA cycle (Tate and Cooper, 2003). Finally, succinic acid can also be formed from the GABA (4-amino butyrate) degradation pathway (Coleman *et al.*, 2001). Again dependent on glutamate, GABA can be produced from glutamate by a glutamate decarboxylase (Gad1p), followed by transamination by the GABA transaminase (Uga1p) to form succinate semialdehyde and then converted by succinate semialdehyde dehydrogenase (Uga5p) to produce succinate. The significance of this pathway during fermentation is unclear. Interestingly *GAD1* is required for tolerance of normal oxidative stress (Coleman *et al.*, 2001), one of the stressors that were applied during the evolution process in this project. Consequently the mechanisms resulting in increased organic acid production could also be responsible for the elevated production of glycerol in stains FM5-M and FM16-M, through the net production of NADH and compensation through increased glycerol production.

Transcriptome data was generated for the single clone FM16-7 compared to L-2056, yielding a short list of differentially expressed genes. The time point chosen, approximately ten hours post-RWD, differs to that of previous analysis. This point was chosen to gain a greater insight to the transcriptional response that could be attributed to the fermentation phenotype observed in FM16-7. The general trend amongst highlighted transcripts was one of a decrease, thus 66 % of such transcripts are seen to be down-regulated in FM16-7.

A commonality with previous arrays was noted, that being the prevalence amongst genes identified (90 %) possessing a Gcr1p transcriptional activator

sequence (CTTCC/CATCC motif (Baker, 1991; Huie *et al.*, 1992)) in their promoter regions (Zho and Zhang, 1999). The *GCR1* gene encodes a positive transcription factor, which is a regulator of glycolysis and is involved in the coordinate expression of glycolytic genes (Holland *et al.*, 1987). The gene product, Gcr1p, has a DNA-binding domain with specificity to CT-box sequences (Huie *et al.*, 1992). Sasaki *et al.* (2005) found that expression of *GCR1* itself was dependent on Gcr1p, and furthermore that decreased transcriptional activity was exhibited when a CT box was deleted from the promoter. This information allows the proposal of a possible mechanism for the decrease in transcript seen in *GCR1*, and inturn genes regulated by its gene product. That is, that over the evolutionary period the four mutations that have occurred in the promoter of *GCR1* (in FM16-7) has lead to altered formation of Gcr1p. The subsequent effect on genes with Gcr1p upstream activating sequences (UASs) has been revealed through cDNA microarray analyses by other groups and which resulted in very similar data to that generated here. For instance, Sasaki and Uemura (2005) showed that when a *gcr1* mutant was grown on glucose an increase in the transcript of the hexose transporter Hxt4p and a decrease in *TDH2* expression is observed. However, a reduced expression of glycolytic genes suggesting a decrease in glycolytic flux is also reputed (Holland *et al.*, 1987; Uemura and Fraenkel, 1999). We suggest that the observed decrease in *GCR1* transcript could be associated with the deregulation of genes considered as non-essential during this stage of glycolysis and therefore allowing for the diversion of energy conferring greater sugar catabolic rates or heightened glycerol production.

TDH2, also down-regulated in FM16-7, encodes a glyceraldehyde 3-phosphate dehydrogenase (McAlister and Holland, 1985a; McAlister and Holland, 1985b), which catalyses the formation of 1,3-diphosphoglycerate from glyceraldehyde 3-phosphate (Boucherie *et al.*, 1995). This down-regulation could be attributed to the change in *GCR1* transcription or promoter sequence. Supporting this, Sasaki and Uemura (2005) found a decrease in *TDH* expression in a null mutant of *gcr1*. A decrease in the activity of Tdh2p could result in elevated concentrations of glycerol as seen in FM16-7, as less carbon

channelled into pyruvate production through 1,3 bisphosphoglycerate could result in more carbon channelled into dihydroxyacetone and subsequently glycerol production.

As seen with the mixed culture (and discussed in Chapter 4) an alteration in hexose transporters is also seen in FM16-7, namely Hxt4p. At this time point the transcript of *HXT4* was shown to be marginally increased (x 1.3). Hxt4p is a transporter of intermediate affinity and is activated by low levels of glucose (Theodoris *et al.*, 1994; Wendel and Bisson, 1994). An increase in its activity may at least in part explain the sustained sugar catabolism seen by FM16-7. Potentially the evolved cells might be able to transport glucose more efficiently, consequently enabling a faster fermentation. However given the modest change in *HXT4* expression at this time point, it would be interesting to determine whether any changes in this transcript during the later parts of fermentation are more dramatic.

IZH2 is regulated at the level of transcription by fatty acids, suggesting a role in lipid metabolism. That is, expression is highly induced in cells grown in the presence of medium chain length fatty acids (Karpichev *et al.*, 2002). Preliminary phenotypic analysis shows that *izh2* mutants are resistant to the sterol-binding antibiotic, nystatin (Karpichev *et al.*, 2002), suggesting that Izh2p has a role in maintaining the sterol composition of the yeast cell membrane (Lyons *et al.*, 2004). This combined with the fact that some vertebrate orthologs function as receptors for steroids (which are structurally related to sterols), identifies ergosterol metabolism as a likely biochemical pathway in which to categorise the Izh proteins (Lyons *et al.*, 2004). Consequently an alteration in the Izh2p function in FM16-7 mutant, seen as an increase in *IZH2* transcript, may result in an amelioration of the restricted sterol biosynthesis and perhaps sub-optimal membrane structure which occurs under anaerobic conditions. Such changes might therefore enable the cell to cope better with the stressful environment of fermentation, particularly that in which ethanol is prevalent.

Mechanisms for the putative involvement of *ADH3* and *ADH4* in the observed phenotype in FM16-7 have already been discussed in Chapter 4.

Previous studies in this laboratory show that deletion of *GID7* from a laboratory strain of *S. cerevisiae* increased the accumulation of glycerol during fermentation (Gardner *et al.*, 2005). Furthermore under conditions of low nitrogen, *gid7* mutants were better able to catabolise all available sugar during fermentation. In this study, transcript of *GID7* was found to be less abundant in FM16-7. *GID7* is one of several *GID* genes, known to be involved in catabolite degradation of fructose 1,6-bisphosphatase (Regelmann *et al.*, 2003). Systematic searches for protein interactions have highlighted putative interactions of Gid7p with other Gid proteins (Ho *et al.*, 2002), but also with the high affinity hexose transporter *HXT7*. This transporter is important at the end of fermentation for the maintenance of hexose transport (Luyten *et al.*, 2002). Perhaps, the decreased expression of *GID7* prevents deactivation of Hxt7p, similar to its action on fructose 1,6-bisphosphate, thus enabling the mutants ability to maintain glucose catabolic activity under the conditions used.

SSA4 is a 70-kilodalton heat shock protein (Boorstein and Craig, 1990) which is rapidly induced in response to stress (Werner-Washburne *et al.*, 1987). Ssa4p is exported from the nuclei under conditions of ethanol stress blocking the export of poly(A)$^+$ RNA (Saavedra *et al.*, 1996; Izawa *et al.*, 2005). The down-regulation of *SSA4* in FM16-7 could suggest that the cells are in fact less affected by ethanol toxicity. Thus, FM16-7 may have an increased tolerance to ethanol, allowing greater export of poly(A)$^+$ RNA, and thus increased or maintained protein production. If this is in fact the case for sugar transporters such as the *HXT* family during the later stages of wine fermentation, an increased or sustained fermentation might be permitted. Hsp104p is another stress tolerance factor which promotes the reactivation of damaged proteins under stress, such as that imposed by increasing ethanol (Sanchez *et al.*, 1992). Further to this, the *SSA* encoded Hsp70p chaperone system plays a critical, dual role in Hsp104p-mediated refolding of proteins (Glover and Lindquist, 1998).

The down-regulation observed in *HSP104* also suggests that FM16-7 may have a greater tolerance to ethanol. Contrasting this is the increase in transcript of *SIP18*, thought to be involved in desiccation and osmotic stress (Zuzuarregui *et al.*, 2006).

MEP2 encodes a high-affinity ammonium ion transporter that has been suggested to also be a sensor of ammonia (Marini *et al.*, 1997; Lorenz and Heitman, 1998). It has also been reported to be important for amino acid transport during fermentation, and is found to be repressed in nitrogen rich media by nitrogen catabolite repression (Bely *et al.*, 1990a). Powers *et al.* (2006) found that *mep2* mutants had a greater ability to withstand heat shock, thought to be attributed to a concurrent increased accumulation of glycogen. The observed decrease in *MEP2* transcript in FM16-7 suggests that there may be a modulation in nutrient sensing and this may reflect the fact that the cells have become somewhat desensitised to decreasing nutrients and potentially increasing stress, thereby allowing prolonged maintenance of fermentation.

The basis for the increase in *GUT1* transcript is difficult to elucidate, since glycerol utilisation proceeds via glycerol kinase (encoded by *GUT1*), catalysing the formation of glycerol 3-phosphate from glycerol (Pavlik *et al.*, 1993; Costenoble *et al.*, 2000). However it is worthwhile to mention that not all alterations in the transcripts abundance will necessarily have a strong impact in the observed phenotype.

Phenotypic array analysis revealed that FM16-7 has a decreased sensitivity to potassium chromate. Chromate [Cr(VI)] ions have been shown to have serious mutagenic, cytotoxic and carcinogenic effects on all yeast cells (Cohen *et al.*, 1993; Gazdag *et al.*, 2003). Cr(VI) ions are taken up by cells by facilitated diffusion through a non-specific permease anion carrier system, which transports a number of anions such as SO_4^{2-} and PO_4^{3-} (Gazdag *et al.*, 2003). Cr(VI) ions entering the cells are reduced to Cr(III) via the unstable Cr(V) and Cr(IV) oxidation states. *In vitro* experiments have demonstrated that the reactive intermediates Cr(V) and Cr(IV) generate harmful reactive oxygen

species (ROS) (Shi and Dalal, 1990a; Liu *et al.*, 1997). Glutathione offers an important antioxidative defense (Jamieson, 1998; Costa and Moradas-Ferreira, 2001), and coordinately glutathione reductase (GR) reduces Cr(VI) directly, using NADPH (Shi and Dalal, 1992; Pesti *et al.*, 2002). It is feasible that the decreased sensitivity to potassium chromate by FM16-7 is a function of reduced transport of chromate into the cell. Alternatively, given the exposure to an oxidative environment during the selection process, it is feasible that this is an adapted oxidative protective mechanism, as a function of the GR/NADPH system (Pesti *et al.*, 2002).

FM16-7 was also found to have decreased respiration when cyclic monophosphates were present, presumably reflecting that this strain is unable to utilise these compounds as a phosphorous source. Putatively the decrease observed in phosphate metabolism could be linked with the reduced sensitivity to potassium chromate of FM16-7. This could be a function of decreased phosphate transporters, thus a decrease in the concentration of chromate transported into the cell, and consequently less oxidative damage to cells.

We have confirmed that both FM5-M and FM16-M are heterogenous cultures, with clonal cultures isolated from either generally displaying similar phenotypes to that of the mixed cultures. Further investigation into two clones derived from FM16-M and considered to be superior, also show marked increase in glycerol production and decreased fermentation duration. The post-RWD transcriptional characterisation of FM16-7 has allowed proposal of putative mechanisms by which to explain the observed fermentation phenotype of FM16-7. Given the widespread occurrence amongst highlighted genes of CT-box sequences, to which the glycolytic transcriptional regulator, Gcr1p, may bind, it is suggested that *GCR1* may be important in the improved phenotype of the evolved clones. The mechanism may be one involving the deregulation of non-essential genes, thereby saving energy, possibly due to mutations in the *GCR1* promoter.

Chapter 6 Determination of the contribution of a sub-set of genes to the desirable adaptive evolution phenotype of FM5 and FM16

6.1 Introduction

We wished to further elucidate the putative genotypic differences between the adaptively evolved cultures and their parents, thought to have occurred as a function of the adaptive evolution process. To do this we chose a subset of genes which were found to be differentially expressed (selected from the microarray data generated (Chapter 4)) and were more obvious candidates to confer the improved fermentation phenotypes of FM5-M, FM16-M and FM16-7. These genes were then manipulated in the appropriate background (C911D or L-2056), that is they were either deleted by replacement of the open reading frame with the *kanMX* cassette, or else were over-expressed by insertion of the constitutive *TDH3* promoter in front of the native open reading frame (ORF). This allowed the impact of each gene modification on fermentation performance to be monitored, with particular focus on fermentation duration and glycerol and ethanol yields.

This chapter describes the identification of several genes that influence the fermentation dynamics of C911D and L-2056 in a similar way to the changes arising out of the adaptive evolution process. Deletion of *HXT1*, *HXT2*, *HXT3*, *GLC3*, *ALD6* and *INO1* from C911D yielded fermentation phenotypes similar to that of FM5-M. Likewise, deletion of *ALD6*, *GPH1* and *RCK1* yielded fermentation phenotypes similar to that of FM16-M and FM16-7. The single clone FM16-7, derived from FM16-M was also included for comparative purposes, as it was highlighted as a potentially significant clone for commercial application.

6.2 Results

Fermentations were conducted as described in Chapter 3. That is, triplicate CDGJM (100 ml) cultures conducted at 30°C.

6.2.1 C911D deletion strains

The entire open reading frames of *HXT1*, *HXT2*, *HXT3*, *GDH2*, *GLC3*, *ALD6*, *INO1* and *HAP4* were disrupted in C911D, using the *kanMX* gene replacement strategy (Wach, 1996). We took advantage of the readily available yeast deletion strains from the Yeast Deletion Project (http://wwwsequence.stanford.edu/group/yeast_deletion_project/deletions3.html). PCR products corresponding to the *kanMX4* module flanked by approx. 200 – 500 bases of the gene of interest were amplified from genomic DNA purified from the corresponding yeast deletion mutants (Table 2.1), with primers *GENE*A and *GENE*D (Table 2.2). C911D was transformed with these PCR products and transformants were selected on YEPD-geneticin (400 μg ml^{-1}) (Gietz *et al.*, 1992).

Deletion of each ORF was confirmed by Southern blot hybridisation with a probe specific for *kanMX4* (data not shown). The *kanMX4* probe was PCR amplified from pFA6-lacKanMX4 (Wach *et al.*, 1994). The probe was generated using DNA from C911D and primers G418F and G418R (Table 2.2). 50 μM DIG labelled dNTPs (Roche cat # 1277065) were also included in the PCR reaction.

6.2.2 Analysis of the fermentation duration, major metabolites and biomass produced of the C911D geneΔ strains

No strains constructed in C911D with gene deletions were found to have a reduced fermentation duration. However, disruption of three of the seven genes in C911D were found to increase fermentation duration as evident in *glc3* (65 hours), *ald6* (81 hours) and *hxt3* (81 hours), contrasting that of C911D (62 hours) (Table 6.1).

There were a number of alterations in metabolites accumulated at the end of fermentation. Four strains had significantly altered succinic acid accumulation (C911D *hxt3*, C911D *hxt2*, C911D *gdh2* and C911D *glc3*), the last three of these being significantly less than the parent. The lactic acid produced by

Table 6.1 Dry weight, fermentation duration and major metabolites present in terminal fermentation samples of C9 deletion strains.

	Duration	Succinic Acid	Lactic acid	Glycerol	Acetic acid	Acetaldehyde	Ethanol	Dry weight
C911D	62.0 ± 2.0 hr	0.73 ± 0.01 g l^{-1}	0.34 ± 0.01 g l^{-1}	7.09 ± 0.08 g l^{-1}	1.39 ± 0.03 g l^{-1}	0.03 ± 0.00 g l^{-1}	102.4 ± 1.2 g l^{-1}	6.2 ± 0.2 g l^{-1}
C911D *hxt1*	100 ± 2.0	95.6 ± 0.1	105.8 ± 0.1	106.5 ± 0.1	106.7 ± 0.1	120 ± 0.1	91.2 ± 4.3	104.6 ± 0.2
C911D *hxt2*	100 ± 2.0	90.5 ± 0.1	96.7 ± 0.2	104.3 ± 0.1	105.7 ± 0.1	130 ± 0.1	92.6 ± 3.9	114.5 ± 0.5
C911D *hxt3*	130.6 ± 2.0	140.7 ± 0.1	179.6 ± 0.1	106.1 ± 0.1	86.8 ± 0.1	60 ± 0.1	94.2 ± 1.4	105.4 ± 01.3
C911D *gdh2*	100 ± 2.0	90.3 ± 0.1	105.3 ± 0.1	102.4 ± 0.1	104.4 ± 0.1	120 ± 0.1	91.5 ± 4.8	111.7 ± 0.3
C911D *glc3*	104.8 ± 2.0	87.7 ± 0.1	104.0 ± 0.1	102.8 ± 0.1	101.6 ± 0.1	105 ± 0.1	88.7 ± 0.2	102.9 ± 0.1
C911D *ald6*	130.6 ± 2.0	114.4 ± 0.1	17.1 ± 0.1	116.8 ± 0.1	20.8 ± 0.1	200 ± 0.1	94.0 ± 2.0	102.4 ± 0.5
C911D *ino1*	100 ± 2.0	113.9 ± 0.1	105.5 ± 0.1	106.3 ± 0.1	102.2 ± 0.1	120 ± 0.1	91.6 ± 4.5	107.2 ± 0.1
C911D *hap4*	100 ± 2.0	95.2 ± 0.1	98.9 ± 0.1	104.2 ± 0.1	98.8 ± 0.1	120 ± 0.1	86.8 ± 4.2	106.5 ± 0.3
FM5-M	100 ± 2.0	97.4 ± 0.1	100 ± 0.1	110.3 ± 0.1	109.1± 0.1	120 ± 0.1	86.5± 0.8	108.6 ± 0.1

Values are the mean of triplicate experiments and are shown as a percentage of the parent (C911D) strain ± standard deviation. Absolute values for C911D are shown. Values which were increased or decreased by > 5 % and were also significantly different to the parent, i.e. $p < 0.05$ (Student's t-test) are highlighted by yellow or red respectively.

C911D *hxt3* was almost double that of the parent (0.61 *vs.* 0.34 g l^{-1}), contrasting to this C911D *ald6* only produced 17 % of the lactic acid of that produced by C911D. Four strains had increased glycerol production (C911D *hxt1*, C911D *hxt3*, C911D *ald6* and C911D *ino1*), of greatest note, deletion of *ALD6* resulted in 1.1 g l^{-1} (17 %) more glycerol than that of C911D.

The yield of acetic acid was significantly reduced in C911D *ald6* and C911D *hxt3*, while it increased in C911D *hxt1*. With regard to acetaldehyde production, again significant variation was seen in C911D *ald6* as well as C911D *hxt3*, with 200 % and 60 % of production noted in C911D, respectively (although these concentrations are probably at the detection limit for this method).

The final concentration of ethanol in terminal samples was found to be significantly less (as low as 88.7 % of the parent) in five of the strains (C911D *hxt2*, C911D *hxt3*, C911D *glc3*, C911D *ald6* and C911D *ino1*). Finally, C911D *hxt2*, C911D *ino1* and C911D *gdh2* had increased dry cell weight at the end of fermentation (7.1, 6.7 and 6.9 mg ml^{-1} respectively *vs.* 6.2 mg ml^{-1}).

6.2.3 Construction of C911D over-expression strains

C911D over-expression strains were constructed based on the self cloning gene expression system, with a two step gene replacement (Hirosawa *et al.*, 2004). In brief, a 0.7 kb DNA fragment of the *TDH3* promoter was amplified using C911D genomic DNA and primers TDH3-1 through TDH3-5 (depending on restriction sites of gene and upstream sequences (Table 2.3)), digested with the appropriate restriction enzymes and inserted into pGG119 to form pCMTDH3A→E (Table 2.4). Fragments of the gene of interest were also amplified from C911D gDNA, with the corresponding primers (Table 2.3), e.g:

ADR1 (+ 1 → + 1273) with ADR1-NCO and ADR1-XMA

ADH2 (+1 → + 987) with ADH2-NCO and ADH2-XBA

VID24 (+ 1 → + 815) with VID24-NCO and VID24-XMA.

The *ADR1* and *ADH2* fragments were digested with *Nco*I and *Xba*I for insertion into the corresponding sites in pCMTDH3A and pCMTDH3B respectively, the *VID24* fragment was digested with *Nco*I and *Xma*I and inserted into the corresponding sites in pCMTDH3E. All genes were located downstream of the promoter. The 5' upstream regions of each gene were also amplified using the following primer configurations (Table 2.3):

ADR1-UPEAG and ADR1-UCAAT (-1981 → -77)
ADH2-UPEAG and ADH2-UCAVR (-1943 → -69)
VID24-SPE VID24-AAT (-1885 → -9).

Upstream fragments and corresponding plasmids were digested with the appropriate restriction enzymes before ligation (Table 2.4). Finally plasmids were restriction digested (see Table 2.4 for appropriate enzymes) before transformation into C911D (Gietz *et al.*, 1992). The transformation mixture was spread on minimal plates containing 1.0 μg ml^{-1} cerulenin. The cerulenin-resistant colonies were streaked on minimal plates containing 0.5 μg ml^{-1} cycloheximide as a double-check (Akada *et al.*, 2002). For counter-selection, transformants were grown at 28°C for 1 day in liquid YPGal medium and then cells were spread on YPGal plates at approximately 100 cells/plate. Colonies that grew on YPGal plates after 36–48 hours incubation at 28°C were selected. Confirmation of integration of the correct product was confirmed by sequencing (data not shown). Several other plasmids constructs were attempted, including those for genes *HSP10*, *SIP18*, *AHP1* and *HXT5*, however these were unsuccessful and due to time limitations were not completed.

6.2.4 *Analysis of the fermentation duration, major metabolites and biomass produced of the C911D over-expression strains*

There were no clear associations between the phenotypes observed of over-expression strains constructed to that of FM5-M, with the exception of the marginal increase in acetic acid concentration in C911D $vid24^{UP}$ (Table 6.2). An increase in lactic acid concentration was noted in C911D $vid24^{UP}$ and C911D $adr1^{UP}$, however this contrasts to that of the phenotype of FM5-M,

Table 6.2 Dry weight, fermentation duration and major metabolites present in terminal fermentation samples of C911D over-expression strains.

	Duration	Succinic Acid	Lactic acid	Glycerol	Acetic acid	Acetaldehyde	Ethanol	Dry weight
C911D	79.0 ± 3.5 hrs	0.36 ± 0.01 g l^{-1}	0.37 ± 0.01 g l^{-1}	7.66 ± 0.36 g l^{-1}	1.67 ± 0.02 g l^{-1}	0.05 ± 0.00 g l^{-1}	104.4 ± 0.6 g l^{-1}	5.27 ± 0.31 g l^{-1}
C911D adh2^{UP}	100.0 ± 2.0	108.3 ± 0.1	112.8 ± 0.1	103.9 ± 0.1	102 ± 0.1	96.7 ± 0.1	102.9 ± 6.2	105.5 ± 0.2
C911D adr1^{UP}	105.5 ± 2.0	93.8 ± 0.1	133.6 ± 0.1	105.4 ± 0.1	103.8 ± 0.1	100 ± 0.1	102.0 ± 4.1	89.1 ± 0.4
C911D vid24^{UP}	116.5 ± 2.0	89.8 ± 0.1	111.9 ± 0.1	105.4 ± 0.1	105.9 ± 0.1	90.9 ± 0.1	100.2 ± 7.1	96.2 ± 0.3
FM5-M	95.0 ± 2.0	98.6 ± 0.1	114.0 ± 0.1	107.1 ± 0.1	108.7 ± 0.1	97.0 ± 0.1	93.7 ± 0.6	112.8 ± 0.1

Values are the mean of triplicate experiments and are shown as a percentage of the parent (C911D) strain ± standard deviation. Absolute values for C911D are shown. Values which were increased or decreased by > 5 % and were also significantly different to the parent, i.e. $p < 0.05$ (Student's t-test) are highlighted by yellow or red respectively.

where no difference in production has been found. There was also a significant increase in fermentation duration observed in C911D vid24^{UP} - this too is dissimilar to reported phenotype of FM5-M.

6.2.5 Construction of L-2056 knock-out strains

Heterozygous deletions were constructed using the same protocol for haploid gene replacement. After gene replacement of a single gene copy, strains were sporulated, dissected and allowed to rediploidise. Homozygous diploid deletion strains were then identified by southern blot analysis (data not shown), as previously mentioned.

6.2.6 Analysis of the fermentation duration, major metabolites and biomass of the L-2056 geneΔ strains

All three homozygous deletion strains (L-2056 *ald6*, L-2056 *gph1* and L-2056 *rck1*) were able to complete fermentation within a shorter duration than the parent (the fastest being 61 % of the duration of L-2056). The glycerol concentrations at the end of fermentation by L-2056 *ald6* and L-2056 *gph1* were increased by 1.1 g l^{-1} and 1.8 g l^{-1} (13.2 % and 21.8 %) respectively compared to the parent L-2056, whereas deletion of *RCK1* decreased glycerol accumulation. The other significant finding was the dramatic increase in acetaldehyde in *ald6* and *gph1* strains (Table 6.3).

6.3 Conclusions

a) Deletion of either *HXT1*, *HXT2*, *HXT3*, *GLC3*, *GDH2*, *ALD6* and *INO1* from C911D resulted in fermentation phenotypes similar to that of FM5-M.
b) Deletion of either *ALD6*, *GPH1*, *RCK1* from L-2056 resulted in fermentation phenotypes similar to that of FM16-M and FM16-7.
c) No single deletion resulted in all of the desirable fermentation characteristics of either FM5-M or FM16.

Table 6.3 Dry weight, fermentation duration and major metabolites concentrations present in terminal fermentation samples of L-2056 homozygous deletion strains.

	Duration	Succinic Acid	Lactic acid	Glycerol	Acetic acid	Acetaldehyde	Ethanol	Dry weight
L-2056	99.0 ± 2.0 hrs	0.42 ± 0.05 g l^{-1}	0.22 ± 0.03 g l^{-1}	8.37 ± 0.49 g l^{-1}	1.64 ± 0.05 g l^{-1}	0.05 ± 0.00 g l^{-1}	97.0 ± 4.8 g l^{-1}	4.41 ± 0.35 g l^{-1}
L-2056 *ald6*	60.6 ± 2.0	78.2 ± 0.1	105.3 ± 0.1	113.2 ± 0.1	102.6 ± 0.1	207.4 ± 0.1	107.3 ± 8.3	98.4 ± 0.1
L-2056 *gph1*	71.2 ± 2.0	90.0 ± 0.1	110.5 ± 0.1	121.8 ± 0.3	98.2 ± 0.1	274.1 ± 0.1	105.3 ± 6.3	100.3 ± 0.1
L-2056 *rck1*	75.8 ± 2.0	82.9 ± 0.1	104.2 ± 0.1	90.6 ± 0.1	102.0 ± 0.1	133.3 ± 0.1	107.3 ± 6.8	100.0 ± 0.3
FM16-M	60.6 ± 2.0	92.6 ± 0.1	93.4 ± 0.1	106.2 ± 0.1	96.1 ± 0.1	140.7 ± 0.1	101.9 ± 10.7	102.3 ± 0.2
FM16-7	59.3 ± 2.0	105.7 ± 0.1	175.4 ± 0.1	121.1 ± 0.3	99.8 ± 0.2	94.4 ± 0.1	107.7 ± 6.7	104.3 ± 0.4

Values are the mean of triplicate experiments and are shown as a percentage of the parent (L-2056) strain ± standard deviation. Absolute values for L-2056 are shown. Values which were increased or decreased by > 5 % and were also significantly different to the parent, i.e. $p < 0.05$ (Student's t-test) are highlighted by yellow or red respectively.

6.4 Discussion

To determine the individual contribution of a subset of genes to the phenotype of the evolved strains during fermentation, the performance of a set of strains harbouring single gene modifications were examined throughout fermentation. Candidate genes were chosen based upon those results from microarray experiments (Chapter 4). Genes of interest were modified by either replacement of the complete open reading frame or integration of the *TDH3* promoter in either of the two parent strains, L-2056 (the commonly used wine strain) or C911D (a haploid derivative of L-2056). It was speculated that this would provide further evidence of the genotypic alterations leading to the adaptively evolved phenotypes of FM5-M, FM16-M and FM16-7. Specifically, we hoped to gain insight to the mechanism(s) which are responsible for the desirable fermentation characteristics such as the increased glycerol and decreased ethanol yield of FM5-M and the increased glycerol yield and decreased fermentation duration of FM16-M and FM16-7.

Deletion of *HXT1*, *HAP4* and *GDH2* as well as over-expression of *ADH2*, *ADR1* and *VID24* in C911D, yielded strains which had minor alterations in fermentation duration, metabolite production and biomass formation. However, the magnitude of these changes was considered as insignificant to the overall phenotype of FM5-M (by means of student's t-test). All three over-expression strains (C911D adh2^{UP}, C911D adr1^{UP} and C911D vid24^{UP}) showed little variation in fermentation characteristics to one another and to that of the parent, C911D. The minimal impact of these modifications could be a consequence of nominal change in normal gene expression, as we did not test if gene expression was actually increased. This could have been achieved by monitoring transcript abundance by QRT-PCR or by detection of functional (and properly located) protein, however due to time constraints this was not achieved.

The deletion of *HXT2* from C911D yielded an alteration in the metabolite yield from terminal fermentation samples. C911D *hxt2* produced less succinic acid and ethanol, as well as an increased biomass, of which both the decreased

ethanol and increased biomass were found in FM5-M. Hxt2p is a high affinity hexose transporter thought to be important at the beginning of fermentation (Wendel and Bisson, 1994; Perez *et al.*, 2005). Deletion of *HXT2* has previously been shown to cause a partial reduction of high affinity glucose transport (Kruckeberg and Bisson, 1990), however, given the ability of C911D *hxt2* to complete fermentation within the same duration as the parent strain this is not apparent in this study.

Hxt3p is the primary low affinity transporter thought to be expressed throughout fermentation (Perez *et al.*, 2005). This is reflected by results here where deletion of *HXT3* yielded significant changes in fermentation, most notably fermentation duration increased by 31 % compared to the parent C911D. The production of major metabolites during fermentation were also found to be alike to FM5-M, for instance there was an increase in glycerol and decrease in ethanol yields. This is presumed to arise as a function of carbon shuffling through glycolysis into organic acid production supported by the increases in lactic and succinic acids (Visser *et al.*, 1995; Pronk *et al.*, 1996).

It is important to note, that a high level of redundancy has been observed within the hexose transporter group. It has been shown that the absence of one or few representatives is not expected to cause dramatic effects on the overall fitness of the strains (Selvi *et al.*, 2003). Consequently, the loss of single hexose transporters has been shown to only have a minor effect on fermentation rate under normal conditions, where the loss of a gene's function, specific to a stage during fermentation, is thought to be substituted by another (Luyten *et al.*, 2002; Perez *et al.*, 2005). This would favour the idea that other effects, such as ethanol inhibition of sugar transport, may be responsible for a large part of the limitation of carbon flux, (e.g. C911D *hxt3* fermentation) (Ansanay-Galeote *et al.*, 2001).

We deleted *ALD6*, the structural gene coding for a cytosolic, Mg^{2+}-activated acetaldehyde dehydrogenase (ACDH) (Meaden *et al.*, 1997) from both C911D and L-2056 which resulted in significant increases in glycerol and acetaldehyde

produced by both strains. There was also a large reduction in the final concentration of acetic acid in the haploid (79.2 % less than C911D) coupled with reduced ethanol production. The homozygous diploid deletant was also able to catabolise available sugar within a shorter duration than the parent, L-2056.

Ald6p, which preferentially uses $NADP^+$ (Meaden *et al.*, 1997; Wang *et al.*, 1998), together with other ACDH's is responsible for oxidation of acetaldehyde to acetate during fermentation (Meaden *et al.*, 1997; Remize *et al.*, 2000; Saint-Prix *et al.*, 2004). In *ald6* mutants, the mitochondrial route of acetate formation is operative due largely to the presence of Ald4p, however the cytosolic route can be markedly decreased (thought to be due to the functionality of *ALD2* and *ALD3* - encoding only a minor form of a cyotsolic, stress induced, glucose repressed ACDH (Norbeck and Blomberg, 1997; Navarro-Avino *et al.*, 1999)). Deletion of *ALD6*, the main cytosolic ACDH, results in a decrease in NADPH formation (Kurita and Ito, 1994). However, the NADP/NAD ratio might also be affected in these mutants, depending on the contribution of other ACDHs that can use NAD (Ald2p and Ald3p (Navarro-Avino *et al.*, 1999)) or NAD and NADP (Ald4p). The increased glycerol production noted in C911D *ald6* and L-2056 *ald6*, therefore, could reflect deregulation mechanisms.

Theoretically, an *ald6* mutant may have to achieve redox balance in individual cellular compartments (i.e. cytosol or mitochondria). The compensation of the mitochondrial enzymes for the loss of *ALD6* would most likely result in redox imbalance. The low concentration of acetic acid in C911D, may be a function of compartmentalisation of ACDH's, that is the inability of other ACDH's to oxidise acetaldehyde. However, the somewhat unchanged concentration of acetic acid in L-2056 *ald6* could be accounted for by Ald4p, as the function of this protein is thought to be strain dependent (Wang *et al.*, 1998). The role of Ald4p may offer a mechanism explaining the duration differences found in the haploid and diploid mutants. It is possible that Ald4p is responsible for the production of acetic acid in the diploid deletant, however inactive in the

haploid, thus an excess of acetaldehyde in cells has lead to toxicity, thus slowing glycolysis (Jones, 1989). The strain variation may be exacerbated by the fact that C911D may not be an isogenic form of L-2056. Preliminary fermentations were conducted comparing the strains from the tetrad set of which C911D was derived from, resulting in no major differences in fermentation duration (data not shown). However, it would be worthwhile to further examine the differences in this tetrad set, by detailed fermentation analysis and comparative genomic hybridisation.

Deletion of *glc3* from C911D resulted in a decreased ethanol yield (96 g l^{-1} *vs.* 102 g l^{-1} for C911D), an attribute shared by FM5-M. This deletant also showed an increase in fermentation duration (3 hours) and a decrease in succinic acid yield (88 % of C911D). *GLC3* encodes the glycogen branching enzyme involved in glycogen biosynthesis (Rowen *et al.*, 1992; Thon *et al.*, 1992). Branching of glycogen is required for cellular accumulation of glycogen, thereby helping the cell cope with heat or osmotic stress (Parrou *et al.*, 1997), as well as enhancing survival upon deprivation of nutrients such as carbohydrates and assimilable nitrogen (Lillie and Pringle, 1980; Parrou *et al.*, 1999). The initiation of glycogen accumulation is a function of glycogenin, a self-glucosylating protein which initiates biosynthesis acting on UDP-glucose (formed from glucose 1-phosphate) (see review by Francois and Parrou, 2001). Consequently disruption of *GLC3* may allow for the shuffling of glucose 6-phosphate into the production of another storage carbohydrate, trehalose (Lillie and Pringle, 1980; Parrou *et al.*, 1997; Parrou *et al.*, 1999).

Trehalose 6-phosphate (T6P), an intermediate in the accumulation of trehalose has been proposed to play a regulatory role in glycolysis, through inhibition of hexokinases, which phosphorylate glucose and fructose (Blazquez *et al.*, 1993). Thus glycolytic capacity of the cell is limited when T6P accumulates and this might explain the increased fermentation duration in the *glc3* deletant. Importantly, an over accumulation of trehalose may explain the reduced ethanol yield of C911D *glc3*. Trehalose 6-phosphate synthase displays high affinity for its substrates (glucose 6-phosphate and UDP-glucose), thus under

normal conditions, accumulation of trehalose is limited (Vandercammen *et al.*, 1989). Therefore in the *glc3* mutant, the diversion of these substrates away from glycolysis into trehalose accumulation may account for reduced ethanol, as a glycolytic safety valve (Blomberg, 2000) and as a cellular protectant.

C911D *ino1* demonstrated increased glycerol and biomass production alike to that observed in FM5-M. Ino1p catalyses the conversion of glucose 6-phosphate to inositol 1-phosphate (Donahue and Henry, 1981) which is the first step in the synthesis of a major membrane component (Carman and Henry, 1989), the phospholipid phosphatidylinositol (Hirsch and Henry, 1986). Consequently disruption of *INO1* would theoretically lead to the diversion of glucose 6-phosphate. An increase in the transcriptional levels of glycolytic genes has been implicated with the heightened accumulation of glucose 6-phosphate (Goncalves and Planta, 1998) consequently we propose that the observed change in ethanol and glycerol concentrations may have arisen through the subsequent alteration of glycoltic flux.

The increase in glycerol yield by FM5-M could have arisen through a stress induced response, due to the inability of cells to produce inositol phospholipids (as in the case of the C911D *ino1*) and the decreased storage carbohydrates (as in C911D *glc3*). Thus cellular glycerol production is increased to function as a replacement osmolyte (Hohmann, 2002) thus diverting flux away from ethanol production.

The homozygous deletant L-2056 *gph1* shows a dramatic increase in the accumulation of glycerol. *GPH1* encodes glycogen phosphorylase (Hwang *et al.*, 1989) and works in combination with Gdb1p, the glycogen debranching enzyme which releases glucose 1-phosphate, shortening the glycogen molecule. The expression of *GPH1* is induced by a variety of stressful conditions, such as growth into stationary phase, heat shock and osmotic shock (Parrou *et al.*, 1997; Teste *et al.*, 2000; Sunnarborg *et al.*, 2001). Disruption of either of these genes results in failure to degrade glycogen, thus a mild hyperaccumulation of glycogen occurs (Hwang *et al.*, 1989). It could be

assumed that the increased glycerol concentration could be a function of an increased requisite of osmolyte by the cell. Increased glycogen accumulation can lead to increased cell viability (Sillje *et al.*, 1999), thus as fermentation became more stressful through the accumulation of ethanol these cells may have better stress tolerance (Hottiger *et al.*, 1987) allowing for continued flux of carbon through glycolysis and thus shortening of fermentation duration.

Deletion of *RCK1* from L-2056 resulted in decreased fermentation duration, as seen for the adaptively evolved strains. In contrast to the evolved strains, however, a notable decrease in glycerol is also evident. *RCK1* is thought to encode a protein kinase (Dahlkvist and Sunnerhagen, 1994), as well as having a general involvement in meiosis (Ramne *et al.*, 2000). Specifically, Rck1p is implicated in the high osmolarity glycerol (HOG) pathway, through Hog1p, which is thought to suppress protein synthesis after osmotic shock as well as promote intracellular osmolyte production. The change in fermentation phenotype of L-2056 *rck1* may be a consequence of the HOG pathway, activating a response to high osmolarity (Schwartz and Madhani, 2004). Rck1p is phosphorylated by Hog1p which is known to down-regulate protein synthesis (Teige *et al.*, 2001). Consequently loss of function of *RCK1* may alleviate the negative regulation of Hog1p under conditions of stress, thus allowing sustained protein production by *HOG1* at a sacrifice of osmolyte production, hence the reduced fermentation duration, coupled with decreased glycerol production. It is noteworthy however, that the deletions in the commercial strain may not be representative, due to the theroretical loss of genomic material through self-diploidisation.

The deletion of a number of genes from C911D and L-2056 (eight and three respectively) affected a number of fermentation parameters emulating the fermentation phenotypes of FM5-M, FM16-M and FM16-7. Specifically, deletion of *HXT* genes offered similar characteristics to that of the evolved strains. Perhaps small changes in the regulation of a number of genes of this family may lead to the observed phenotypes. It is also highly feasible that a change in reserve carbohydrates may account for the increased glycerol yield

by the haploid and diploid cultures and the decreased fermentation duration by the diploid, as well as an increased cell viability. The down-regulation of *ALD6* could confer the elevated glycerol and acetaldehyde production in the evolved strains, to balance redox potential. The change in duration of fermentation observed in L-2056 *ald6* may also play an important role in the fermentation characteristics of the FM16- cultures.

It is most likely that a number of cellular activities have been altered in the adaptively evolved cultures and that this is the result of several genetic alterations. To further evaluate the impact of each of the genetic manipulations investigated here it would be worthwhile to examine strains with multiple modifications. This is certainly of interest, however, it falls outside of the scope of the current project.

Chapter 7 General discussion and future directions

Wine fermentations are a complex system, whereby yeast, which are responsible for the conversion of sugars to ethanol and importantly the production of desirable metabolites, play an integral role in wine quality. As the international market place becomes increasingly competitive, winemakers require yeast with more diverse properties as tools which can be exploited during fermentation to yield processing efficiencies and wines of greater quality. Such improvements include assured completion of fermentation (for example as a function of greater stress tolerance and reduced nitrogen dependency) or increased aroma production.

Over recent decades, strain improvement programs have been undertaken by numerous groups utilising an array of different techniques as reviewed by (Pretorius, 2000). We sought to evaluate the suitability of the use of adaptive evolution to generate improved industrial yeast strains. By applying the environmental constraint of a stressful anaerobic fermentation we generated from the commercial wine yeast strain L-2056 and a haploid derivative thereof both diploid and haploid cultures which could be considered adaptively evolved. Evolved mixed cultures were initially selected based on an increase in fermentative capacity, that is, they were able to complete fermentation within a shorter duration than their respective parent (Chapter 3). Further phenotype and transcriptome analysis was conducted to gain insight in to how the desirable phenotypes were conferred, thus shedding light on the potential genomic alterations that had led to the occurrence of FM5 and FM16 cultures.

Multiple changes were observed when comparing evolved and parent cultures. Notable phenotypic alterations included the ability of haploid derivative (FM5-M) to produce more glycerol and less ethanol than the parent (C911D). Similarly an increase in glycerol and decrease in fermentation duration was further validated in the evolved diploid culture (FM16-M) in comparison to its parent (L-2056). Global gene expression profiles were monitored at three sample points during fermentation for both cultures, of which a large subset of

genes were found to be differentially expressed. The comparison of haploids generated large datasets at mid exponential phase and prior to the RWD, whereas comparison of the diploid cultures only revealed a large transcriptional difference before the RWD.

Strains were then constructed with single gene manipulations, to evaluate the effect of a subset of genes on the fermentation phenotype of the parent and thus provide some insight to the contribution of each gene toward the phenotype of the evolved cultures. The subset of genes was selected based on data generated from the mixed culture microarrays (Chapter 4). Interestingly, deletion of either *HXT1*, *HXT3*, *GLC3*, *ALD6* and *INO1* in C911D yielded phenotypes similar to the evolved FM5 culture. Similarly deletion of either *ALD6*, *GPH1* and *RCK1* in L-2056 yielded phenotypes similar to that of FM16 cultures (Chapter 6). This supports our hypothesis that a number of mutations may be responsible for the altered fermentation phenotypes of evolved cultures, given that no one strain harbouring a single gene manipulation emulated the phenotype of the evolved culture.

It was decided that clonal analysis was necessary to determine the extent of heterogeneity in the mixed culture and thus the impact of such on the fermentation dynamics. It is widely accepted that industrial wine fermentations are considered heterogenous populations (Fleet and Heard, 1993; Fleet, 2003), and are thought to enhance flavour complexity and consequently the appeal of the wine through metabolic interactions (Lambrechts and Pretorius, 2000; Fleet, 2003; Cheraiti, *et al.*, 2005). Initial testing of 10 clonal isolates from both FM5-M and FM16-M yielded data confirming a high degree of heterogeneity, where all but one of each of the haploid and diploid evolved clones performed similarly or better than the mixed cultures from which they were isolated.

Due to the potential commercial applicability of the evolved diploid culture (since it was derived from an unaltered commercially available wine yeast), we sought to confirm the observed phenotypes of the best performing clones of FM16. Clonal cultures, FM16-5 and FM16-7 were chosen and evaluated more

rigorously, confirming their superior fermentation fitness (a shorter fermentation duration) and production of significantly more glycerol than the parent, L-2056. Transcriptome analysis was also undertaken on FM16-7, but because of the fermentation profile of this culture (Figure 5.6) and the assumption that the phenotype of this clone was a function of patterns of gene expression occurring at a close approximate time frame, sampling for RNA analysis occurred at 40 hours (Figure 5.7). This analysis generated a list of genes with altered transcript abundance. Interestingly 95 % of these genes possessed a common binding site for the Gcr1p transcriptional activator in their promoter region. Also expression of the self regulated *GCR1* gene was also decreased. Subsequent sequencing of the promoter of *GCR1* found 4 base pair differences between L-2056 and FM16-7 (Chapter 5), although these alterations need to be confirmed by further replicated sequencing. Evaluation of the functionality of the *GCR1* promoter in this strain would certainly be of benefit in future investigation of the basis for the altered phenotype of FM16-7. In particular it would be of interest to determine whether the introduction of the mutant *GCR1* promoter to L-2056 confers the desirable fermentation phenotypes of FM16-7, and if so, which of these mutations (alone or in combination) are necessary.

A more detailed genomic approach may also lead to the resolution of other mutations in the evolved cultures. Comparative genomic hydridisation as described by (Winzeler, *et al.*, 2003), is one approach which could be utilised. The Affymetrix platform offers approximately 125,000 unique 25 base-pair probes for hybridisation. These short probes offer greater target specificity due to the volume and size of probes. It has been suggested that this technique should resolve single base pair changes (A, Hayes; University of Manchester; pers. comm. 2003). Additionally, recent attempts have been made to include these strains in a large yeast sequencing project, however due to the nature and size of such a project, the similarities between our evolved strains preclude their inclusion.

Although analysis of minor metabolites has yielded favourable attributes in both FM5 and FM16 under controlled laboratory conditions, a more detailed analysis of metabolite production is required, particularly under more wine-like conditions. It will be of importance to not only evaluate the impact of adaptive evolution on desirable metabolites such as esters and higher alcohols, but also those metabolites which are thought to yield detrimental changes, such as acetic acid, whose production has been seen to change adversely upon strain optimisation procedures by other approaches (de Barros Lopes, *et al.*, 2000). As such a metabolomics or fluxomic approach would be highly suited for such future work. Profiling of both the internal metabolites (fingerprinting) (Oliver, 2001) and metabolites extruded into the media (footprinting) (Allen, *et al.*, 2003) may offer complementary diagnostics and as such, insight to the phenotypic differences between parent and evolved cultures. Quantification of fluxes through metabolic pathways may aid the elucidation of the basis for increased glycerol yield (Nielsen, 2003).

An industrial scale wine fermentation trial using grape must instead of defined media would also be beneficial to evaluate evolved cultures, hopefully further validating our reported findings. This would also allow for detailed determination of attributes of sensorial importance. The successive step from here could allow the commercialisation of the evolved culture. The application of such technology is simplified given the evolved cultures are derived from an already commercially available background, thus highlighting the simplicity and marketability of such a strain.

The approach of adaptive evolution therefore offers an excellent dynamic tool for the development of optimised yeast strains. Combined with genomic, transcriptomic and metabolomic analysis the elucidation of mechanisms responsible for desirable fermentation traits will be increasingly possible, thus leading to a better understanding of the dynamics of fermentation by these strains. Certainly a direct consequence of the development of such strains is the potential improvement of wine quality with also a much better understanding of biochemical and molecular processes which yield specific phenotypes.

Appendix I Solutions (additional to those outlined in text)

Media

Chemically Defined Grape Juice Medium (CDGJM)

Carbon	D-Glucose		100 g l^{-1}
	D-Fructose		100 g l^{-1}
Nitrogen			
	Ammonium	$(NH_4)_2SO_4$	2.68 g l^{-1}

Salts		
	Potassium sodium tartrate	2.5 g l^{-1}
	L-malic acid	3.0 g l^{-1}
	Potassium phosphate, dibasic	1.14 g l^{-1}
	Magnesium sulphate	1.23 g l^{-1}
	Calcium chloride	0.44 g l^{-1}
Trace Minerals		
	Manganese chloride monohydrate	189.2 μg l^{-1}
	Zinc chloride	135.5 μg l^{-1}
	Ferric chloride	32.0 μg l^{-1}
	Copper chloride	13.6 μg l^{-1}
	Boric acid	5.7 μg l^{-1}
	Cobaltous nitrate hexahydrate	29.1 μg l^{-1}
	Sodium molybdate dihydrate	24.2 μg l^{-1}
	Potassium iodide	10.8 μg l^{-1}
Vitamins		
	Myo-inositol	100 mg l^{-1}
	Pyridoxide-HCl	2 mg l^{-1}
	Nicotinic acid	2 mg l^{-1}
	Calcium pantothenate	1 mg l^{-1}
	Thiamine-HCl	0.5 mg l^{-1}
	ρ-amino benzoic acid	0.2 mg l^{-1}
	Riboflavin	0.2 mg l^{-1}
	Biotin	0.125 mg l^{-1}
	Folic acid	0.2 mg l^{-1}

pH adjusted to 3.5

Miscellaneous Buffers

TE Buffer

100 mM Tris base (pH 7.4 or pH 8.0, adjusted with hydrochloric acid)
10 mM EDTA (pH 8.0)
pH either 7.4 or 8.0

PBS
0.2 g l^{-1} KCl
8 g l^{-1} NaCl
0.2 g l^{-1} KH_2PO_4
1.15 g l^{-1} Na_2HPO_4

TAE Running Buffer
4.84 g l^{-1} Tris Base (Sigma 7-9)
1.142 ml glacial acetic acid
1 mM EDTA (pH 8.0)

TAE agarose gels were made in TAE Running Buffer with the appropriate addition of Agarose 1 (Amresco), typically 0.8 - 2.0 %.

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