Identifying genes that confer ethanol tolerance in *Saccharomyces cerevisiae*

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DECLARATION

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

Signature Date

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ABSTRACT

S. cerevisiae has evolved the ability to tolerate high concentrations of ethanol, a trait that has contributed to this yeast being a cornerstone of beverage and biofuel industries. The key genes involved in conferring ethanol tolerance in *S. cerevisiae* are unknown. One strategy used to identify genes conferring ethanol tolerance, was to create ethanol-tolerant (ET) mutants from a laboratory yeast strain and use these to identify ethanol-tolerance conferring loci. Dr. Dragana Stanley (Victoria University) created two ET mutants, a spontaneous (SM) and a chemical (CM) mutant from W303, via adaptive evolution (Stanley 2008). Transcriptome analysis of the resultant ET mutants found that expression levels of hundreds of genes were altered, relative to the parent under ethanol-stress conditions.

The primary objective of this thesis was to identify genes that confer ethanol tolerance in SM and CM. As a starting point, the mutants backcrossed and aporulated to characterize the genetics of the ethanol-tolerance mutations. A Rapid Ethanol Tolerance Assay (RETA) was developed from this work, enabling accurate quantification ethanol tolerance levels in the numerous progeny and spores generated. The segregation ratios of ethanol tolerance in CM indicated that a single gene was responsible for conferring the phenotype. In SM, ethanol tolerance segregated in a less straightforward fashion, suggesting more than two genes were responsible for conferring the phenotype.

In attempt to identify the genetic mutations conferring ethanol tolerance in the mutants, Affymetrix Tiling Microarrays were applied. However, this method generated large amounts of background noise and was unable to resolve the mutations. Genomis sequencing of CM was then used revealing a large number of mutations across the genome. Candidate loci were screened, leading to identification of an intergenic region containing four SNPs, and this was found to confer ethanol tolerance when transformed into the parent. This Ethanol Tolerance Conferring Sequence (ETCS), was further characterized to reveal that only SNP1 (Chromosome II: 697,850 – SNP1 = C→A) and SNP3 (Chromosome II: 697,907 – SNP1 = C→T) were required to confer ethanol tolerance.

Interestingly, ETCS resides in a transcribed, intergenic region between ORFs *YBR238C* and *ERT1,* genes which have not been previously associated with ethanol tolerance. Upstream of *ERT1* is *THI2*, and transcriptome data from Stanley et al (2010) indicated that expression of these two genes is up-regulated under ethanol-stress conditions in CM. The intergenic region is highly conserved across a number of industrial *S. cerevisiae* strains. When industrial wine strains were made homozygous for ETCS, it was found that the effect of ETCS on the ethanol tolerance phenotype was genetic background dependent. Future work is required to elucidate the mechanism by which ETCS confers the ethanol tolerance in *S. cerevisiae.*

PUBLICATIONS AND PRESENTATIONS

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Note

An electronic PDF version of the thesis is located in the Appendix CD.

ABBREVIATIONS

Abbreviation	Shortened Word
% v/v	Percentage volume per volume
bp	**B**ase **p**air
CIP	**C**alf **I**ntestinal **P**hosphatase
CORE	**CO**unterselectable **RE**porter
DIG	**Dig**oxygenin
EDTA	**E**thylene**d**iamine**t**etraacetic **a**cid
EMS	**E**thyl**m**ethane **S**ulphonate
ET	**E**thanol **T**olerant
ETCS	**E**thanol **T**olerance **C**onferring **S**equence
gDNA	**G**enomic **D**eoxyribo**n**ucleic **A**cid
hrs	**H**ou**rs**
ITS	**I**nter-**t**ranscribed **S**pacer
LB	**L**uria **B**roth
min	**Min**utes
nET	**n**ot **E**thanol **T**olerant
PCI	**P**henol **C**hloroform **I**so-amyl alcohol
PEG	**P**oly**e**thylene **G**lycol
PMPP	**P**lasma **M**embrane **P**roton **P**ump
RETA	**R**apid **E**thanol **T**olerance **A**ssay
SC	**S**ynthetic **C**omplete
SGD	***S**accharomyces* **G**enome **D**atabase
SNPs	**S**ingle **N**ucleotide **P**olymorphisms
SSC	**S**aline **S**odium **C**itrate
ssDNA	**S**almon **S**perm **DNA**
TBE	**T**ris **B**orate **E**DTA
TE	**T**ris-**E**DTA
VIS	**Vis**ible
YPD	**Y**east **P**eptone **D**extrose
YPG	**Y**east **P**eptone **G**lycerol

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Literature Review

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1.1 *Saccharomyces cerevisiae:* an industrial workhorse with limitations

Angiosperms emerged during a massive adaptive radiation event in the Cretaceous era. These flowering and fruiting plants were rich sources of sugar, paving the way for the evolution of new survival strategies by herbivorous animals and some fungi. Amongst these fungi, *Saccharomyces cerevisiae,* has evolved an incredible capacity for fermentation, where glucose is converted into ethanol, even in the presence of oxygen. *S. cerevisiae* produces ethanol, forfeiting maximum energy production, giving it a competitive advantage over other species since it is able to tolerate ethanol concentrations that other organisms cannot (Piskur *et al.,* 2006); in other words it kills off competitive species.

Fermentation efficiency and tolerance to relatively high levels of ethanol are traits that have made *S. cerevisiae* the cornerstone of modern alcoholic fermentation industries. The level of ethanol tolerance in *S. cerevisiae* is, of course, limited and varies from strain to strain. When ethanol reaches toxic concentrations, it has multiple detrimental effects on the cell, leading to decreased vitality and viability (Bauer and Pretorius 2000; Attfield *et al.*, 1997; Van Uden *et al.*, 1989; Casey and Ingeldew 1986; Stanley *et al.* 1993, D'Amore *et al.* 1991), which are problematic in an industrial setting.

Industrial fermentations occasionally fail to go to completion or progress at a slow rate; these problems are referred to as stuck and sluggish ferments respectively and, generically, as sub-optimal ferments. Several factors can contribute to sub-optimal fermentations but ethanol toxicity in *S. cerevisiae* appears to be a major cause (Alexandre *et al.,* 1998; Bisson *et al.,* 1999; Ribereau-Gayon *et al.,* 2000; Santos *et al.,* 2008).

Different yeast strains vary in the maximum concentration of ethanol they can tolerate before their activity diminishes, and this variation is genetically determined (Kalmokoff *et al.,* 1985; Chi *et al.,* 2000; Bisson *et al.* 2002; Pretorius *et al.,* 2003; da Silva *et al.,* 2007). Ivorra *et al.,* (1999) reported an inverse correlation between stress resistance of yeasts and their propensity to become stuck during fermentation. That is, the more stress tolerant a yeast strain, the less likely it is to cause a stuck or sluggish ferment. Thus, determining the genetic mechanisms required for tolerance to ethanol stress may be the key to developing novel yeast strains that minimize the risk of sub-optimal fermentations caused by ethanol toxicity.

1.2 The toxic effects of ethanol on *S. cerevisiae*

Ethanol freely diffuses across biological membranes, affecting the functions of vital cellular components and thereby stressing the cell. The toxic effects of ethanol lead to a loss in cell viability and decrease growth rate (Stanley *et al.,* 2010), which has been associated; with impairment of the cell cycle (Kubota *et al.,* 2004, Dinh *et al.,* 2008). However, the mechanism by which ethanol impacts on yeast are complex and much is yet to be elucidated.

1.2.1 Effects of ethanol on plasma membrane structure and function

Whilst ethanol potentially impacts on all cellular membranes, research to-date has primarily focused on effects of ethanol on the plasma membrane. The plasma membrane functions to physically separate intracellular components from the surrounding environment. It is a highly complex, organized matrix, consisting mainly of a phospholipid bilayer, sterols and proteins, and it is dynamic and fluid in nature; the cell stringently regulates its composition and therefore structure.

The plasma membrane controls the transport of substances into and out of the cell, but cannot fully protect the cell against harmful, passively diffusing substances such as ethanol. Ethanol is able to freely diffuse because it is amphipathic and is therefore soluble in the phospholipid bilayer, leading to increased membrane fluidity; using fluorescence anisotropy (Salgueiro *et al.,* 1988; Lloyd *et al.,* 1993; Swan *et al.,* 1997; Marza *et al.,* 2002) and electron spin resonance (Lloyd *et al.,* 1993), the fluidity of the plasma membrane has been shown to increase with increasing ethanol concentrations. This increased fluidity causes loss of structural integrity and

leads to increased permeability and leakage (Jones *et al.,* 1987; Salgueiro *et al.,* 1988; Zeng *et al.,* 1993; Alexandre *et al.,* 1994; Quintas *et al.,* 2000; Marza *et al.,* 2002; Mannuzzu *et al.,* 2008). Under ethanol stress conditions various researchers have determined permeability by measuring the flux of propidium iodide (PI) uptake by the cell using flow cytometry (Alexandre *et al.,* 1994; Marza *et al.,* 2002; Mannazzu *et al.,* 2008). A direct correlation between ethanol exposure and PI permeability was found.

The impact of ethanol on structural integrity of the plasma membrane is clearly important for cellular function. However, the physical changes in membrane structure that result from ethanol toxicity, remain to be fully elucidated. Tierney *et al.,* (2005) presented evidence that the fatty acid components of phospholipids in the membrane become interdigitated when ethanol reaches a critical concentration. Interdigitation is the overlapping of fatty acid tails from opposite sides of the plasma membrane leading to tighter packing and shortening of the distance across the membrane (illustrated in Figure 1.1). Along with this, the spacing between phospholipid head groups is increased, which would presumably contribute to increased leakage across the membrane.

1.2.1.1 Effect of ethanol on the Plasma Membrane Proton Pump (PMPP): H^+-ATPase

The plasma membrane proton (H^+-ATPase) pump has two major functions; to maintain a proton motive force (required for nutrient uptake) and to regulate intracellular pH. As previously mentioned ethanol causes increased permeability of the plasma membrane leading to increased leakage of such species as protons into and out of the cell. At low ethanol concentrations (<3% v/v ethanol), the PMPP is able to remove excess protons from the cell, maintaining intracellular pH homeostasis (Li *et al.,* 2010, Rosa *et al.,* 1991). However, at inhibitory concentrations of ethanol, the PMPP is impaired, and therefore the passive influx of protons results in intracellular acidification (Manderia *et al.,* 2010; Pascual *et al.,* 1987; Cartwright *et al.,* 1987; Rosa *et al.,* 1996; Leao *et al.,* 1984; Zeng *et al.,* 1993).

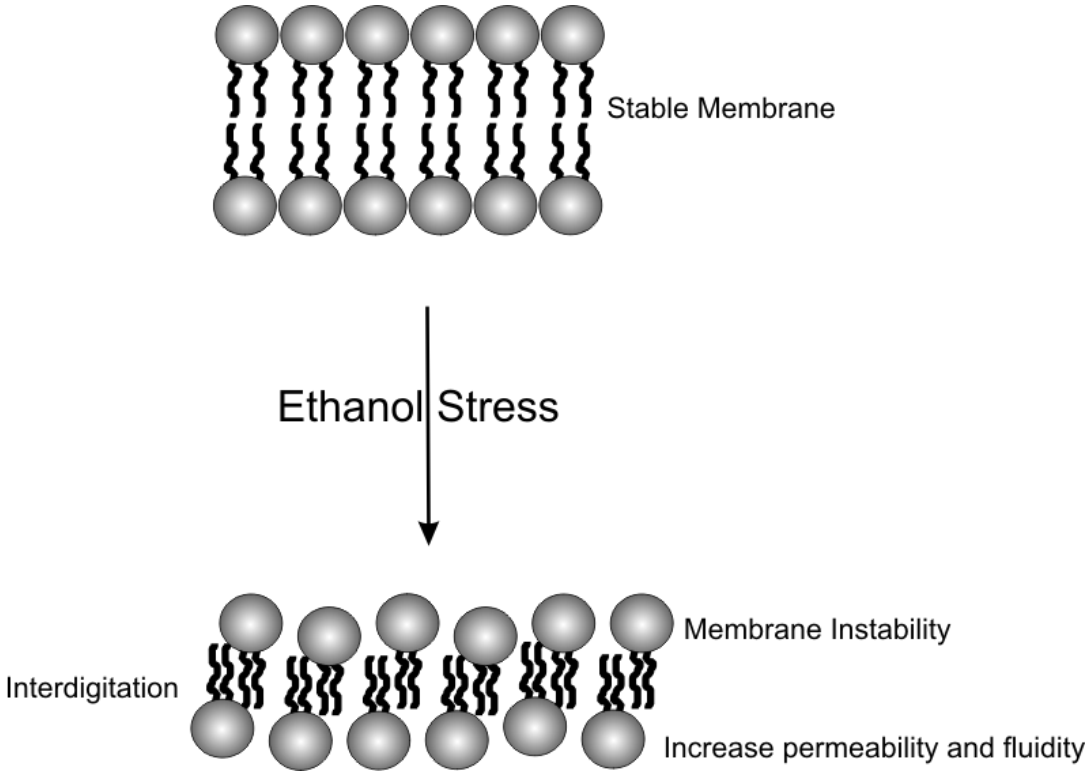


Figure 1.1: Schematic diagram illustrating interdigitation of fatty acids in the membrane phospholipid bilayer, hypothesized to occur under ethanol stress conditions (based on Tierney *et al.,* 2005).

1.2.1.2 Effect of ethanol on plasma membrane sugar transporters and fermentation rate

Another indicator of ethanol toxicity is decreased fermentation rate (Pascual *et al.,* 1987; Salmon *et al.,* 1989; Ansanay-Galeote *et al.,* 2001; Karpel *et al.,* 2008). This has been attributed in part to ethanol toxicity preventing the uptake of sugars by hexose transporters (Busturia *et al.,* 1986; Mauricio *et al.,* 1992). Leao *et al.,* (1982) observed the effect of ethanol on sugar transport across the membrane. These authors used xylose to determine impacts of ethanol on flux of a monosaccharide since the strain used could not metabolize this sugar. A negative correlation between increasing ethanol concentrations and xylose uptake was observed.

In addition to the inhibition sugar transporter activity ethanol also causes inhibition of glycolysis. Several authors (Pascual *et al.,* 1987; Dombek *et al.,* 1987) have observed the effects of inhibitory ethanol concentrations on the activity of major glycolytic enzymes. At low concentrations of ethanol the activity of the enzymes is unaffected but at higher concentrations the activity declines rapidly. Millar *et al.,* (1982) and Hallsworth *et al.,* (1988) discussed how ethanol induces water stress causing a decrease in water activity, leading to disruption of hydrogen bonds of glycolytic enzymes, leading to denaturation and resulting in decreased fermentation and growth rates. However, the work conducted by Millar *et al.,* (1982) was performed *in vitro*, which may not fully reflect what happens *in vivo*.

In summary, the plasma membrane is a highly organized structure performing a number of essential functions. Ethanol is an amphipathic molecule able to freely diffuse across the plasma membrane. At toxic concentrations it disrupts the structure of the membrane, with evidence to suggest it causes phospholipids to become interdigitated, leading to increased fluidity and permeability. This leads to passive influx of protons, in conjunction with ethanol-induced inactivation of the PMPP, ultimately resulting in intracellular acidification. Intracellular acidification and increasing ethanol concentrations in the cell have many detrimental effects on such things as central metabolism and function of plasma membrane sugar transporters. In all, this leads to a decrease in fermentation rate and decline in growth, and presumably, in an industrial context, this would lead to sluggish or stuck ferments.

1.3 Ethanol Tolerance: the response of *S. cerevisiae* to ethanol stress

The effects of ethanol on the cell are multi-faceted and the response by *S. cerevisiae* is equally complex with alterations in gene expression, plasma membrane composition, vacuole morphology, and nuclear localization of proteins. The following will describe the major cellular alterations that occur in response to, or as a defense against, ethanol toxicity.

1.3.1 Ergosterol content in the plasma membrane plays an important role in S. cerevisiae ethanol tolerance

Ergosterol is one of the main sterols found in the plasma membrane of fungi, including *S. cerevisiae*. Increased ergosterol content in the plasma membrane has been reported to positively correlate with increased ethanol tolerance (Thomas *et al.,* 1978; Agudo Del Castillo *et al.,* 1992; Alexandre *et al.,* 1994; Alexandre *et al.,* 1996; Swan *et al.,* 1998; Aguilera *et al.,* 2006). The supplementation of fermentations with ergosterol appears to improve cell viability and growth rates under ethanol-stress conditions (Swan *et al.,* 1998; Tran *et al.,* 2010). Ergosterol has been shown to stabilize the membranes under hypertonic pressure (Hossack *et al.,* 1976), which suggests an explanation for the increased viability observed upon ergosterol supplementation to ferments. Perhaps, in the case of ethanol stress, ergosterol also counteracts the instability of membranes caused by ethanol toxicity.

The following will discuss how ergosterol is thought to help counteract the detrimental effects of ethanol on plasma membrane structure and function.

1.3.1.1 Ergosterol delays interdigitation of membrane fatty acid groups under ethanol-stress conditions

As discussed earlier, a possible effect of ethanol on plasma membrane structure, is interdigitation of the phospholipid bilayer. Tierney *et al.,* (2005) demonstrated that in the presence of ethanol, 1,2-dipalmitoylphosphatidylcholine (DPPC) fatty acid components of phospholipids on opposite sides of the lipid bilayer become interdigitated, destabilizing the structure. When the authors introduced varying concentrations of ergosterol in DPPC vesicles, interdigitation was delayed. The mechanism by which ergosterol prevents interdigitation is largely unknown, but is likely to be due to steric hindrance; ergosterol is a rigid, planar molecular which intercalates between fatty acid chains of membrane phospholipids.

1.3.1.2 Ergosterol decreases membrane permeability under ethanol-stress conditions

Dickey *et al.,* (2009) reported that increased ergosterol content of DPPC vesicles under ethanol-stress conditions was correlated with decreased permeability. The authors observed the permeability of DPPC vesicles at various ergosterol concentrations, and found they were much more permeable at low concentrations than at high concentrations. However, above 15% v/v ethanol the (common upper threshold of wine fermentations), ergosterol did not counteract increased permeability caused by ethanol. The authors suggested that competition for ethanol binding sites within the bilayer may explain the decreased permeability in vesicles enriched with ergosterol (Hossack *et al.,* 1976).

1.3.1.3 Increased ergosterol content in the plasma membrane increases PMPP activity

Ethanol toxicity causes deactivation of PMPP, and this is thought to be a consequence of the impact of ethanol on destabilization of membrane structure and increased permeability (Alexandre *et al.,* 1996). Aguilera *et al.,* (2006) found that activity of the plasma membrane ATPase positively correlates with increasing ergosterol content in *S. cerevisiae;* yeast species that produce less ergosterol had reduced PMPP activity compared to *S. cerevisiae.* Thus, ergosterol may alleviate at least some of the detrimental effects of ethanol on PMPP activity. However, more direct evidence is required to substantiate this and it is still not known whether the presence of ergosterol prevents the intracellular acidification resulting from ethanol stress.

1.3.1.4 Impact of expression of ergosterol biosynthesis genes on ethanol tolerance levels

Since the presence of ergosterol has a positive impact on cell growth under ethanol-stress conditions, it is not surprising that data have shown that strains with higher ethanol tolerance also have increased expression of genes associated with ergosterol biosynthesis (Shobayashi *et al.,* 2007).

The *S. cerevisiae* genome has several genes involved in the biosynthesis of ergosterol. For example *ERG6,* a gene encoding Delta (24)-sterol C-methyltransferase, is essential for the synthesis of ergosterol. Inoue *et al.,* (2000)

created ethanol sensitive mutants from a *S. cerevisiae* sake strain using ethyl methanesolfonate (EMS). A number of ethanol sensitive strains were isolated. One strain, es5, was chosen for further work. The authors introduced *ERG6* into this strain and found that it recovered ethanol tolerance levels similar to the parental strain. However, it is important to note that not only were the mutants ethanol sensitive but the fermentation rate and cell viability were less than that of the parental strain in non-stressed conditions. This indicates that the strains had lower fitness levels not only in the presence ethanol toxicity.

The authors then proceeded to introduce a plasmid overexpressing *ERG6* into a laboratory strain and found that the growth of the strain was inhibited under ethanol-stress conditions. Since *ERG6* encodes only one of the genes involved in ergosterol biosynthesis, overexpression may have led to excess amounts of intermediates (rather than ergosterol) being produced which has been known to inhibit cell growth (Gachotte *et al.,* 1998).

Shobyashi *et al.,* (2005) compared the ergosterol content of an ethanol-tolerant (ET) *S. cerevisiae* sake strain (K9) to a non-ethanol tolerant (nET) laboratory strain (X2180) during fermentation. K9 was found to have higher ergosterol content prior to and during exposure to ethanol. The authors also found that expression of ergosterol biosynthesis associated genes of K9 was higher than in X2180. The authors suggest that increased expression levels of ergosterol genes in K9 allow the strain to produce higher concentrations of ergosterol thereby conferring higher ethanol tolerance levels on K9. Similarly, Stanley (2008) performed transcriptome microarray studies comparing an ET mutant to its parental strain under ethanol-stress conditions and found many genes involved in ergosterol biosynthesis were up-regulated.

Consistent with the above studies, ethanol tends to down-regulate the expression of ergosterol genes in less ET strains. For example, Li *et al.,* (2010) and Rossignol *et al.,* (2003) found that during a batch ferment, where ethanol concentration is accumulative, there is a down-regulation of ergosterol genes in an industrial *S. cerevisiae* strain. Chandler *et al.,* (2004) compared transcriptional profiles of a laboratory *S. cerevisiae* strain in ethanol-stress and non-stress conditions. The authors found that in this relatively ethanol-sensitive strain expression of some genes involved in ergosterol biosynthesis were down-regulated in this strain under ethanol stress.

In summary, ergosterol is hypothesized to counteract at least some of the toxic effects of ethanol by increasing membrane stability and perhaps preventing interdigitation. In strains of high ethanol tolerance, the expression of ergosterol genes is either up-regulated or, at least, not down-regulated, allowing for the production of membranes with higher ergosterol content. These observations may explain why the supplementation of ergosterol in growth media increases the cells ability to tolerate higher concentrations of ethanol (Tran *et al.,* 2010).

1.3.2 Role of unsaturated fatty acids (UFAs), particularly oleic acid, on ethanol tolerance levels of S. cerevisiae

1.3.2.1 Unsaturated fatty acids influence membrane fluidity and permeability

Fluidity and permeability of the plasma membrane is influenced by unsaturated fatty acid (UFA) content, and UFAs form the main structural component of the membrane (Alexandre et al 1994). The membrane is largely composed of two UFAs: palmitoleic ($C_{16:1}$) and oleic acid ($C_{18:1}$) (You *et al.,* 2003).

Thomas *et al.,* (1978) determined the effects of oleic and palmitoleic acid supplementation on cell viability at lethal ethanol concentrations. The experiments were conducted in anaerobic conditions, since oxygen is required for the biosynthesis of UFAs (Rosenfield *et al.,* 2003). The authors found that supplemented oleic acid was incorporated into the plasma membrane, and resulted in increased cell viability under ethanol stress. Casey *et al.,* (1984) supplemented oleic acid in anaerobic brewing fermentations and found an increase in cell biomass as well as ethanol production.

In more recent studies Landolfo *et al.,* (2010) observed the effect of oleic acid supplementation on permeability of the plasma membrane to propidium iodide (PI) under oxidative stress. Under these conditions oleic acid led to decreased permeability. Since ethanol stress causes increased membrane permeability, perhaps this is how oleic acid increases ethanol tolerance.

1.3.2.2 Supplementation of ferments with oleic acid for ole1 *mutant increase ethanol tolerance*

The above experiments were conducted in anaerobic environments, which cause the release of intermediates that are toxic to cells. As an alternative approach, the enzyme Δ^9 fatty acid desaturase (encoded by *OLE1*), required for the biosynthesis of both oleic and palmitoleic acid, was deleted and the effects of oleic acid supplementation were observed. Swan *et al.,* (1999) compared a *S. cerevisiae* strain defective for Δ^9 fatty acid desaturase (KD115) to S288C, a commonly used laboratory strain, under sub-lethal ethanol (17% v/v) for 60 min by quantifying viability. The authors found that when the defective strain was supplemented with oleic acid, it had increased viability. However, it is unclear whether KD115 was derived from S288C or has a different genetic background. In addition, the authors did not show the ethanol tolerance levels of KD115 without supplementation and growth in the absence of ethanol. Thus it is not clear whether KD115 had increased viability levels in the absence of ethanol compared to S288C.

In a similar study, You *et al.,* (2003) introduced plasmids expressing fatty acid desaturases (required for oleic acid synthesis) into an *S. cerevisiae ole1* knockout (L8-14C). The authors observed the effects of the various desaturase-encoding genes, on the ethanol tolerance phenotype of L8-14C under 5% ethanol stress. L8-14C expressing desaturase showed increased ethanol tolerance phenotype which corresponded to increased oleic acid content in the transformants relative to ethanol tolerance levels. The authors suggested that the ethanol tolerance of a strain was dependent on the oleic acid content. To really test the authors' hypothesis, it would have been better to observe whether increasing expression levels of desaturase correlated to increasing ethanol tolerance levels. Nonetheless, this work further supports previous findings that ergosterol influences ethanol tolerance levels in a positive manner.

1.3.2.3 Expression levels of OLE1 *impacts on ethanol tolerance levels of S. cerevisiae*

In light of the above observations, it is not surprising that ET strains have elevated *OLE1* expression levels compared to non-ethanol tolerant (nET) strains under ethanol-stress conditions. Yamada *et al.,* (2005) compared gene expression of an ET sake *S. cerevisiae* strain (K7) to a nET laboratory strain (X2180) under 8% v/v ethanol-stress conditions using GeneFilters®. The gene expression data and

northern analysis, confirmed that *OLE1* was one of the genes in K7 with elevated expression under ethanol stress. The authors then quantified the oleic acid content of the two strains and attributed increased oleic acid content to increased ethanol tolerance levels in K7. However the authors did not perform statistical analysis nor measure the influence of oleic acid content on growth rates or cell viability.

In a similar study, Kajiwara *et al.,* (2000) overexpressed *OLE1,* which led to increased oleic acid production and elevated ethanol tolerance. When re-inoculated into fresh medium, the growth rate of the wild-type was similar to that of the overexpressing strain. This is likely due to the cell having been pre-adapted to the environment and thus, differences in ethanol-tolerance levels between the overexpressing and wild-type were not observed. The results suggest that, since the parental strain contained less oleic acid it took an extended time to adapt to the new culture environments compared to the transformant. The authors concluded that overexpression of *OLE1* increased oleic acid content in the membrane and also led to increased ethanol tolerance levels. However, they did not expose the cells to ethanol stress, thus it is unclear whether the increased adaptation rate also applies to ethanol-stress conditions. Interestingly, strains overexpressing *OLE1* did not have increased unsaturated fatty acid levels. Thus, the authors' suggests that it is the expression of *OLE1* and not increased unsaturated fatty acid content per se, which induces increased ethanol tolerance.

However, from the publications discussed above the weight of evidence would suggest that elevated *OLE1* expression and increased oleic acid content is required by *S. cerevisiae* to maintain cell viability and growth under ethanol-stress conditions. The impact and mechanism of elevated oleic acid on membrane permeability, fluidity and structural integrity, remains unclear.

1.3.3 Inositol content of the plasma membrane influences ethanol tolerance

The plasma membrane contains various types of phospholipids including; phosphatidylinositol (PhI), phosphatidylcholine (PhC) and phosphatidylethanolamine (PhE). PhI in particular has been shown to positively influence ethanol tolerance levels. For example Chi *et al.,* (1999) induced increased content of PhI in the membrane, by the supplementation of inositol in the medium. The increased PhI content led to increased biomass and ethanol production by 0.8%. The authors then exposed the cells to lethal (18% v/v) ethanol concentrations and found that

supplementation with inositol led to decreased death rates and increased ethanol tolerance levels of *S. cerevisiae*. However there was no statistical analysis of the data to support the author's claims.

The biosynthesis of PhI requires expression of *INO1*, which encodes Inositol-3-phosphate synthase. Furukawa *et al.,* (2004) performed a similar study to Chi *et al.,* (1999) however this group, observed the effects of low (10 μM) and high (90 μM) amounts of inositol supplementation on ethanol tolerance levels of an *ΔINO1* mutant. Similar to reports by Chi *et al.,* (1999), Furukawa *et al.,* (2004) found a decrease in death rate with inositol supplementation under lethal ethanol concentrations. Also, under sub-lethal (12% v/v) ethanol stress, cultures supplemented with higher inositol concentrations had increased viability. The authors extended the study by observing the effects of inositol supplementation on membrane permeability by quantifying extracellular nucleotide, phosphate and potassium concentrations under increasing amounts of ethanol stress. The permeability of the membrane was lessened as concentrations of inositol increased. Thus, the presence of inositol increased cell viability under sub-lethal ethanol concentrations and decreased permeability and death rate under lethal ethanol concentrations.

OPI1 encodes a negative transcriptional regulator of *INO1.* Krause *et al.,* (2007) deleted *OPI1* to allow for the biosynthesis of inositol in a laboratory *S. cerevisiae* strain. The authors compared cell viability of the Δ*opi1* to the wild-type (WT) under ethanol-stress in conjunction with inositol supplementation. Under non-stress and in the absence of inositol addition, both WT and Δ*opi1* mutants had identical growth profiles. With the addition of inositol, the WT strain had increased growth rate compared to the *opi1* mutant. However, under ethanol-stress, inositol supplementation improved growth rates and cell viability of the *opi1* mutant.

1.3.4 Trehalose in ethanol tolerance

Trehalose is a disaccharide produced by *S. cerevisiae* as a means of storing glucose. However, when *S. cerevisiae* is exposed to heat, osmotic or ethanol stress, intracellular accumulation of trehalose is induced (Attfield *et al.,* 1987; Wiemken *et al.,* 1990; Ribeiro *et al.,* 1999; Carvahleiro *et al.,* 1999; Sharma *et al.,* 1997). However, whether accumulation of trehalose has a role in stress tolerance remains somewhat controversial.

Some data suggest that trehalose accumulation has a role in conferring ethanol tolerance, possibly by i) maintenance of membrane integrity and/or ii) preventing protein denaturation. The following will discuss the possible effects of trehalose accumulation and controversies in the literature relating to a role for trehalose in stress responses.

1.3.4.1 S. cerevisiae *accumulates intracellular trehalose upon ethanol stress*

Upon exposure to ethanol-stress, *S. cerevisiae* accumulates trehalose intracellularly. Attfield (1987) quantified trehalose concentrations during fermentation with the addition of 1.6 M ethanol. The authors found that there was a cumulative increase in trehalose concentrations with increasing time of exposure to ethanol. In a similar vein, Vianna *et al.,* (2008) compared a variety of *S. cerevisiae* strains and found that there was a gradual increase in trehalose concentrations with increasing ethanol concentrations. However, up to 15% v/v ethanol, trehalose concentrations appeared to decrease in some strains. This may have been due to decreased cell viability, which would not necessarily be reflected in cell weight. It would, perhaps, have been better to determine whether the increased trehalose concentrations corresponded to increased growth rates or cell viabilities.

Odumeru *et al.,* (1993) and Majara *et al.,* (1996) performed studies where *S. cerevisiae* strains were exposed to 10% v/v ethanol for 60 min. The authors quantified trehalose concentrations after this treatment comparing the stressed cultures to the non-stressed cultures. Trehalose concentrations in the ethanol-stressed cells increased compared to the control cultures.

Thus, the accumulation of intracellular trehalose appears to be associated with ethanol-stress. The possible role(s) of trehalose on cell physiology under ethanol-stress will be discussed in the following.

1.3.4.2 Impact of increased intracellular trehalose concentrations on cell viability under ethanol stress

Some authors have reported that increased trehalose levels are associated with increased cell viability under ethanol-stress conditions. Sharma *et al.,* (1997) reported the correlation between increased trehalose levels and cell viabilities with

increasing exposure time to osmotic and ethanol stress. Mahmud *et al.,* (2009), also found a correlation with increased trehalose accumulation and cell viabilities in 6% v/v ethanol stress conditions.

However, some authors have reported that increased trehalose concentrations do not necessarily correspond to increased cell viabilities under ethanol-stress. Alexandre *et al.,* (1998) for example found that a mutant lacking one of the key genes required for the production of the PMPP (*pma1-1*) was unable to maintain intracellular pH homeostasis at 10% v/v ethanol. And, even though the authors found that the pma1-1 mutant produced higher amounts of trehalose under ethanol-stress, this did not correlate with increased cell viability. Nonetheless, the absence of a functional PMPP in this mutant would have made it particularly vulnerable to ethanol stress.

Bandara *et al.,* (2009) gave insight into the influence of trehalose on ethanol tolerance. The authors found that under non-lethal ethanol concentrations the accumulation of trehalose had no influence on cell viabilities or fitness levels. However, under lethal concentrations the death rate of strains containing higher intracellular trehalose concentrations were diminished. Thus, the concentration of ethanol appears to influence the impact of trehalose on ethanol tolerance.

1.3.4.3 Maintenance of membrane integrity by trehalose

The presence of trehalose has been found to decrease leakiness caused by ethanol toxicity. For example, Mansure *et al.,* (1994) determined leakage levels of *S. cerevisiae* strains under ethanol-stress conditions by monitoring the flow of ions in solution. The authors found that, in the presence of trehalose, leakage was diminished.

Trehalose has been shown to reduce endocytosis inhibition caused by ethanol toxicity. Lucero *et al.,* (2000) compared a mutant that was unable to synthesize trehalose to wild-type levels and observed endocytosis of maltose under 2 – 6%v/v ethanol. The authors reported that the mutant, in the presence of ethanol, had diminished maltose uptake rates compared to the wild-type.

A possible role of trehalose in the cell is the maintenance of membrane integrity, which is particularly important under ethanol-stress conditions. In a review by Crowe (2008), the author discussed the chemical properties of trehalose and its possible roles in stabilization of membranes, proposing that it binds to, and sits between, the phospholipid head groups. However, there is no emperical data to support this.

1.3.4.4 Expression of trehalose biosynthesis and degradation genes under ethanol stress

In response to ethanol stress the cell accumulates trehalose and when this stressor is removed, the cell rapidly degrades trehalose. The cell does this by regulating expression of trehalose biosynthesis and degradation associated genes.

Analysis of global gene expression profiles of ethanol stressed yeast has consistently found an up-regulation of both trehalose biosynthesis and degradation genes. Ogawa *et al.,* (2000) applied GeneFilters® to determine expression levels of an ET sake mutant compared to its parental strain. The authors found that the ET mutant had up-regulated expression levels of trehalose biosynthesis genes *TPS1* and *TPS2* as well as elevated intracellular trehalose concentrations. Abe *et al.,* (2009) used RT-PCR to determine the expression levels of *TPS1* and *TPS2* in an ET mutant of a laboratory yeast strain under 10% v/v ethanol stress. The authors found that the yeast strains with higher *TPS* gene expression levels were also more tolerant to ethanol.

Similar to the work conducted by Ogawa *et al.,* (2000), Ma *et al.,* (2010) used quantitative RT-PCR arrays to determine gene expression levels of an ET mutant *S. cerevisiae* strain compared to it parental strain exposed to 8% v/v ethanol stress. The authors found expression of *TPS1, TPS2, TSL1, ATH1* and *NTH1* was consistently up-regulated under ethanol-stress and this was associated with increased trehalose production. The up-regulation of *TPS1* and *TPS2*, explains trehalose accumulation upon ethanol stress. The increased expression of trehalose degradation genes such as *ATH1* is likely to regulate trehalose levels, which is consistent with the rapid degradation of trehalose once ethanol stress is removed.

Upregulation of trehalose biosynthesis and degradation genes was also observed by Alexandre *et al.,* (2001). The authors used microarrays to get a snapshot of the transcriptome of laboratory *S. cerevisiae* strain S288C, after 30 min exposure to 7%

ethanol stress. The authors found that genes involved in both the biosynthesis (*TPS1, TPS2, TSL1*) and degradation (*NTH1)* of trehalose were up-regulated in ethanol-stress conditions. Thus, trehalose associated genes are up-regulated as a response to ethanol stress and this is associated with increased ethanol tolerance.

1.3.5 Role of the vacuole in ethanol stress response

Ethanol stress induces changes to vacuolar morphology; the vacuole goes from fragmented vesicles, to an observably single enlarged organelle (Izawa *et al.,* 2005; Maeden *et al.,* 1999; Pratt *et al.,* 2007; Matsuura *et al.,* 2005; Abe *et al.,* 2009). Whether the enlarged vacuole is associated with ethanol tolerance is however unclear.

Genes encoding components of the vacuolar H^+-ATPase pump such as *VMA2, VMA6, VMA8, VMA10,* and *VMA21* are essential under ethanol-stress conditions (Fujita *et al.,* 2006; Kubota *et al.,* 2004, Auesukaree *et al.,* 2009; Ma *et al.,* 2010). Since ethanol stress impairs the plasma membrane H^+-ATPase, it is possible that the cell attempts to control intracellular pH using the vacuole (Carmelo *et al.,* 1997, Veigas *et al.,* 1998); possibly the vacuolar H^+-ATPase pump offsets the decline in intracellular pH caused by ethanol toxicity.

Deletion of genes involved in vacuole biogenesis and in regulation of vacuolar pH such as *VPS15* (alias *PEP3)* and *VPH1* (respectively), causes the cell to become highly sensitive to ethanol and heat (Sugden *et al.,* 1983; van Voorst *et al.,* 2006; Kubota *et al.,* 2004; Matsuura *et al.,* 2005). However the authors did not determine the fitness (i.e. growth) of the mutant and parental strains in the absence of ethanol. Thus, the reduced growth phenotype in ethanol assays might simply reflect on overall diminution of fitness.

Interestingly, Vacuolar protein sorting (*VPS*) genes, in particular *VPS34,* have been found in five independent studies to be required for cell viability in ethanol stress conditions (Van Voorst *et al.,* 2006; Kubota *et al.,* 2004; Kumar *et al.,* 2008; Auesukaree *et al.,* 2009; Takahashi *et al.,* 2001).

1.3.6 Nuclear localization proteins under ethanol-stress conditions

The reversible re-localization of proteins to the nucleus has been observed in ethanol-stressed *S. cerevisiae*. These proteins are hypothesized to play a role in selective mRNA export specifically in response to ethanol.

1.3.6.1 Alcohol sensitive ring/PHD finger (Asr1) re-localizes to the nucleus specifically under ethanol stress conditions

Proteins that shuttle from the cytoplasm and nucleus specifically in response to alcohol stress were first reported by Betz *et al.,* (2004). A protein named Alcohol Sensitive Ring/PHD finger, Asr1p, was found to accumulate in the nucleus upon exposure to 7.5% v/v ethanol stress and then shuttle back into the cytoplasm once the stress was removed. Re-localization of Asr1p was not evident under oxidative stress suggesting that this not a general stress response. Later work performed by Izawa *et al.,* (2006) reaffirmed the re-localization of the Asr1 to the nucleus but this did not appear to impact on ethanol tolerance.

However, work conducted by Bandara (2009) suggests that *Asr1* plays an important role in the viability of strains under lethal (>14% v/v) ethanol concentrations; whereas under sub-lethal concentrations (6-9% v/v) of ethanol, deletion of *ASR1* had no observable influence on cell viability. In a competitive growth curve analysis at 7.5% v/v ethanol, the △*Asr1* strain begins to decline after 30 generations, whereas the wild-type continues. This was not observed when the cells were exposed to sub-lethal ethanol concentrations in a competition growth curve. Thus, *ASR1* may play an essential role in ethanol tolerance at lethal ethanol concentrations.

1.3.6.2 Dead Box Protein (Dbp5 alias Rat8) re-localizes to the nucleus specifically under ethanol stress conditions

mRNA export and translation is highly regulated in *S. cerevisiae*. The Dead Box Protein (Dbp5 alias Rat8) is involved in mRNA export and translation termination and is located on the cytoplasmic side of the nuclear envelope (Estruch *et al.,* 2003). Ethanol stress perturbs the export of poly(A)$^+$ mRNA from the nucleus. It is hypothesized that Dbp5p relocates to the nucleus specifically upon ethanol stress and might function to selectively export mRNA, particularly encoding heat shock proteins (Saavedra *et al.,* 1997; Izawa *et al.,* 2005; Izawa *et al.,* 2009). When Dbp5

was over-expressed, there was no nuclear poly(A)$^+$ mRNA retention. Takemura *et al.,* (2004) observed the localization of Dbp5 in *S. cerevisiae* industrial Japanese sake strains. However, Rollenhagen *et al.,* (2004) reported that Dbp5 also re-localizes under heat stressed conditions and is not specific to ethanol stress.

1.3.7 Functional genomics and transcriptome wide-analysis of the S. cerevisiae *ethanol stress response*

The advent of gene array technology and the availability of the *S. cerevisiae* gene deletion collection have enabled global analysis of cellular responses to external stimuli. The following is a summary of what we have learnt about ethanol stress and ethanol tolerance by the application of these technologies.

1.3.7.1 Functional genomic analyses of ethanol tolerance in S. cerevisiae

Completion of the *S. cerevisiae* genome sequencing project in 1996 (Goffeau *et al.,* 1996), led to the development of laboratory yeast (BY4743 homozygous diploid as well as BY4741 haploid) genome deletion libraries, completed in 2001. These collections of deletion mutants were constructed by systematically replacing ORFs with a KanMX cassette. The libraries allow for the identification of genes that impact on ethanol tolerance; a summary of findings, amalgamating results of deletion library screening studies from multiple laboratories is given in Table 1.1.

The first functional genomics study of ethanol tolerance was by Kubota *et al.,* (2004), where the deletion library was exposed to 8 and 11% v/v ethanol in agar. Any deletion mutants inhibited by the presence of ethanol compared to the wild-type BY4743, potentially have a role in ethanol tolerance. The authors identified a total of 256 genes required for growth in the presence of ethanol. Fujita *et al.,* (2006) screened the deletion library on solid medium containing 10% v/v ethanol. One hundred and thirty seven genes were found to impact on ethanol tolerance. Van Voorst *et al.,* (2006) performed a similar study with similar findings to Fujita *et al.,* (2006). More recently, Auesukaree *et al.,* (2009) performed the same screen as Fujita *et al.* (2006), however only found 95 genes to be associated with ethanol tolerance. The variation in number of genes identified as potentially conferring ethanol tolerance is likely due to differences between the screening assays used. All used a drop plate method, where the deletion mutants were grown overnight in liquid medium then serially diluted. Droplets (~5 μL) of diluted cultures were then

spotted onto ethanol containing medium. This method has several factors that would introduce variation in results between laboratories and inaccuracies for example, deciding on a cut-off (or end point) for growth/ no growth in the presence of ethanol is arbitrary and therefore one would expect different laboratories to generate different results (as seen in Table 1.1).

Despite the variation in data obtained across functional genomic screens Table 1.1 illustrates that some genes have been consistently identified as being essential for growth in ethanol containing environments. In particular the deletion of *VPS34*, a gene involved in vacuolar protein sorting and encoding a Phosphatidylinositol 3-kinase (required for membrane-associated signal transduction) has been identified by four independent authors as being required for growth in the presence of ethanol. This is consistent with the observations of Takahashi *et al.,* (2001); using transposon mutagenesis the authors found that transposon insertion into VPS34 led to increased sensitivity to ethanol. However, the function of *VPS34* in ethanol tolerance is yet to be determined and mutants have increased sensitivity to a range of stressors (see entry for this in SGD http://www.yeastgenome.org/); *VPS34* is not ethanol-stress specific.

A limitation of the functional genomic studies is the inability to distinguish between strains with decreased fitness levels and sensitivity to ethanol. Genes such as *BUB1* have been identified by three independent studies at being required for ethanol tolerance (see Table 1.1), however *BUB1* encodes a protein kinase involved in a cell cycle check point. As indicated on the SGD, the deletion of *BUB1* renders the mutants with decreased viability and therefore in the drop-plate assays used in the functional genomic studies will appear as more sensitive to ethanol compared to the parental strain.

1.3.7.2 Global gene expression under ethanol-stress conditions

The availability of *S. cerevisiae* genome sequence led to the development of whole genome microarrays. This enabled measurements of global gene expression levels in yeast exposed to ethanol stress. Alexandre *et al.,* (2001), Chandler *et al.,* (2004) and Stanley (2008) identified genes that are upregulated upon exposure to ethanol stress (Table 1.2). These authors observed gene expression levels during the lag phase when cells are aclimitising to ethanol stress. Hundreds of genes were identified as up or down regulated upon exposure to ethanol stress. This is probably

due to *S. cerevisiae* having general stress responses such as those regulated by STREs (STress Response Elements), found in the promoters of many genes (Gasch *et al.,* 2000; Ruis *et al.,* 1995; Schuller *et al.,* 1994). The general stress response is induced by many stressors; it is not specific to ethanol.

Some genes have consistently been identified by various studies to have increased expression upon exposure of yeast cells to ethanol stress. For example, Heat Shock Protein (HSP) encoding genes *HSP12, HSP28, HSP30 and HSP104,* have been found in five independent studies to be up-regulated after exposure to ethanol stress (see Table 1.2). There is evidence to show that *HSP12*, *HSP26* and *HSP104* have a direct role in ethanol stress tolerance. For example, Sales *et al.,* (2000) demonstrated that Hsp12 resides in cell membranes decreasing fluidity caused by ethanol; strains lacking the ability to express *HSP12* have diminished ethanol tolerance. Jiminez *et al.,* (2010) showed that increased expression of *HSP26* in wine strains was also associated with increased stress, indicating ethanol tolerance as well as improved fermentative capacity. Sanchez *et al.,* (1992) demonstrated that a non-functional Hsp104 in a laboratory *S. cerevisiae* strain had diminished ethanol tolerance levels. Interestingly none of these *HSP* genes were identified in functional genomic screens as required for growth under ethanol stress.

The hyper-osmolarity response gene, *HOR7*, has been found in five independent studies to have up-regulated expression under ethanol stress. However, this is perhaps not surprising since when ethanol is added to yeast growth medium it increases the osmotic potential of the medium and therefore it would be expected to induce an osmotic shock. Again *HOR7* did not impact on ethanol tolerance in functional genomic screens.

It is clear from Tables 1.1 and 1.2, that functional genomics screens and transcription approaches do not identify the same genes being associated with ethanol tolerance.

GENE	Voorst *et al.*, (2006)	Fujita *et al.*, (2004)	Kubota *et al.*, (2008)	Auesukaree *et al.*, (2009)	Takahashi *et al.*, (2001)	TOTAL
VPS34	1		1	1	1	4
ATP1	1		1	1		3
BUB1		1	1	1		3
BUD27		1	1	1		3
DOA4		1	1	1		3
ERG28		1	1	1		3
GIM4	1	1		1		3
GIM5	1	1		1		3
HPR1		1	1	1		3
PAC10		1	1	1		3
PAT1	1	1			1	3
RAD27		1	1	1		3
SEC66	1		1	1		3
SIT4	1		1	1		3
SLG1	1	1		1		3
SMI1	1	1	1			3
STP22		1	1	1		3
SUR4	1		1	1		3
TRS33		1	1	1		3
UME6		1	1	1		3
VMA6		1	1	1		3
VMA8		1	1	1		3
VMA10	1	1		1		3
VMA21		1	1	1		3
VPS20		1	1	1		3
VPS36	1	1	1			3
AKR1		1		1		2
ALG6		1	1			2
ARD1		1		1		2
ATP11			1	1		2
BDF1			1	1		2
BEM1		1	1			2
BEM2	1				1	2
BEM4		1		1		2

Table 1.1: Genes identified in functional genomics studies as being required for survival under ethanol stress. The table shows which and how many publications identify genes associated with ethanol-stress tolerance. The remainder of the table can be found in Appendix 1.1.

GENE	Alexandre et al., (2004)	Fujita et al., (2004)	Chandler et al., (2004)	Ogawa et al., (2004)	Zuzuarregui et al., (2004)	Stanley (2008)	TOTAL
HOR7	1	1	1	1		1	5
HSP12	1	1	1	1		1	5
HSP104	1	1	1	1	1		5
CTT1	1	1	1	1			4
HSP26	1	1	1			1	4
TDH1	1	1	1			1	4
ALD4	1		1			1	3
DAK1	1	1	1				3
GLK1	1	1	1				3
GPD1	1			1	1		3
GRE3	1	1	1				3
HSP30	1	1	1				3
HSP78	1	1	1				3
HXK1	1	1	1				3
PGK1		1	1			1	3
SSA3	1	1			1		3
SSA4	1	1	1				3
ACT1				1	1		2
AHP1	1					1	2
ARG4		1	1				2
CIT1	1	1					2
CIT2	1		1				2
CYC7		1		1			2
DDR2	1		1				2
GDB1	1	1					2
GLC3	1	1					2
GMP2	1	1					2
GPH1	1	1					2
GRX1	1		1				2
GSY2		1	1				2
HSP42	1		1				2
HSP82	1	1					2
MCR1	1		1				2
OPI3			1			1	2
PGM2	1		1				2
PYC1	1		1				2
RPN4	1					1	2
SER3		1	1				2
SNZ1		1	1				2
SPI1			1	1			2
SSE2		1	1				2
TPS1			1	1			2
TPS2			1	1			2
TRX3		1		1			2
TSL1	1		1				2

Table 1.2: Genes identified in microarray studies as having up-regulated expression under ethanol stress. The table shows which and how many publications identify genes as ethanol-stress responsive. The remainder of the table can be found in Appendix 1.2.

1.3.8.3 Genes which directly increase ethanol tolerance levels in *S. cerevisiae*

Functional genomics and global expression analyses have identified hundreds of genes that are, in one way or another associated with the ethanol-stress response and/or ethanol-tolerance in *S. cerevisiae*. However, there are also many publications describing non-'omics based approaches that have identified genes and gene functions associated with ethanol tolerance (see Table 1.3). Some of these genes and their possible roles in ethanol tolerance, including *ERG6, HOR7, HSP12, HSP104, OLE1, TPS1, ATH1* and *VPS34,* have been discussed earlier in this literature review, others will be discussed in the following.

Alper *et al.,* (2006) screened a gTME (global Transciption Machinery Engineering) mutant libraries of *SPT15.* The mutant libraries are generated via error-prone PCR and transformed into the laboratory *S. cerevisiae* strain BY2742. The authors then screened the transformants for increased tolerance and isolated an ET mutant *spt15-300.* The authors later found that *spt15-300* carried three mutations leading to amino acid substitutions Phe177Ser, Tyr195His, and Lys218Arg. Later work by Baerends *et al.,* (2009) attributed the increased ethanol tolerance in *spt15-300* to be due to increased leucine uptake in chemically defined medium with limited nutrients. Baerends *et al.,* (2009) did not observe increased ethanol tolerance when the mutant was grown in complex rich medium; it was only observed when defined medium had low concentrations of leucine. Thus, *spt15-300* appears to enable the cell to overcome ethanol-induced impacts on leucine transport across the plasma membrane.

ORF	References that link ORF to ethanol tolerance	Brief description of ORF and/or protein encoded by ORF*
ATH1	Kim *et al.,* (1996); Bandara *et al.,* (2010)	Acid trehalase required for utilization of extracellular trehalose
BTN2	Espinanzo-Romeu *et al.,* (2008)	possible role in mediating pH homeostasis between the vacuole and plasma membrane H(+)-ATPase
CPR1	Kim *et al.,* (2006)	Cytoplasmic peptidyl-prolyl cis-trans isomerase
CTT1	Schuller *et al.,* (1995); Ogawa *et al.,* (2000)	Cytosolic catalase T, has a role in protection from oxidative damage by hydrogen peroxide
ERG6	Inoue *et al.,* (2000)	Delta(24)-sterol C-methyltransferase, converts zymosterol to fecosterol in the ergosterol biosynthetic pathway
GAL6	Yazawa *et al.,* (2007)	Cysteine aminopeptidase with homocysteine-thiolactonase activity
HSP12	Ogawa *et al.,* (2000); Sales *et al.,* (2000)	Plasma membrane protein involved in maintaining membrane organization in stress conditions
HSP30	Piper *et al.,* (1994)	Hydrophobic plasma membrane localized, stress-responsive protein that negatively regulates the H(+)-ATPase Pma1p; induced by heat shock, ethanol treatment, weak organic acid, glucose limitation, and entry into stationary phase
HSP104	Sanchez *et al.,* (2000); Lucero *et al.,* (2000); Parsell *et al.,* (1991)	Heat shock protein that cooperates with Ydj1p (Hsp40) and Ssa1p (Hsp70) to refold and reactivate previously denatured
MPR1	Du et al 2007	L-azetidine-2-carboxylic acid acetyltransferase, reduces intracellular ROS and contributes to L-proline analog resistance and tolerance to ethanol and freezing
OLE1	Kajiwara *et al.,* (2000); You *et al.,* (2003); Yamada *et al.,* (2005)	Delta(9) fatty acid desaturase, required for monounsaturated fatty acid synthesis
SPT15	Alper *et al.,* (2006)	TATA-binding protein, general transcription factor that interacts with other factors to form the preinitiation complex at promoters, essential for viability
TPS1	Alexandre *et al.,* (1998); Soto *et al.,* (1999); Kwona *et al.,* (2003); Bandara *et al.,* (2009)	Synthase subunit of trehalose-6-phosphate synthase/phosphatase complex
URA7	Yazawa *et al.,* (2007)	Major CTP synthase isozyme (see also URA8), involved in phospholipid biosynthesis

Table 1.3: List of genes that have been shown to have a direct influence on the ethanol tolerance phenotype in *S. cerevisiae*.

* Description of gene as indicated in SGD (http://www.yeastgenome.org).

Interestingly Yazawa *et al.,* (2007), found that the deletion of either *GAL6* or *URA7* increased ethanol tolerance of a laboratory strain. *GAL6* and *URA7* encode a cysteine aminopeptidase and major CTP synthase isozyme respectively. The mechanisms by which deletion of these genes confer ethanol tolerance are unclear. However, both mutations lead to increased oleic acid content, which as discussed in Section 1.3.2.2, would be expected to facilitate ethanol tolerance. It is unclear as to how the deletion of these genes leads to increased oleic acid content and ethanol tolerance.

Espinanzo-Romeu *et al.,* (2008) found that *BTN2* (encoding a v-SNARE binding protein) is required for ethanol tolerance and that overexpression of this genes gave slightly improved ethanol tolerance. As discussed earlier the vacuole and maintenance of intracellular pH homeostasis (Sections 1.35 and 1.2.1.1 respectively) significantly influence ethanol tolerance in *S. cerevisiae*. *BTN2* may have a role in mediating pH homeostasis between the vacuole and the PMPP, this may partially explain its role in ethanol tolerance (SGD-http://www.yeastgenome.org).

Another gene *CTT1* which encodes a cystolic catalase that protects *S. cerevisiae* from oxidative damage caused by hydrogen peroxide, was found by Schuller *et al.,* (1995) to have a role in ethanol tolerance. Similar observations were made for superoxide dismutase genes *SOD1* and *SOD2* (Periera *et al.,* 2001). Thus, it appears that sub-lethal ethanol concentrations cause an oxidative stress, as discussed by Costa *et al.,* (1993).

Du *et al.,* (2007) reported that *MPR1*, (which encodes an enzyme that reduced intracellular Reactive Oxygen Species (ROS)), found in *S. cerevisiae* Σ1278, and industrial wine and brewery strains (Borneman et al., 2011) contributes to ethanol tolerance. The authors found that making Σ1278 null for *MPR1* caused the strain to become hypersensitive to ethanol and strains overexpressing *MPR1* had increased ethanol tolerance. The authors propose that, since ethanol causes an increase in ROS, *MPR1* will have a role in limiting the accumulation of this toxic product.

Kim et al., (2006) found that a stress tolerant strain of *S. cerevisiae* KNU5377 was rendered sensitive to ethanol when *CPR1* was deleted. However, in BY4742, *CPR1*

deletion had no impact on ethanol tolerance. A possible role for *CPR1* (encodes a cytoplasmic peptidyl-prolyl cis-trans isomerase), in ethanol tolerance in KNU5377 are not at all obvious, but this work does highlight the influence of genetic background on the ethanol tolerance phenotype.

1.4 Generation and characterization of ethanol-tolerant *S. cerevisiae* ET mutants

The cellular mechanisms involved in conferring ethanol tolerance remain unclear. To improve knowledge and understanding in this field, Dr. Dragana Stanley (former PhD student, Victoria University) successfully generated two ET *S. cerevisiae* mutants. Both mutants could tolerate up to 9% ethanol where the parental strain would begin to decline in viability (Stanley 2009). Under lethal ethanol concentrations the ET mutants were found to have a decreased death rate compared to the parental strain. Physiological characterization showed that the ET mutants had altered metabolism compared to the parental strain and they also differed from each other. The mutants produced higher concentrations of glycerol in both the presence and absence of ethanol stress (Stanley 2009), and produced lower amounts of acetic acid, which was hypothesized to be due to improved glycolytic flux under ethanol stress relative to the parental strain. Transcriptional analysis was performed comparing the expression profiles of the ET mutants and parental strains. Hundreds of differentially expressed genes were found; a vast difference in the expression profiles across all three strains was apparent (Stanley *et al.,* 2010). Thus, whilst work on these mutants provided some insights into how ethanol stress tolerance can be improved in *S. cerevisiae*, they did not identify the genetic determinants responsible for ethanol tolerance.

1.5 Aim of work described in this thesis

This project aimed to identify the genetic determinants that confer ethanol tolerance on the ET mutants generated by Stanley (2008).

Chapter 2

Materials and Methods

2.1 Yeast strains

A haploid laboratory strain, *S. cerevisiae* W303-1A (WA) (Mat **a** *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15)* is the parental strain from which the ET mutants, CM and SM, were derived (generated by Dr. Dragana Stanley see Stanley 2008). Strains generated in this project are listed in Table 2.1.

2.2 General equipment

Application	Equipment Model Name	Company
Centrifugation >2 mL volumes	Universal 32R Zentrifugen	Hettich
Bench-top Centrifugation <2 mL	Centrifuge 5415D	Eppendorf
PCR and 1mL short spin	Qik Spin	Qik Spin
PCR	PTC-100 PCR	MJ Research Inc.
PCR	Icycle iQ	BioRad
PCR	FTS-960	Corbett Research
Microplate Reader	Multiskan Ascent	Thermo
Microplate Reader	SpectraMax M2	Molecular Devices
Optical Density	DU® 530 Life Science	Beckman and Coulter
Microscopic viewing of cells	BX51	Olympus
Incubator	Orbital Mixer	Ratek
Incubator	Laboratory Incubator	Thermoline
Water bath	180 Series Water bath	Precision
Lysis of cells	Bead Beater	Biospec Products
DNA Quantification	Fluorometer Qubit™	Invitrogen
Autoclave	GE L Series	Gentige

Table 2.1: *S. cerevisiae* strains used throughout this project.

Strain Name	Shorthand used in this thesis	Genotype	Mating Type	Source
W303-1A	WA	*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	**a**	D. Stanley, Victoria University
W303-1B	WB	*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	α	J. Bellon, Australian Wine Research Institute
FY4	FY4	Wild-type strain closely related to S288C - *GAL*	α	J. Bellon, Australian Wine Research Institute
DBY745	DBY	*ura3-52, leu2-3, 112ade1-100*	α	I. MacCreadie, Commonwealth Scientific and Industrial Research Organization
SM	SM	*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	**a**	D. Stanley, Victoria University
CM	CM	*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	**a**	D. Stanley, Victoria University
W303-1A	WBU	*leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15*	α	Generated in this project (Chapter 4)
SM	SML	*112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	**a**	Generated in this project (Chapter 4)
CM	CMH	*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1*	**a**	Generated in this project (Chapter 4)
C3-7C	C3-7C	*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1*	**a**	Generated in this project (Chapter 5)
C3-2A	C3-2A	*112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	**a**	Generated in this project (Chapter 5)

2.3 Media

All media was produced using recipes listed below and prepared in MilliQ water and sterilized by autoclaving at 121°C for 15 minutes unless stated otherwise.

2.3.1 YPD (Yeast Peptone Dextrose)

Reagent	Concentration (g/L)
Yeast Extract	10
Bacteriological Peptone	20
Dextrose*	20
Bacteriological Agar**	15

2.3.2 YPG (Yeast Peptone Glycerol)

Reagent	Concentration (g/L)
Yeast Extract	10
Bacteriological Peptone	20
Glycerol*	30
Bacteriological Agar**	15

2.3.3 Sporulation media

Reagent	Concentration (g/L)
Yeast Extract	0.5
Amino acid drop-out	0.5
Potassium Acetate	3
Dextrose**	0.5

2.3.4 LB (Luria Broth)

Reagent	Concentration (g/L)
Bacteriological Tryptone	15
Yeast Extract	5
NaCl	10

2.3.5 SC (Synthetic Complete)

Reagent	Concentration (g/L)
Yeast Nitrogen Base (with Ammonium sulphate)	6.7
Drop-out mix****	0.67
Dextrose	20
Bacteriological Agar	15

***The following table lists the ingredients added to SC medium to select for progeny from mating experiments in which parental strains have complementary auxotrophies the cognate nutrients were omitted.

Nutrient	Mass (g)
Adenine hemisulfate	2
Arginine HCl	2
Histidine HCl	2
Isoleucine	2
Leucine	4
Lysine HCl	2
Methionine	2
Phenylalanine	3
Serine	2
Threonine	2
Tryptophan	3
Tyrosine	2
Uracil	1.2
Valine	9

2.4 General culturing method

Yeast strains were inoculated from <3 week old YPG plates into 20 mL of liquid YPD in 50 mL Erlenmeyer flasks. Flask cultures were shaken at 150 rpm and incubated at 30°C overnight in aerobic conditions. A 2 mL inoculum was transferred to 200 mL of fresh medium and cells allowed to reach exponential phase (OD_{600} 1.5-2.0).

2.5 OD_{600} to monitor yeast growth

Optical density of yeast cultures were determined at 600nm using a Beckman Coulter™ DU® 530 UV/Vis Spectrophotometer using Greiner Bio-One 3 ml Semi-Micro-Cuvettes, (10 x 10 x 45 mm), manufactured from crystal clear polystyrene. The spectrophotometer was calibrated against sterile medium of the same composition as was used for yeast incubations. At high culture densities, samples were diluted with filtered water (0.2 mm filter MiliQ™) to give OD_{600} readings in the range of 0.1 - 0.5. Medium used for spectrophotometer calibration was diluted with filtered MiliQ™ water according to the dilution factor used for sample preparation.

2.6 Yeast Mating

Mating was conducted on YPD agar plates. Strains to be mated were transferred from frozen stocks onto YPD agar plates and incubated at 30°C for 24 hrs. Similar amounts of cells were then loop-transferred onto fresh YPD plates, to create two patches in close proximity. These were subsequently mixed with each other on the surface of the plate using a sterile loop, and spread as an even layer. The mixed cultures were incubated for 24 hrs at 30°C, after which they were harvested with a sterile loop, and streaked onto selective drop-out medium. Progeny from crosses were confirmed using mating-type PCR.

2.7 Long term storage of strains

Strains were streaked onto YPG plates, and single colonies were inoculated into Protect vials (TSC Ltd), inverted several times, and excess liquid removed leaving inoculated beads. The vials were stored at -80°C, and a single bead was removed and placed onto YPD plates when a fresh culture was required.

2.8 Dissection of tetrads

Cells were streaked onto sporulation plates and allowed to grow at 30°C for 2 days, then left at room temperature for a further 2 days. Plates were checked by

microscopy at 100x magnification with a light microscope. Following sporulation, asci walls were removed using 15 mg/mL zymolase (ICN Zymolase – 20T Arthrobacter Luteus 2000 U/g). A small colony from sporulation plates were inoculated into 50 μL zymolase solution and incubated at 37°C for 15 min. 50 μL of 1 M sorbitol and 50 μL sterile water were added to the cell suspension. A small amount of the suspension was gently streaked onto a level 10 mL YPAD plate and left to dry. The plate was inverted and tetrads dissected at 20x magnification using the Singer MSM Micromanipulator. Dissection plates were incubated at 30°C for 3 days. Colonies were replica plated onto selective media to determine auxotrophic requirements and mating-type was determined was PCR verified. The ET of spores was determined using RETA described in Chapter 3.

2.9 Yeast DNA extraction

Yeast was grown overnight in 10 mL YPD and centrifuged at 4,000 rpm for 5 min. The cells were washed in 1 mL sterile water and resuspended in 200 μL breaking buffer (2% TritonX-100, 1% SDS, 100 mM NaCl, 10 mM Tris.Cl, 1 mM EDTA). A solution PCI (Phenol/Chloroform/Iso-amyl alcohol to ratio of 25:24:1 respectively) and 0.3 g of 500 μm acid-washed glass beads were transferred to a screw cap eppendorf tube. The pellet was homogenized in the Beckman Bead Beater on low for 3 min. 200 μL of TE was added and vortexed for 40 sec and centrifuged on a bench top centrifuge (Beckman) at 13,200 rpm for 5 min. The top layer of the supernatant was transferred to a fresh tube and 1 mL of absolute ethanol was added. The tube was mixed by inversion and placed on ice for 30 min to precipitate the DNA. The DNA pellet was centrifuged at 13,200 rpm for 5 min, and washed in 500 μL of 70% (v/v) ethanol. Ethanol was removed by pipetting, and DNA dried using a vacuum. The DNA pellet was resuspended in 300 μL of TE, 3 μL of RNaseA (10mg/mL) added, and incubated for 5 min at 37°C. 300 μL of PCI was added and the solution vortexed for 40 sec. The pellet was centrifuged at 13,200 rpm for 5 min, and the top layer removed to a fresh tube where 130 μL ammonium acetate and 1 mL of absolute ethanol was added, mixed by inversion and incubated on ice for 30 min. The pellet was centrifuged at 13,200 rpm for 5 min in a microfuge, and washed in 500 μL of 70% (v/v) ethanol after which residual ethanol was removed by vacuum. The final purified gDNA was resuspened in 30 μL TE (or sterile water if to be used in restriction digests) and allowed to dissolve overnight at 4°C. The DNA

was stored at -20°C, and its integrity determined by running on a 0.8% agarose gel alongside a commercial *S. cerevisiae* gDNA standard at 237 ug/mL.

2.10 Ethidium Bromide Staining of gels

Gels were stained for 15 min in 1μg/mL ethidium bromide solution and destained in water for 10 min. Gel images were taken by exposing stained gels to UV transilluminator (Kodak).

2.11 Determining concentration and purity of genomic DNA

The purity and concentration of DNA was determined from $A_{260/280}$ measurements (?) in a UV-VIS Spectrophotometer (Beckman). DNA samples were diluted 1:100 in water and read in a quartz micro cuvette. Determination of DNA integrity was visualized on a 0.8% agarose gel using a commercially available *S. cerevisiae* gDNA standard (Promega). In later experiments, a fluorometer (Invitrogen Qubit) became available, which allowed more accurate and rapid quantification of DNA. The Quant-iT ds DNA BR Assay kit was used in conjunction with the fluorometer to accurately quantify DNA.

2.12 Purification of DNA for sequencing

Amplified DNA fragments to be sequenced were purified using UltraClean™ PCR Clean-up DNA Purification Kit (MO BIO Laboratories, Inc.). DNA samples were re-suspended in 200 μL of SpinBind buffer (supplied with the kit), transferred to spin filter units and centrifuged at 16,000 g for 30 sec. The spin filter basket was removed and liquid flow-through discarded from the collection tube by decanting. Next, the spin filter basket was replaced in the same tube, 300 μL of 80% (v/v) ethanol was added and spun for 60 sec at 16,000 g. The flow-through was discarded by decanting and the spin filter basket placed back into the same collection tube and spun again for 60 sec at 16,000 g to remove residual ethanol. Subsequently, spin filter baskets were transferred to new collection tubes and 50 μL of sterile MiliQ™ water was added and left for 60 sec. Tubes were spun for 60 sec at 16,000 g and spin filter baskets discarded. The solution containing DNA to be sequenced was collected in collection tubes.

2.13 DNA sequencing and analysis of sequencing data

DNA sequencing was conducted at The Australian Genome Research Facility, Ltd. (AGRF). Purified DNA, sterile MiliQ™ water and sequencing primers were aliquoted into 1.5 µL sterilized micro-centrifuge tubes. The final concentrations of DNA and primers were prepared according to the requirements of AGRF. Sequencing data obtained from AGRF was processed using Vector NTI software. Chromatograms of each sequence were examined manually for quality and only unambiguous, clear sequencing data was used to assemble the fragments.

2.14 Standard PCR Protocol

PCR reaction master mixes were prepared as listed in the table below, unless otherwise stated.

Component	Stock Concentration	Volume (µL)
Sterile Water	N/A	12.8
Reaction Buffer	10x	2.5
Magnesium Chloride	25 mM	2.5
dNTPs	1.25 mM	4.0
Primer 1	100 mM	1.0
Primer 2	100 mM	1.0
Taq polymerase*	5 U/µL	0.2
Template DNA	~500 ng/µL	1.0

*Astral and AB gene *Taq* polymerase kit were used for routine PCR. For high-fidelity PCR, the Roche High Fidelity PCR kit was used.

2.15 Colony pick PCR

Colony Pick PCR is a rapid method without the requirement of genomic DNA isolation. Fresh (<2 day old) single colonies of approximately 1 x 1 mm were suspended in 15 µL sterile water and heated to 95°C for 20 min in a PCR cycler. Tubes were centrifuged for 1min at 13,200 rpm. 10 µL of the supernatant was added to the PCR reaction mixture.

2.16 Verification of strain identity

Strain identities were verified routinely throughout the project using Mating-type (Methods 2.17.1) and Transposon PCR methods (2.17.2).

2.16.1 Mating-type PCR

To determine the mating type of yeast strains PCR, primers specifically targeting the mating type locus, Mat **a** and Mat α, were used (Illuxley C., 1990). The PCR reaction mix was made as follows:

Solution	Stock Concentration	Volume (μL)
Sterile Water	N/A	12.3
Reaction Buffer	10x	2.5
Magnesium Chloride	25 mM	2.5
dNTPs	1.25 mM	4.0
Mat Locus primer	100 mM	0.5
Mat **a** primer	100 mM	0.5
Mat α primer	100 mM	0.5
Taq polymerase (Astral)	5 U/μL	0.2
Template DNA	~500 ng/μL	1

Mating type primers	Sequence 5'-3'
Mat locus	AGTCACATCAAGATCGTTTATGG
Mat **a** specific	GCACGGAATATGGGACTACTTCG
Mat α specific	ACTCCACTTCAAGTAAGAGTTTG

The PCR cycle was as follows: 92°C for 2 min, followed by 30 cycles of 92°C for 1 min, 58°C for 2 min, 72°C for 2 min then 72°C for 10 min. PCR products were visualized on a 1.5% agarose gel run at 90 V for 90 min and stained in 0.001 mg/mL of ethidium bromide for 15 min and destained in water for 30 min. Gel images were taken with a UV Transilluminator.

2.16.2 Ty1 transposon PCR

Transposon PCR was used to verify strain identity. Transposon primers, listed below, were used to target Ty1 elements and amplified using the standard PCR protocol (Methods 2.15). PCR amplified fragment were loaded on a 1.5% agarose

gel and stained with ethidium bromide. The banding patterns were compared to known standards.

Transposon Primers	Sequence 5'-3'
Fwd	CAAAATTCACCTATA/TTCTCA
Rvs	GTGGATTTTTATTCCAACA

2.17 Yeast Transformation Protocol

Yeast transformations were performed essentially as described in Gietz et al (2002). Briefly, cells were grown overnight in 10 mL 2x YPD in 50 mL Erlenmeyer flasks. Cells were then inoculated into fresh 2x YPD in order to obtain high-density exponential phase cells. Cells were centrifuged at 2,000 rpm for 5 min and washed in sterile water. The transformation mixture was made up as listed in the table below.

Reagents	Volume (μL)
PEG 3350 (50% w/v)	240
1.0 M Lithium Acetate	36
Denatured salmon sperm DNA	50
PCR DNA product (~5 μg DNA)	34

PEG 3350 (Sigma) was dissolved in water and sterilized using 0.22 μm filter. Salmon sperm carrier DNA was denatured prior to making the reaction mix, by placing it on ice for 2 min and then in boiling water (x3) for 0.5 min. Washed cells were re-suspended in transformation reaction mixture and heat shocked at 42°C in a water bath for 40 min. Cells were centrifuged at 13,200 rpm for 30 sec and the supernatant removed. Cells were gently re-suspended in 500 μL sterile water, centrifuged again and re-suspended in 2x YPD and then recovered overnight. Then 200 μL of the transformation mix was spread onto selective medium. Plates were incubated at 30°C until colonies appeared, which was usually within 3 days. Colonies of transformants were streaked onto fresh plates.

2.18 Southern blot

Southern blots were performed as described in Current Protocols in Molecular Biology, Unit 2.9A. All Southern blot solutions listed below apart from Blocking solution were autoclaved at 121˚C for 15 min.

Denaturing Solution

Reagent	Concentration (M)
NaOH	0.5
NaCl	1.5

Neutralization Solution

Reagent	Concentration (M)
Tris.HCl	0.5
NaCl	3

20xSSC

Reagent	Concentration (M)
Sodium Citrate	0.3
NaCl	3

10x Maleic Acid Solution

Reagent	Concentration (M)
Maleic Acid	0.1
NaCl	0.15

Detection Buffer

Reagent	Concentration (M)
Tris.HCl	0.1
NaCl	0.1

10x Blocking Solution

Reagent	Amount
Blocking Powder	5 g
10 x Maleic Acid*	45 mL

2.18.2 Digoxygenin (DIG) probes for Southern blots

Probes for Southern blots targeting auxotrophic markers *HIS3*, *LEU2* and *URA3* were made by amplifying the markers from a S288C gDNA. The following table lists the primers used to amplify the markers using PCR.

Auxotroph primer	Sequence 5'-3'	Product size (bp)
Histidine Fwd	CACCCCGTAATTGGTCAAC	2089
Histidine Rvs	ATCCTCGGGGACACCAAATA	
Leucine Fwd	GCGGAACCGGCTTTTCATAT	1274
Leucine Rvs	TAACTTCTTCGGCGACAGCA	
Uracil Fwd	AAGAACGAAGGAAGGAGCACA	1127
Uracil Rvs	TTGGTTCTGGCGAGGTATTG	

For the amplification of the auxotrophic markers, the following listed PCR master mix was used. A DIG/dNTP mix was made up to a ratio of 1:2. The PCR cycle consisted of the following 92°C for 2 min, (92°C for 1 min, 58°C for 2 min, 72°C for 2 min) x 30, and 72°C for 10 min in a Thermocycler.

Solution	Stock Concentration	Volume (μL)
Sterile Water	N/A	10.8
Reaction Buffer	10x	2.5
Magnesium Chloride	25 mM	2.5
DIG/dNTP mix	10 mM	5.0
Primer 1	100 mM	1.0
Primer 2	100 mM	1.0
Taq polymerase	5 U/μL	0.2
Template DNA	~500 ng/μL	2.0

2.18.3 DNA Restriction digests

Genomic DNA was isolated form yeast using the method described in Section 2.10. Samples were diluted with Tris buffer, so that all DNA extracts were equivalent concentrations and digested with suitable restriction enzymes; restriction enzymes were chosen on the basis that they did not have any sites within the genes of interest according to the SGD (http://www.yeastgenome.org). Fragments containing genes of interest would be of known size. 10 μg of DNA was digested for 12 hrs with 10 units of the restriction enzyme and x1 buffer.

2.18.4 Gel electrophoresis for resolution of restriction digests

Digested DNA (50 μL) was run on a 1% agarose gel in 1x TBE. Following this the gel was stained in a solution of 1 μg/mL ethidium bromide and visualized on a UV transluminator.

2.18.5 Southern blot assembly

The Southern blot was assembled as shown in the figure below (http://www.currentprotocols.com/protocol/mb0209a).

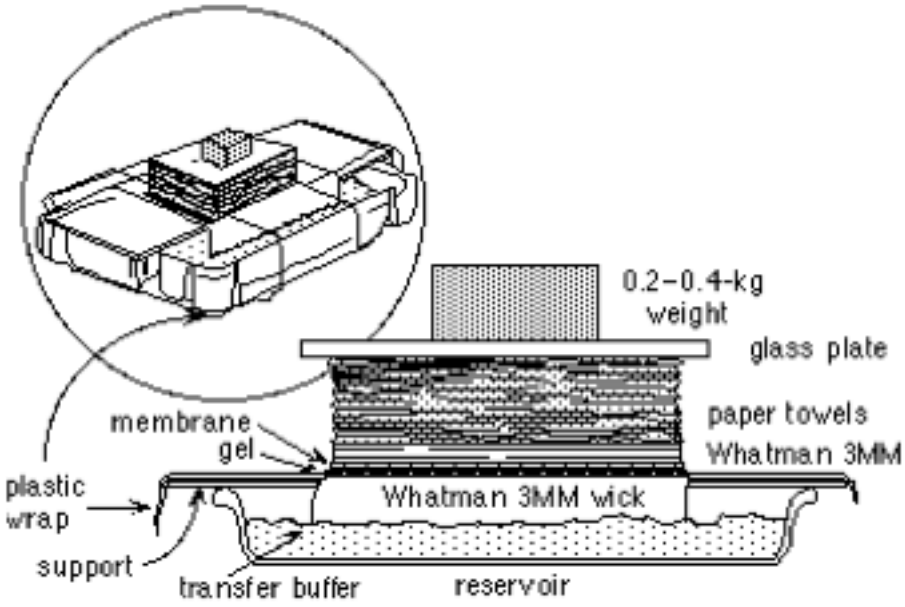


Figure 2.1 Schematic diagram of Southern Blot assembly

2.18.6 Probing the southern blot

The Southern blot membrane was placed between two mesh sheets (Diversified Biotech) previously soaked in Easy Hyb (Roche) solution. The sandwiched membrane was placed into a roller bottle (Schott) and 20 mL Easy Hyb added. The membrane was pre-incubated in the Easy Hyb solution for 2 hrs at 42°C. The DIG probes (10 ng/mL) were denatured at 100°C for 10 min then placed on ice until ready for use. The Easy Hyb solution was then removed from the roller bottle and the denatured DIG probe were added to the appropriate membrane accordingly. The blot was hybridized overnight at 44°C.

2.18.7 Stringent washes and probe detection

All washes and incubation steps described below were performed at room temperature unless stated otherwise. The southern blot membrane was removed from the roller bottle and washed twice in 2x SSC/0.1 % SDS solution with gentle

rocking for 5 min. The blot was then washed twice in 0.5x SSC/0.1 % SDS at 55°C for 5 min. The membrane was then placed in 1x Maleic acid buffer/ 0.03 % Tween 20 for 15 min. The membrane was then placed in blocking solution for 45 min. The blocking solution was decanted and 50 mL 0.075 U/mL Anti-Digoxygenin-Alkaline Phosphatase (Roche) was added. The membrane was incubated in the antibody for 60 min with gentle rocking. The antibody solution was then discarded and washed twice in 1x Maleic acid buffer/0.03 % Tween-20 for 15 min. The membrane was incubated in detection buffer for 5 min and then placed between two sheets of plastic. The membrane was then incubated in CPD-Star (Roche) left for 30 min and then exposed to X-ray film (Amersham) for 1 min. The film was developed using the Kodak imager X-ray film developing machine (Cowanman 2000 IR).

2.19 Construction of a genomic library

Genomic DNA was isolated as described in Section 2.10. The DNA was digested with various concentrations of *Sau*3AI (NEB) and run on an agarose gel. DNA fragments between 5-12 kb were isolated and purified using a gel purification kit (Qiagen). The P416 plasmid was isolated and dephosphorylated using CIP (Calf Intestinal Phosphatase). The isolated inserts and plasmids were added and T4 ligase (NEB) was added and incubated overnight at 4˚C. The ligation mixture was then transformed into *E. coli* DBH10 using an Electroporator (BioRad) at 1.5V 5 sec and plated onto ampicillin LB plates. Plasmids from transformants were isolated and cut with *Eco*RI (NEB) to produce a linear fragment, which was run on an agarose gel at 95 V for 1 hour and stained with ethidium bromide.

2.20 Affymetrix tiling microarrays

Affymetrix Tiling Arrays are spotted with 25-mer probes in a tiled format of the *S. cerevisiae* genome. The probes are tiled in such a way that there is a 20 bp overlap with a 5 bp resolution. Genomic DNA was isolated from the yeast strain to be analysed and sent to The Australian Genome Research Facility (AGRF) for hybridization to Tiling microarrays. The genomic DNA was sheared and hybridized onto Affymetrix GeneChip *S. cerevisiae* 1.0R Tiling Arrays. AGRF returned output signal intensities of the probes were in .cel format and analysed using both SNP Scanner (Gresham et al. 2006), Integrated Genome Browser (IGB) and Tiling Analysis Software (TAS). (For further information refer to

http://www.affymetrix.com/estore/browse/products.jsp?navMode=34000&productId=131499&navAction=jump&aId=productsNav#1_1)

2.21 Whole genome Illumina sequencing

Genomic sequencing was outsourced to Geneworks, which used Illumina Genome Analyser-Solexa sequencing techniques. Genomic DNA was isolated as described in Section 2.10. For the Solexa Sequencing technique the genomic DNA is randomly sheared and adapters are ligated to the ends. The DNA strands are then attached to the surface of a flow cell. The DNA then undergoes bridge amplification forming clusters. A DNA polymerase then uses the hybridized DNA fragments as a template and incorporates nucleotides labeled with different fluorescence with the 3'-OH group blocked only allowing a single incorporation event. The unincorporated nucleotides are washed away and emissions of the incorporated nucleotides are sequentially recorded by the Genome Analyzer (Illumina). After the emission of fluorescence nucleotides are recorded, the blocking group is chemically removed for the next nucleotide to be incorporated. This procedure is repeated for discrete read lengths of a=25-35 bp. The output of the sequencing is 35 bp reads which are assembled against a W303-1A reference sequence (kindly donated by Dr. Chris Harris, Sanger Institute) in CLC Genomics Workbench (For further information http://www.illumina.com/support/documentation.ilmn)

Chapter 3

Development of a Rapid Ethanol Tolerance Assay (RETA) to quantify ethanol-tolerance in *S. cerevisiae*

3.1 Introduction

The general aim of this project was to identify genes that confer the ethanol tolerance phenotype in the ET mutant strains of *S. cerevisiae* W303-1A (WA) [1]. The mutants were generated by Dr. Dragana Stanley, a former PhD student at Victoria University (Stanley 2009). An adaptive evolution strategy, with increasing ethanol concentrations as the selection pressure, was used by Dr. Stanley to generate two ethanol-tolerant mutants. The Chemical Mutant (CM) was isolated from a chemically (ethyl methane sulphonate, EMS) mutagenised population of WA, whereas the Spontaneous Mutant (SM) was generated without the aid of mutagenic agents.

A classical genetics approach, backcrossing, was used to characterize the genes responsible for conferring ET in SM and CM (see Chapter 4). Numerous progeny were generated from this work, and therefore a high throughput method to assess ethanol tolerance was required.

The method used to determine ethanol tolerance of progeny needed to reproducibly and accurately distinguish between the ET mutants and the parental strain. The most distinguishing feature of the ET mutants compared to the parental strain is growth rate under ethanol stress conditions, which can be determined by optical

[1] Shorthand nomenclature for strains used in this and subsequent chapter.

Short-hand Nomenclature	Identity of Strains
WA	Wild-type **W**303-1**A** parental strain
SM	**S**pontaneous ethanol-tolerant **M**utant
CM	**C**hemical-induced, ethanol-tolerant **M**utant

density (OD) readings or viable counts. However, such distinguishing features are not obvious in all growth monitoring assays, such as the drop plate method (Hu,et al. 2007). The drop-plate method is a qualitative comparison of colony appearance rates when yeast cultures are dropped in controlled volumes of media onto YPD-plates containing various ethanol concentrations. The toxic effect of ethanol on the cells causes them to have extended growth rates and thus, an extended amount of time is required before colonies will appear on the plates. This method can be problematic, since the volatility of ethanol can result in significant variation in ethanol concentrations across the plate. Also, the drop-plate method does not provide a quantitative data; by dropping small volumes of culture it is difficult to obtain viable cell numbers due to merging of colonies and, even when individual colonies are observed, often there are small numbers leading to statistical inaccuracies.

A variation in the ethanol drop-plate method is the exposure of cells to medium containing ethanol for a limited time period and then plating the cells to observe strain survivability (Hu et al. (2007) Ogawa et al. (2000)). This method however is not quantitative for determinations of growth rate.

ET is a complex phenotype to quantify, being defined by various phenotypic parameters including:

Parameter	Reference
Cell viability	e.g. Stanley et al. 2009a
Adaptation (lag) period	e.g. Chandler et al. 2004
Growth rate	e.g. Novotny et al. 1994
Death rate	e.g. Santos et al. 2008
Biomass production	e.g. Alper et al. 2006
Ethanol production	e.g. Arguesco et al. 2009

Shake-flask cultures are used in many studies to differentiate ET phenotypes, however, it was impractical to use such an approach to quantify the ethanol tolerance phenotypes of the numerous progeny generated for work in this thesis. With this in mind, it was important in the initial stages of this project to develop a rapid, sensitive and reliable ET assay that was suitable for screening numerous mutants simultaneously. The assay needed to be:

1. *Capable of reliably distinguishing between ethanol tolerance levels of W303-1A parent and the ET mutants.*
2. *Reproducible*
3. *Efficient*- Erlenmeyer flask cultures are time-consuming, laborious and limited in the number of strains that can be screened simultaneously. The new method needed to be rapid, straightforward and allow a relatively large number of strains to be screened simultaneously.
4. *Minimise ethanol loss during assaying*- Ethanol evaporation can result in unreliable and varied ethanol concentrations during the assay.

Microplate-grown cultures may, under some circumstances, be used as an alternative to flask-grown cultures for characterizing microbial phenotypes. A number of recent studies used microplate cultures to determine the growth profiles of microbial strains in the presence of inhibitory substances (Duetz, 2007). The results in the literature were shown to be reproducible and reflective of results generated by shake-flask cultures. For example, Weiss et al. (2004) demonstrated that microplate cultures can be used to characterize the impact of oxidative stress agents on the growth of laboratory yeast strains. A number of other workers have also used microplate cultures to assay yeast growth under various conditions (Baranyi, 1995, Warringer, 2003, Weiss, 2004, Toussaint, 2006). Liccoli et al 2010 successfully applied microplates cultures for the screening of wine strains for phenotypes of interest. Microtitre plate cultures offer the advantage of high-throughput, quantitative assays compared to the drop-plate method. The applicability and ability of the microplates to cover the aforementioned criteria is discussed in this chapter.

Microplate cultures are amenable to optical density measurements to determine biomass levels, and therefore provide a means of determining culture growth. Although, optical density measurements cannot ascertain cell viability and are influenced by changes in cell size, they are convenient, rapid and, with an appropriate design and the right controls, can provide sufficient resolution to accurately distinguish between different ethanol-tolerance levels.

This chapter describes the development of the Rapid Ethanol-Tolerance Assay (RETA) for use as a screening tool to determine differences in ET phenotype.

3.2 Results

3.2.1 Growth validation of the ethanol tolerance phenotype of ET mutants

Stanley (2009) created ethanol tolerant mutants from a laboratory yeast strain WA. This projects aim is to identify the genes that confer ethanol tolerance in the ET mutants. It was important to confirm the ethanol tolerance phenotype of SM and CM. This was done by performing a growth curve assay shown in Figure 3.1. The ethanol tolerance phenotype of the ET mutants were validated.

3.2.2 Development of a Rapid Ethanol Tolerance Assay (RETA): the basic parameters

Microplate cultivation has had a controversial history regarding its ability to deliver reproducible results (Shekarachi et al., 1984, Gellert et al., 1999, Silberblatt et al. 2000). For example issues such as static incubation can limit culture oxygenation and can cause cells to settle to the bottom of the wells. Differences in evaporation rate across plates can lead to significant edge effects. This is where higher ventilation rates at the plate edges result in uneven evaporation of volatiles. However, developments in sealing membrane technology and microplate readers have been able to minimize the impact of the edge effects (Zimmermann, 2003). For example Breathe-easy^{TM} membranes (Diversified Biotech) are gas permeable membranes that, while permitting gas exchange, are impervious to culture volatiles minimizing their evaporative loss. Microplate readers have also been improved by the installation of top and bottom plate heaters to ensure uniform temperatures across the plate.

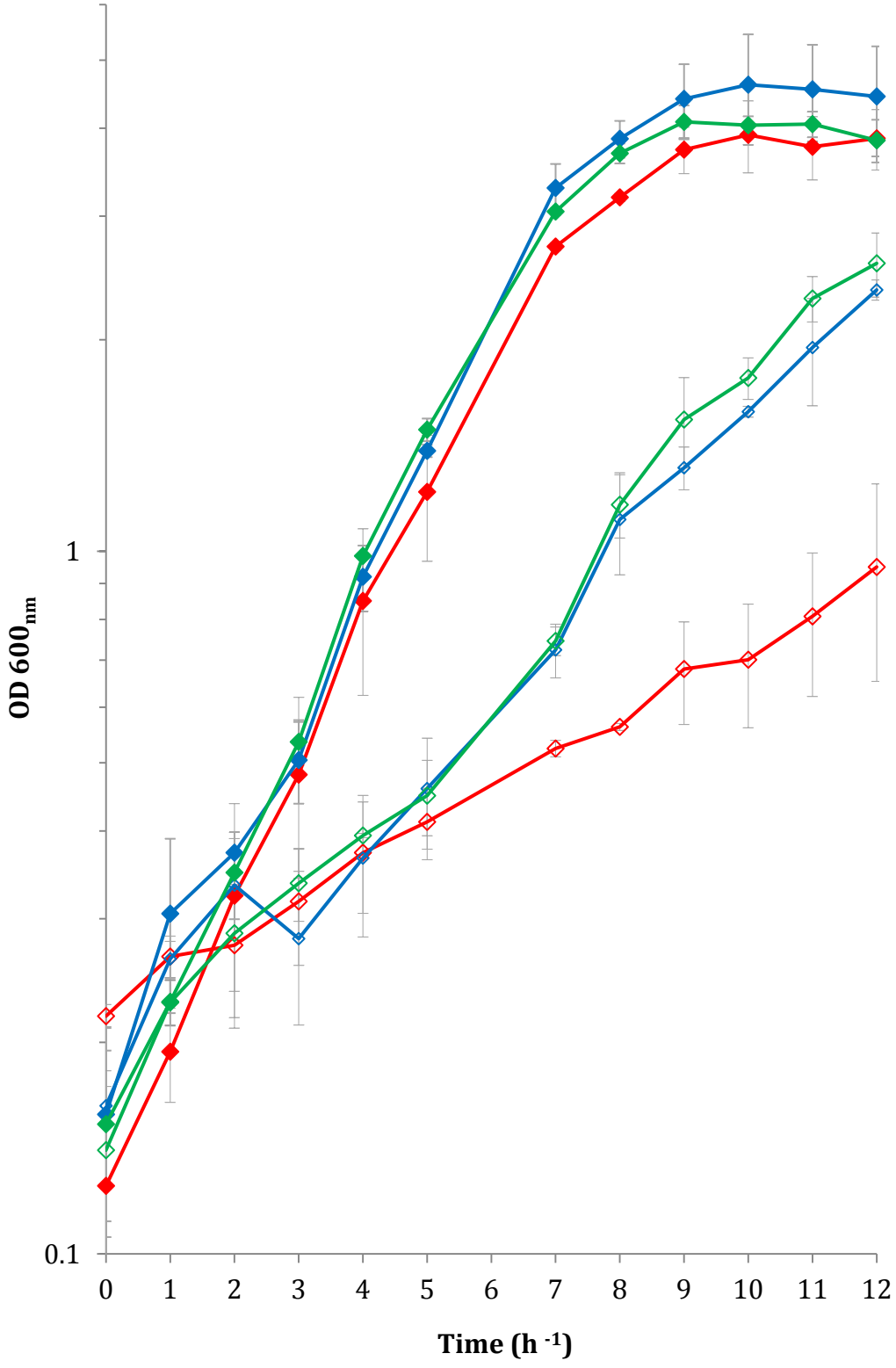
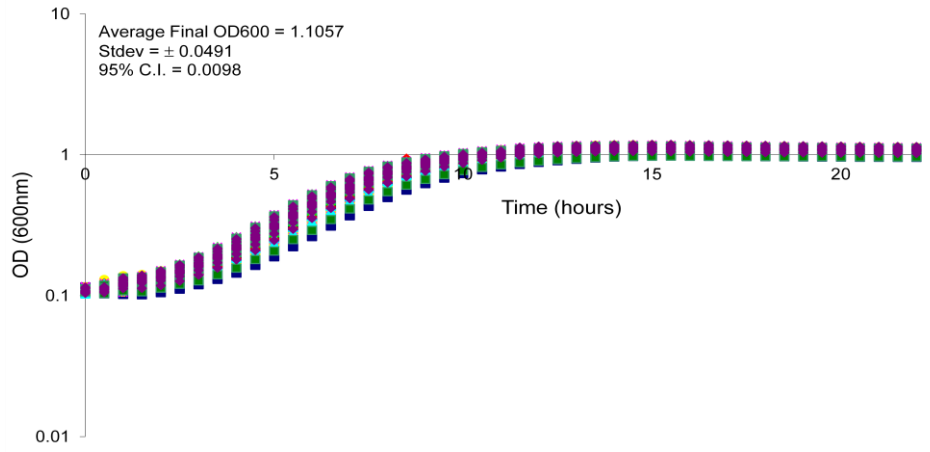


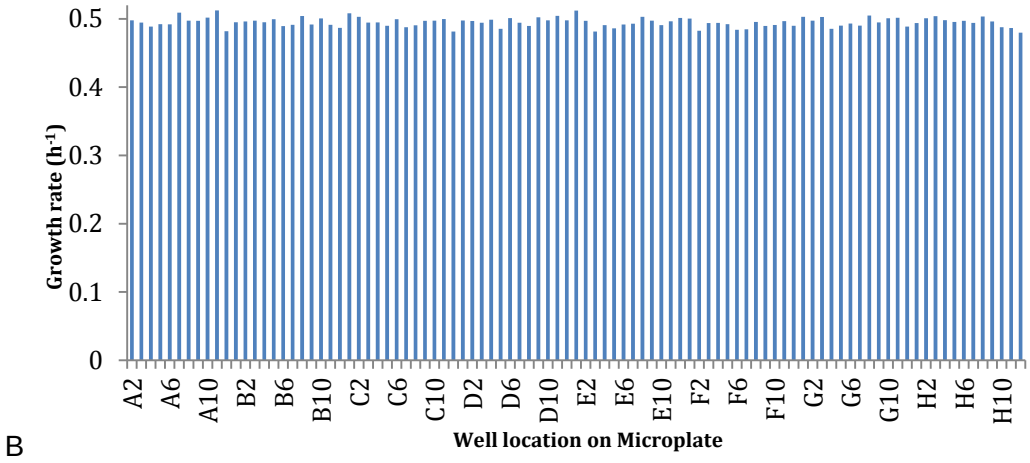
Figure 3.1: Growth profiles of nET parental WA (■) and ET mutants SM (■) and CM (■) in the absence (closed symbols) and presence (open symbols) of 6% (v/v) ethanol. Cultures were incubated in YPD at 30°C/130 rpm.

Firstly variance of growth rates across the wells in a single plate and across independent plates to determine the reproducibility of using microplates as a culture vessel for the assay. This was achieved by measuring the variability in growth curve profiles across all wells subjected to identical environmental conditions. Mid-exponential phase WA was inoculated (all inocula were derived from the same parent culture) into the wells of a single microplate; each well had an inoculum of OD_{600} of 0.1 and sealed with Breathe-easy^{TM} membrane. The plate was incubated at 30^{o}C in an Ascent Multiskan microplate reader with automated OD_{600} readings every 30 min. The growth curves for individual wells and their corresponding growth rates are shown in Figure 3.2. The average growth rate was determined using an automated macro in Excel called DMfit (Baranyi et al. 1995). This macro was used to determine the growth rate at specified time point (i.e. exponential phase). The average growth rate of the amalgamated growth curves in all wells was 0.4733 ± 0.021 h^{-1}, however, there was some variation in the growth rates at the outer edges of the plate (Figure 3.2B). ANOVA was used to determine whether the variation significantly affected the outcome. It was found that the environmental effect, most likely due to evaporation, was not statistically significant (α=0.05).

The next experiment was designed to determine whether microplate cultivation could differentiate growth in the absence and presence of ethanol. Earlier work using shake flask cultures with the current strains showed that a substantial OD difference in the growth profile of nET and ET strains occurs in the presence of 6% (v/v) ethanol (Stanley, 2009). With this in mind, cultivations in the absence and presence of 6% (v/v) ethanol were performed in microplates to determine the significance of growth rate variation across the wells using the ANOVA student's t-test (Figure 3.3). The statistical program JMP was used to generate a diamond box plot with an associated student's t-test. The green diamond represents the average of the data and the circles on the right hand side group the subjects using ANOVA. If the subjects are statistically significant, two separate circles are represented. If the group circles overlap, or there is only a single circle, then the two subjects are not statistically significant.



A

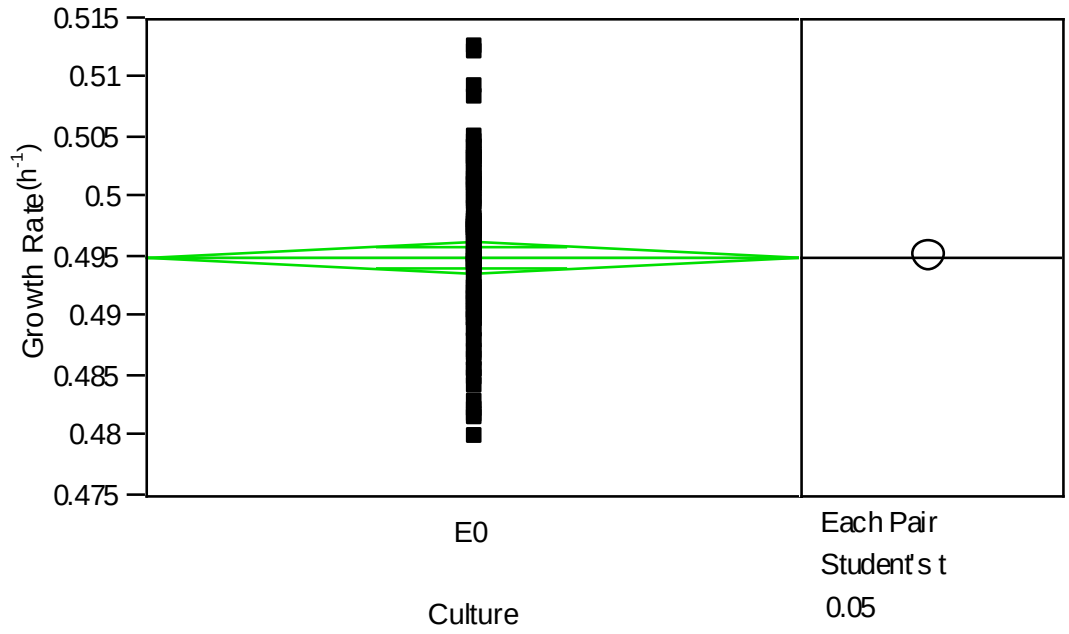


B

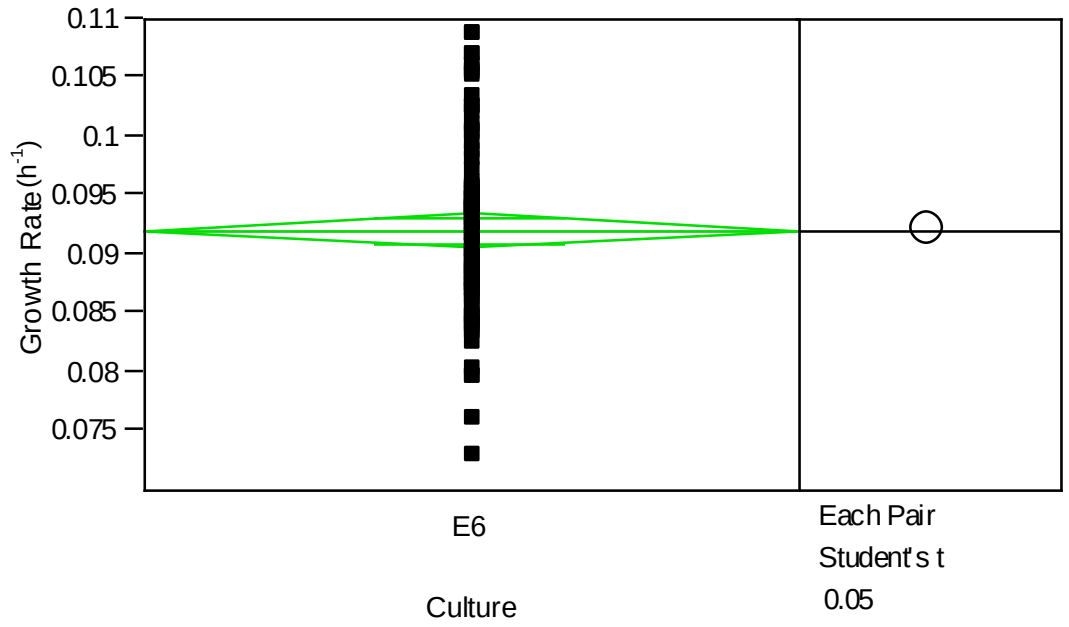
A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

C

Figure 3.2: Growth curve results in microplate wells inoculated with W303-1A in YPD and statically incubated at 30˚C. (**A**) Growth profiles of cultures in each well of a 96-well microplate. One strain was inoculated across 96-wells. Each time point captures all of the data across the plate with a different colour for each of the 96-wells. (**B**) Growth rates of each well culture; the average growth rate was 0.4733 h^{-1} ± 0.021. (**C**) Schematic diagram of 96-well microplate.



A



B

Figure 3.3: ANOVA plot of triplicate microplates inoculated with WA in all 96 wells. Microplate wells contained the following: (**A**) YPD only (E0) and (**B**) YPD containing 6 % (v/v) ethanol (E6) in triplicate. Microplates were statically incubated at 30°C in the Multiskan plate reader and covered with the Breathe-easy™ membrane. The growth rates were determined for each well. The mean growth rate for E0 was 0.4951 $h^{-1} \pm 0.0067$ and for E6 was 0.0921 $h^{-1} \pm 0.0071$ (n = 95).

The mean growth rate for cultivations in the absence of ethanol stress was found to be 0.4951 ± 0.0067 h^{-1} (Figure 3.3A). Since data points for all wells were grouped in the same circle, growth rate differences between wells were considered to be insignificant. This experiment was conducted in triplicate for cultures containing added ethanol (6% v/v), the outcomes being of particular interest due to the high volatility of ethanol. It was found that after 24 hours, growth rates in all wells (excluding the blank A1) were statistically the same at 0.0921 ± 0.0071 h^{-1} (Figure 3.3B).

A clear distinction was observed between the growth rate of cultures incubated in the presence or absence of ethanol. These results provide evidence that the edge effect was insignificant when the microplates were placed in an incubated plate reader using the Breathe-easy™ membrane and that under these conditions the microplate is able to accurately and reproducibly distinguish between the growth rates of *S. cerevisiae* W303 incubated in the presence and absence of ethanol.

3.2.3 Development of RETA: Effects of inoculum density and ethanol concentration on the growth of nET and ET strains

ET phenotypes of the parent strain WA and mutants SM and CM were used to benchmark the terms nET (WA) and ET (SM and CM). The phenotypes of these strains have been extensively studied, including their relative ethanol tolerance (Stanley, 2009). These strains were used as reference strains to validate RETA.

It was important to determine an inoculum size and ethanol concentration that would provide the clearest difference between nET and ET strains. In Erlenmeyer-flask experiments, mid-exponential phase cells inoculated to an initial OD_{620} of 0.1 and 6% (v/v) ethanol resulted in a substantial difference in growth profile between the ET and nET strains. Although these results provided some guidance on which conditions to use, it was recognized that such parameters were not necessarily optimal for use in microplate cultivation. Therefore, based on previous physiology studies (Stanley et al., 2010) and preliminary work in the current project, the three reference strains were inoculated into the microplate wells containing YPD with 6-14% (v/v) added ethanol and initial microculture OD_{620} readings of 0.05, 0.1 and 0.2; mid-exponential phase parent cultures were used to inoculate the microplate wells. incubation.

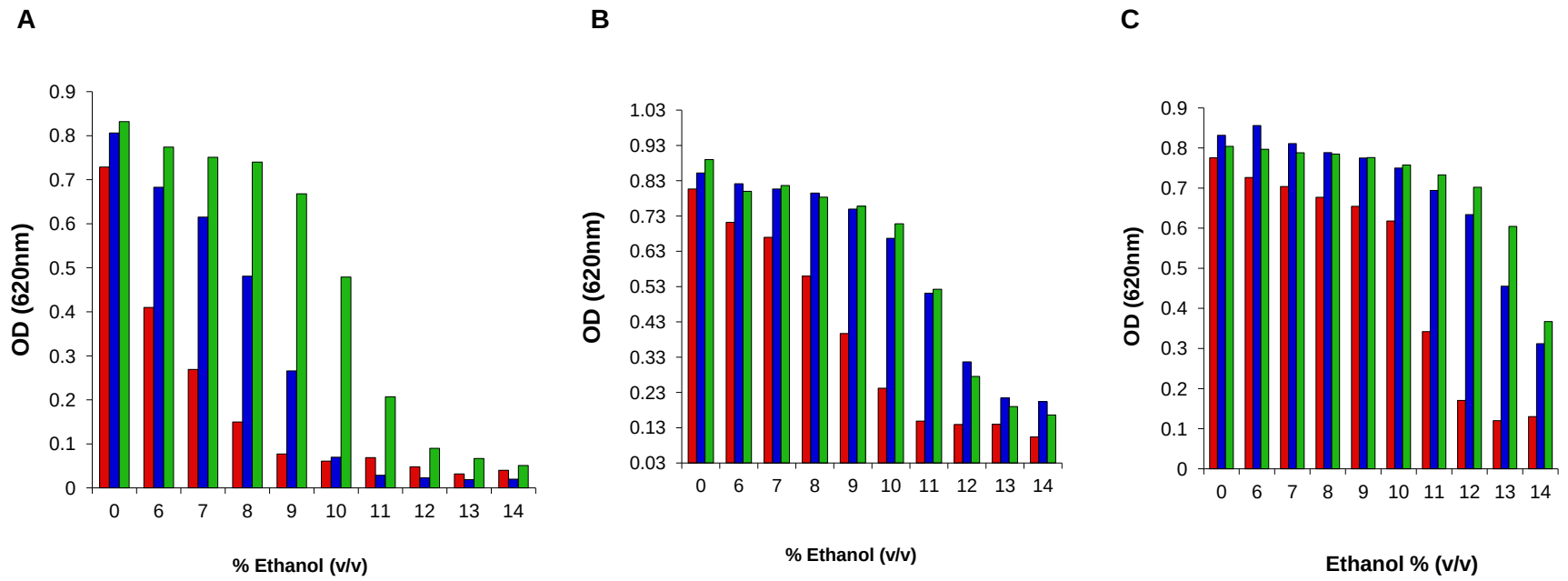


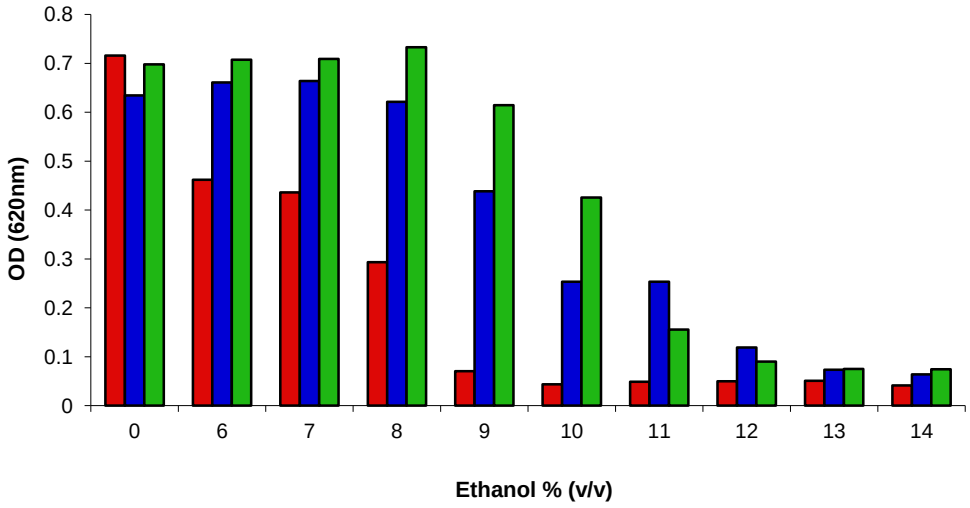
Figure 3.4: Final OD_{620} results obtained after 24 hrs of incubation in various concentrations of ethanol using initial OD_{620} of (**A**) 0.05 ± 0.0046 (**B**) 0.1 ± 0.0028 and (**C**) 0.2 ± 0.0076. Cultures of WA (■), SM (■) and CM (■) were diluted and inoculated into microplate wells to obtain the target initial OD_{620} and final ethanol concentration indicated. Each cultivation was conducted in triplicate wells in a single 96-microplate that was statically incubated at 30°C.

The BIO-RAD microplate reader was used in these preliminary experiments to obtain optical density measurements. This spectrophotometer was not automated therefore only a single set of readings were taken at 24 hours incubation for each microplate. Subsequent experiments (Section 3.3.3) used the Spectramax and Ascent Multiskan automated microplate readers that took automated readings at regular intervals during the growth curve.

Although shake flask cultivation showed substantial differences between ET and nET growth profiles when inoculated at OD_{620} 0.1 and exposed to 6% (v/v) ethanol (Stanley et al, 2010), this difference in growth was not observed in microcultures inoculated at the same density and exposed to the same concentration of ethanol for 24 hours. The optical densities of microplate cultures grown in the presence or absence of ethanol after 24 hrs incubation are shown in Figure 3.4. Strains WA, SM and CM, were inoculated at OD_{620} 0.05, 0.1 and 0.2 and exposed to ethanol concentrations between 6-14% (v/v). The difference in final OD_{620} readings of the WA and ET mutant microcultures were not as apparent at OD 0.1 (Figure 3.4B) and 0.2 (Figure 3.5C) compared to 0.05 (Figure 3.4A). Given these results, it was established that differences in the ET mutants and WA phenotype were most obvious (based on OD_{620} readings after 24 hours incubation) using micro-cultures with an initial OD_{620} of 0.05 and ethanol concentrations in the range of 6-9% (v/v).

3.2.4 Development of RETA: Effect of inoculum culture growth phase on resolution of the RETA

The experiments described in the previous section used mid-exponential phase inocula to demonstrate that microplate cultures could demarcate between nET and ET phenotypes using an initial OD_{620} of 0.05 and ethanol concentrations of 6 – 9% (v/v). The effect of growth phase on the ability of the assay to predict a nET or ET phenotype was an unknown but important aspect of the experimental design, making it important to determine the sensitivity of the assay to growth phase to minimize any potential influence of this parameter on assay accuracy.



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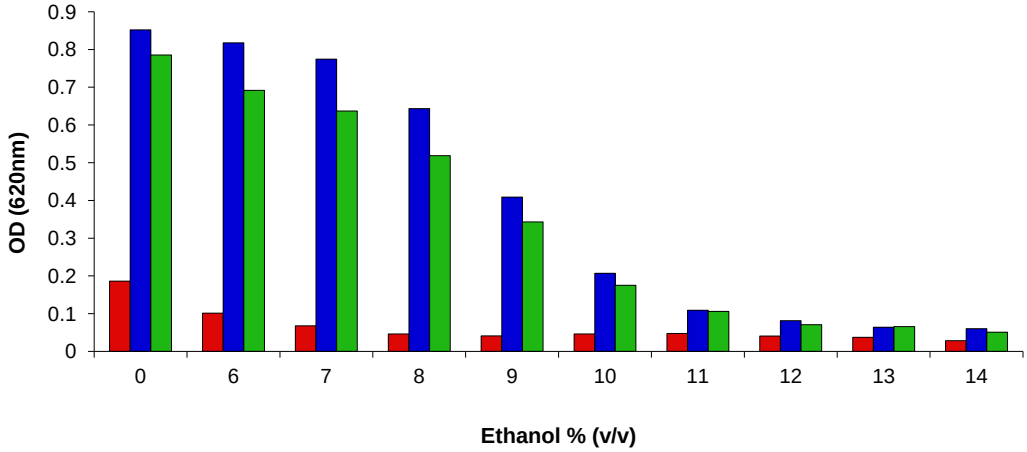


B

Figure 3.5: OD_{620} readings obtained after incubation for 24 hours of mid-exponential phase inocula (**A**) or stationary phase inocula (**B**) in microplate wells containing various ethanol concentrations. Cultures of WA (■), SM (■) and CM (■) were inoculated to an initial OD_{620} of 0.05 into YPD with or without added ethanol. Triplicate 96-microplate cultures were statically incubated at 30°C.

This was investigated by inoculating the wells with cells at mid-exponential (OD_{620} of 1-1.5) or stationary phase (OD_{620} of 2.5-3.5) to determine the influence of growth phase on the ability of RETA to distinguish between nET and ET phenotypes. Strains WA, SM and CM were exposed to various ethanol concentrations in YPD (as described in the previous section) and incubated 30°C.

3.2.5 Development of RETA: Ability of RETA to distinguish between nET and ET strains

It was found that growth phase affects the 24 hour OD_{620} readings, with inocula from stationary phase parent cultures showing little growth for the strain WA, either in the presence or absence of ethanol (Fig 3.5). The small differences in readings of WA microplate cultures after 24 hours of incubation in the presence or absence of ethanol considerably reduced the sensitivity of the assay. For this reason it was concluded that it is important to use mid-exponential phase inocula for RETA to accurately discriminate between nET and ET phenotypes. The results described in the previous sections provided preliminary evidence that microplate cultures can differentiate nET and ET phenotypes provided that specific inoculation and incubation parameters are used. It was important however to statistically verify the significance of the difference between strains WA, SM and CM in ET phenotypes identified by RETA.

Principal Component Analysis (PCA) is a statistical method for determining patterns in data sets that may not be apparent by visual inspection. PCA analysis entire raw data set, not just the average growth rate. It interprets complex data sets containing many variables; in this case OD_{620} readings, time, different strains and ethanol stress conditions. PCA can also determine variables that significantly influence the data. PCA transforms growth curve data into eigen vectors that are plotted onto the PCA diagram. Using the program Unscrambler, the raw data used for the growth curves (48 optical density measurements x 96 wells) was analysed for maximum variation between strains.

PCA was first performed on the data set from microplate experiments using 8% (v/v) ethanol since the maximum variation between nET and ET strains was observed in these growth curves; these data sets were entered into the Unscrambler program and PCA performed on the strains. The PCA plot shows that 90% of the variability in the data can be explained by the 1st Principal Component (Figure 3.6).

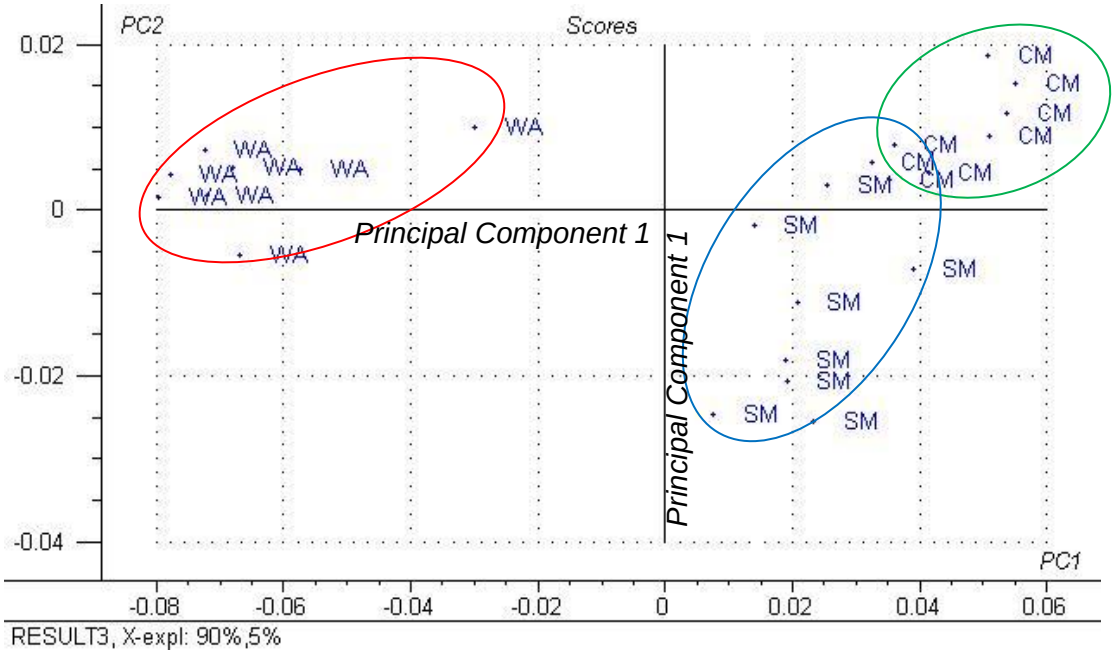
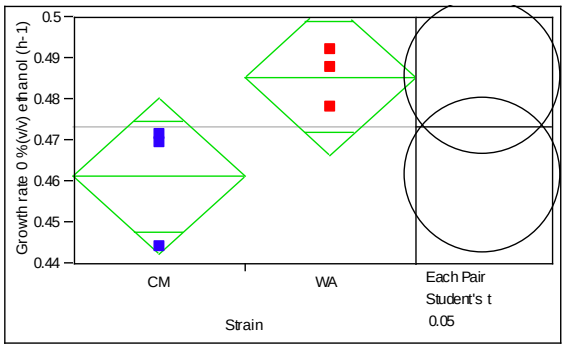


Figure 3.6: PCA of quadruplicate data from two independent microplate cultivations inoculated at OD_{620} 0.05 in YPD containing 8% (v/v) ethanol and incubated for 24 hours at 30°C. The PCA plot transformed the raw growth curve data into eigen vectors; the eigen vector scores are coordinates derived by grouping the data, taking into account data variance. The abscissa shows PC1 (Principal Component 1) where the maximum difference between the data points can be seen. The RESULT3 shown in the bottom left-hand corner indicates that 90% of the variance between the strains is shown in PC1 and 5% in PC2. The ordinate shows PC2 (Principal Component 2). The nET parent strain WA (Red Circle) eigen vectors are grouped together, with eigen vectors for ET strains SM (Blue Circle) and CM (Green Circle) forming two separate distinct groups.

The percentage value shown indicates variability in the data set according to the particular principal component. Grouping each strain into clusters by the PCA plot shows a distinction between the ET and nET strains; it is known from the growth curve results that these groups refer to ET and nET strains.

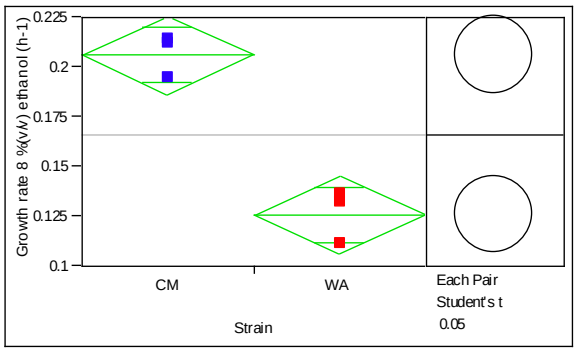
ANalysis Of VAriance (ANOVA) was performed to determine the statistical significance of the differences observed between ET and nET strains. ANOVA is a statistical method used to verify if the differences between the strains identified by RETA are significant to 95% confidence (α = 0.05) It compares variation amongst replicates of a particular sample and, for example, if the variation overlaps then the analysis would likely yield a non-significant outcome. ANOVA comparison of RETA results for WA vs CM and WA vs SM during exposure to 0 and 8% (v/v) ethanol stress are shown in Figure 3.7. In the absence of ethanol stress (*i.e.* control conditions), strains WA, SM and CM are grouped together, identifying growth rate values as not significantly different from each another. In the presence of 8% (v/v) ethanol stress, each strain is grouped separately identifying differences in ET phenotype of the strains as statistically significant.

The effect of inoculum size on the growth of the strains in various ethanol stress concentrations was described in Section 3.3.2. It was found that initial OD_{620} readings exceeding 0.05 decreased assay resolution. The growth phase of the parent culture used to prepare the inoculum was also found to impact on assay resolution, with the use of stationary phase cells decreasing resolution achieved by the RETA *i.e.* the ability to discriminate between ethanol tolerance levels of ET and nET strains was reduced.



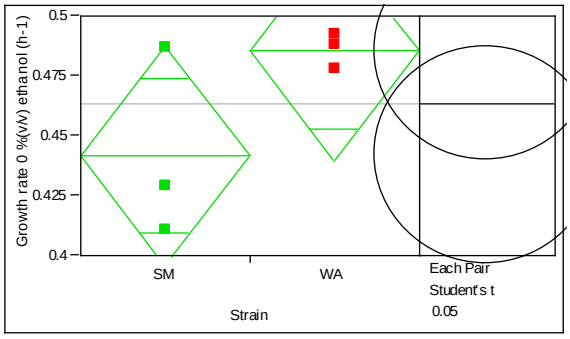
Strain	Group	Mean
WA	A	0.4856
CM	A	0.4615

A



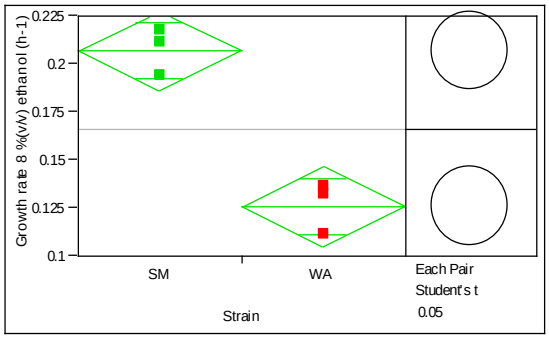
Strain	Group	Mean
CM	A	0.2063
WA	B	0.1260

B



Strain	Group	Mean
WA	A	0.4856
SM	A	0.4420

C



Strain	Group	Mean
SM	A	0.2070
WA	B	0.1260

D

Figure 3.7: ANOVA plots of growth rate (h^{-1}) comparing strain WA (nET) with strains (A and B respectively) CM (ET) or (C and D respectively) SM (ET) in the absence of ethanol or in the presence of 8% (v/v) ethanol. The ANOVA summary is shown in the tables where the strains have been classified into groups; groups not assigned the same letters are statistically significantly different. The strains were inoculated into YPD in the absence or presence of ethanol and incubated at 30^{o}C. Microplate cultures were performed in triplicate.

Overall, microplate inoculation and incubation conditions that showed maximum differenced between nET and ET phenotypes using RETA were:

1. Microplate cultures with an initial OD_{600} of 0.05.
2. An inoculum comprising mid-exponential phase cells.
3. Ethanol concentrations of 6 - 10% (v/v).
4. Static cultures incubated for 24 hrs at 30°C, with OD_{600} readings taken every 30 minutes[2].

3.2.6 Reproducibility of RETA

RETA was developed to provide a rapid screen for ET phenotypes against a background of nET phenotypes and for this reason it was essential that the assay was reliable and reproducible. The reproducibility of RETA was tested by screening cultivations of single strains, in triplicate, on nine different microplates and on nine different occasions. *S. cerevisiae* strains with known ethanol tolerance levels were used. Strain WA was inoculated at an initial OD_{620} of 0.05 in triplicate into 9 individual microplates. The growth rates of these micro-cultures were determined and the results subjected to ANOVA analysis. The variation in growth rate of nine amalgamated microplate experiments was found to be not statistically significant (Figure 3.8). The inter-experimental variation was also not statistically significant, providing evidence that RETA is reproducible.

[2] NB. All subsequent microplate cultivations were incubated at 30°C in static conditions in YPD medium. The microplates were sealed with a Breathe-easy membrane and optical density measurements were performed with Ascent Multiskan and Spectra Max microplate reader.

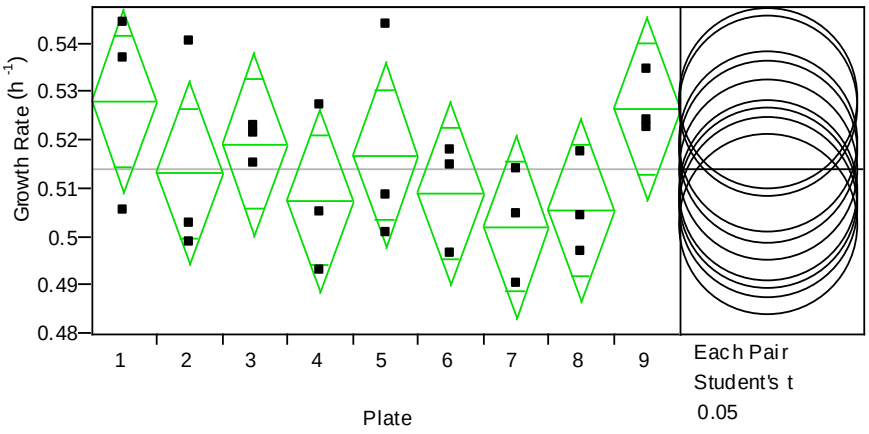


Plate	Group	Mean
1	A	0.5284
9	A	0.5268
3	A	0.5196
5	A	0.5173
2	A	0.5136
6	A	0.5094
4	A	0.5080
8	A	0.5058
7	A	0.5025

Figure 3.8 Reproducibility of RETA. WA was inoculated into YPD in triplicate into nine different microplates and assayed individually. The growth rates and final biomass yields were determined and subjected to ANOVA. Groups in the table not connected by the same letter are significantly different. The cultures were statically incubated at 30°C and OD_{620} readings were taken at 30 minute intervals.

3.3 Discussion

Shake flask cultures have been traditionally used to assay ethanol tolerance in various microorganisms and this approach continues to be used in circumstances where only a small number of strains are characterized and/or the ethanol-tolerance phenotype needs to be determined in considerable detail *e.g.* lag period, specific growth rate, cell yield and metabolite production. ET assays using agar plates containing added ethanol have also been used however this method can be unreliable due to the volatility of ethanol impacting on the reproducibility of the results and inability to quantify the ethanol tolerance phenotype (Lorenz et al. 2000; Mitchell et al. 1998).

The above methods were unsuitable for the current project which required a rapid and reliable screening assay to determine the relative ethanol tolerance of variants arising from backcrossing experiments. A literature search revealed a lack of suitable ET phenotype assays for this project necessitating the development of a high-throughput method capable of determining the relative ethanol tolerance of numerous backcrossing progeny.

In particular, the assay needed to reproducibility distinguish nET from ET strains. This chapter described the approach used to develop RETA (Rapid Ethanol Tolerance Assay), the derivation of parameters for obtaining optimum resolution of ET phenotypes using RETA and validation of RETA's accuracy and reliability.

Ethanol evaporation from agar plates and microplate wells, have been issues affecting the resolution of previously published ET assays. For example, Mitchell et al. (1998) studied the cytotoxicity of ethanol on rat cells incubated for 48 hours and found that the volatility of ethanol resulted in variation in the concentration of ethanol across the plate wells. With regard to RETA, different plate membranes and relatively short incubation times were used resulting in statistically equivalent ethanol concentrations in all wells at the end of the incubation. The major differences in the phenotype of the SM, CM and WA strains is their respective growth rates under ethanol stress; the drop plate method as described in the literature (Ogawa et al., 2000; Hu et al., 2007) was considered to be incapable of rapidly, accurately and reliably determining the differences in ethanol tolerance phenotype. Microplate assays described in the literature at the time of commencing this project had not been developed for determining the relative ethanol tolerance of

different *S. cerevisiae* strains; the majority of published results (see Section 3.1) related to studies on the effects of other inhibitory substances on the microbial growth phenotype (Schmitt et. al. 2004, Toussaint et al., and Conconi et al. 2006). Another benefit of RETA is that it is an automated procedure, thus requiring less manpower compared shake flask-based growth curve profiling. RETA is also more flexible by allowing a larger number of variables and replicates to be assayed at any given time.

Several ethanol concentrations were tested to verify and optimize the RETA. It was found that ethanol concentrations in the range 8-9% (v/v) were most effective in distinguishing between ET mutant and nET strains. Outside these concentrations differences in growth rates were not statistically significant due to either the lack of, or excessive inhibition by, ethanol. The most significant differences between the growth rates of nET and ET strains were observed at 8% (v/v) ethanol. Previously performed growth curve assays with these strains using shake flask cultures observed that 6 and 8% (v/v) ethanol were the most effective concentrations for distinguishing growth rate differences between the nET parent and ET mutant strains (Stanley et al., 2009a). Shake flask cultures are definitive by providing information on cell viability and various features related to the entire growth profile whereas the microplate assay uses optical density measurements to monitor biomass, which is not a reliable indicator of viable cell population. As a screen however the microplate assay is convenient, flexible, efficient and capable of accurately and reproducibly distinguishing between the ET of parent and mutant strains.

As a result of the work described in this chapter RETA was developed, which allowed for accurate and reproducible assays of the ethanol tolerance for numerous *S. cerevisiae* isolates, and was sufficiently sensitive to clearly distinguish between nET parent and ET mutants, SM and CM.

Chapter 4

Genetic characterisation of the ethanol-tolerance phenotypes in *S. cerevisiae* SM and *S. cerevisiae* CM

4.1 Introduction

This chapter describes experiments to characterise mutations conferring ethanol tolerance in CM and SM mutants of *S. cerevisiae* W303-1A. Mutations occur at various levels, from changes in single nucleotides and genes, to whole chromosomal rearrangements and changes in ploidy. Classical genetics, involving crosses and following segregation patterns of traits, remains a vital part of characterising genetic determinants that shape a particular phenotype. Backcrossing, for example, can be used to determine the number of genes responsible for a particular trait and whether the trait is dominant or recessive. This chapter describes backcrossing of the chemically-induced (CM) and spontaneous (SM) ET mutants to *S. cerevisiae* W303-1B (WB). To complement the classical approach, Clamped Homologous Electric Field (CHEF) at chromosome resolution, was used to determine whether there are major chromosomal re-arrangements in the ET mutants compared to their parental strain.

4.2 Results

4.2.1 Comparative karyotype analysis of ET mutants and the parental strain

CHEF (methods Section 2.3) was used to determine whether major chromosomal rearrangements were present in ET mutants SM and CM, relative to their parent, WA. Shifts in chromosomal banding pattern of ET mutants compared to the parental strain would indicate chromosomal mutations, and, in the context of this thesis, may contribute to the ethanol tolerance phenotype.

Figure 4.1 shows chromosomal banding patterns of the parental strain and ET mutants. Comparing the banding patterns of WB, WA, CM and SM, there are no apparent differences except for chromosome XII which appears to be missing in WA and CM. CHEF analysis was repeated several times using different whole chromosomal preparations, and chromosome XII was variously present or absent for all strains (data not shown) thus, the inconsistent absence of this band was regarded as experimental error.

*Shorthand of nomenclature for strains used in this chapter and thesis.

Nomenclature	Identity of Strains
WA	*S. cerevisiae* **W**303-1**A** parental strain Mat-**a**
WB	*S. cerevisiae* **W**303-1**B** strain Mat-α
WBU	**WB**, ***U****RA3* prototrophy
SM	**S**pontaneous ethanol-tolerant **M**utant of WA
SML	**SM**, ***L****EU2* prototrophy
CM	**C**hemical-induced, ethanol-tolerant **M**utant of WA
CMH	**CM**, ***H****IS3* prototrophy
DB	*S. cerevisiae* **DB**Y745
FY	*S. cerevisiae* **FY**4 is derivative of S288C with nil auxotrophies

Transcriptome analysis of WA, CM and SM performed by Stanley et al. (2009) and Comparative Genome Hybridisation microarray data (discussed in Chapter 5), confirmed that chromosome XII was indeed present in all strains. There were no consistent differences between karyotypes of the ET mutants compared to the parental strain.

4.2.2 Genetic characterization of the ethanol-tolerance phenotypes of CM and SM

4.2.2.1 Generating complementary auxotrophic markers across the parent and ET mutants to enable easy selection of progeny

Backcrossing experiments were performed to characterize the genetics of ethanol-tolerance in CM and SM. However, the parental strains, WA, and ET mutants have identical auxotrophic markers, thus it was preferrable to develop complementary auxotrophies between WB and the ET mutants so that progeny could be easily selected on drop-out plates.

SM, CM, WA, and WB, have the genotype: *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*. It was decided to change auxotrophies in all three strains by returning a different prototrophy, *HIS3*, *LEU2* or *URA3*, into each background. Prototrophic alleles for each of these genes were amplified from *S. cerevisiae* FY4 (a derivative of S288C) and introduced into the host strains by transformation (Section 2.4), and. transformants were selected on appropriate drop-out plates; for example transformants rescued for *URA3* prototrophy were selected on SC Ura^- drop-out plates.

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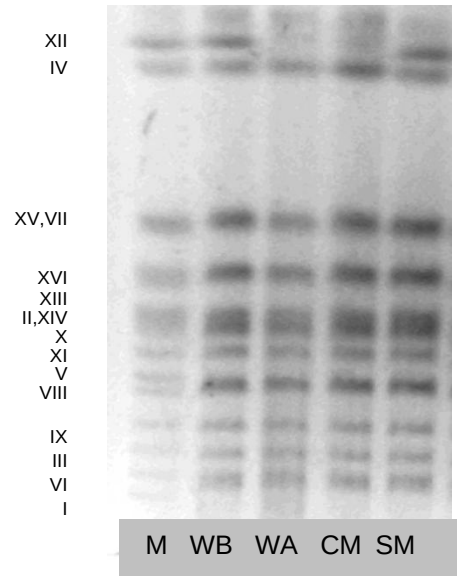


Figure 4.1: CHEF gel of whole chromosomal preparations from strains WB, WA, CM and SM. M is a *S. cerevisiae* chromosomal marker (BIORAD), roman numerals represent chromosomal numbers.

To ensure that ET phenotypes were not affected by marker rescue, transformants with recovered prototrophies were assessed for ethanol tolerance using RETA. Growth rates of transformants under various ethanol concentrations are shown in Figures 4.2 to 4.4. While, in most cases, ethanol tolerance was not affected by rescuing auxotrophic markers, some transformants did show an altered ET phenotype, which will be discussed later in this chapter. Transformants WB-U02 (WBU), SM-L00 (SML) and CM-H02 (CMH) were chosen for backcrossing experiments, since they had similar ET phenotypes to the strains they were derived from. However, before commencing backcrossing experiments with these strains, the insertion site of the rescued marker was verified.

Primers were designed to target inside and outside of the rescued marker loci. Using various combinations of these primers, segments of each locus were PCR amplified and sequenced such that the entire locus and flanking regions were sequenced. The sequences of the prototrophic transformants were compared to the auxotrophic wild-type strains they were derived from (See Appendix 4.1). As Figure 4.5 illustrates, the SNP mutations conferring auxotrophic phenotypes were substituted to produce a strain with a prototrophic phenotype in the transformants. Thus, confirming correct sequence integration of the marker. Sequences of flanking regions were also the same as for the parent.

A limitation of the above approach for confirmation of the location auxotrophic marker rescue sequences is that it will not detect multiple insertions of the genetic marker in the genome. Even though such events are rare in *S. cerevisiae,* it was important to ensure that auxotrophy rescue genes were not incorporated at additional loci. Southern Blotting was used for this; restriction enzymes targeting regions surrounding the known marker insertion site to produce bands of known molecular weight. If the marker inserted into other sites in the genome, then the Southern Blot should yield additional bands.

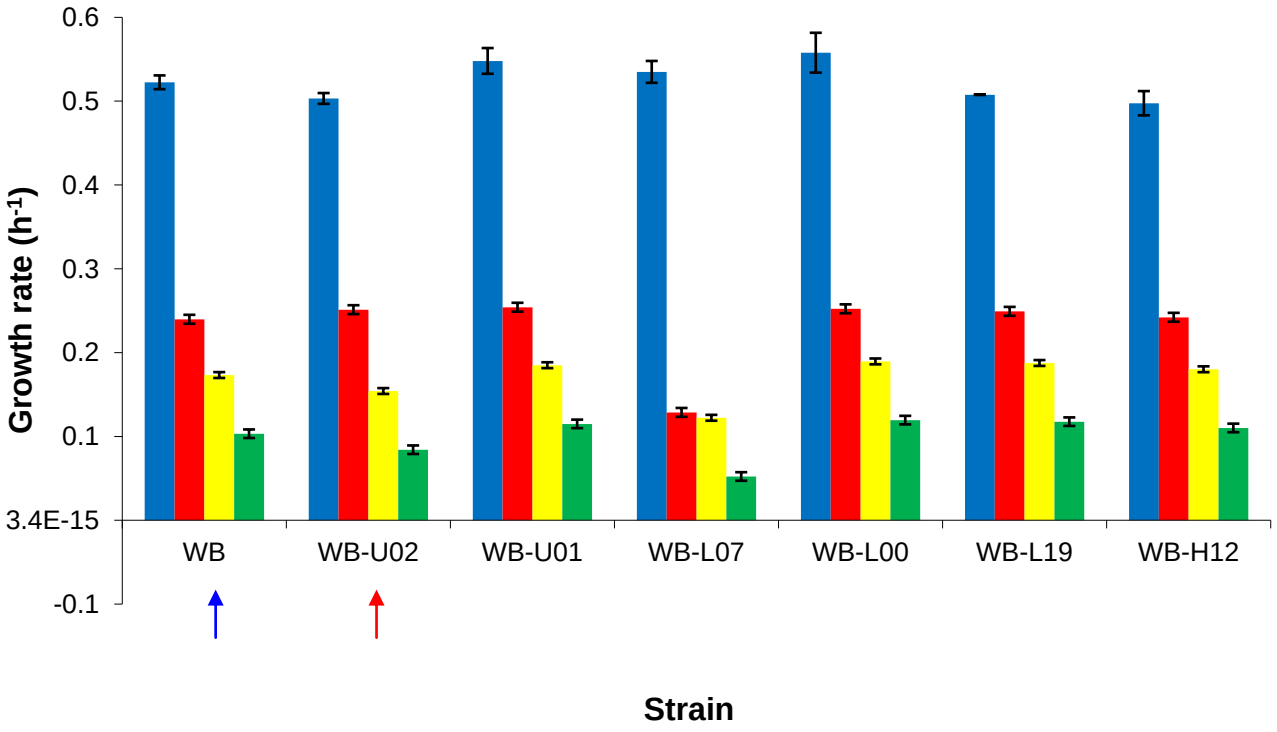


Figure 4.2: Growth rates of WB (↑) and transformants thereof. Ethanol tolerance was determined using RETA with ethanol concentrations of 0 (■), 6 (■), 8 (■), and 10 (■) % (v/v). Letters following hyphens represent the marker rescued; H (*HIS3*), L (*LEU2*) and U (*URA3*). Numbers identify the particular transformant. ↑ indicates the strain chosen for subsequent backcrossing experiments.

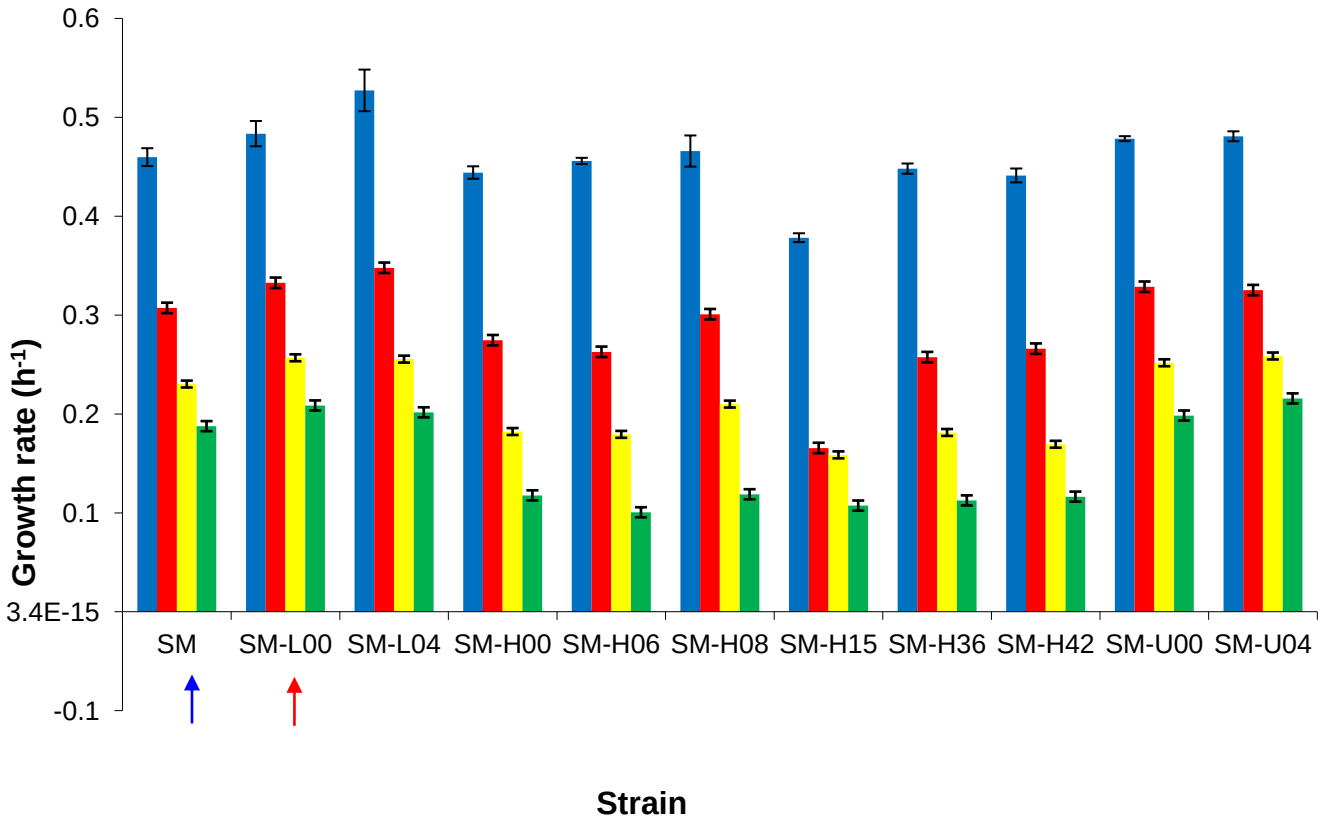


Figure 4.3: Growth rates of SM (↑)and transformants thereof. Ethanol tolerance was determined using RETA with ethanol concentrations of 0 (■), 6 (■), 8 (■), and 10 (■) % (v/v). Letters following hyphens represent the marker rescued; H (*HIS3*), L (*LEU2*) and U (*URA3*). Numbers identify the particular transformant. ↑ indicates the strain chosen for subsequent backcrossing experiments.

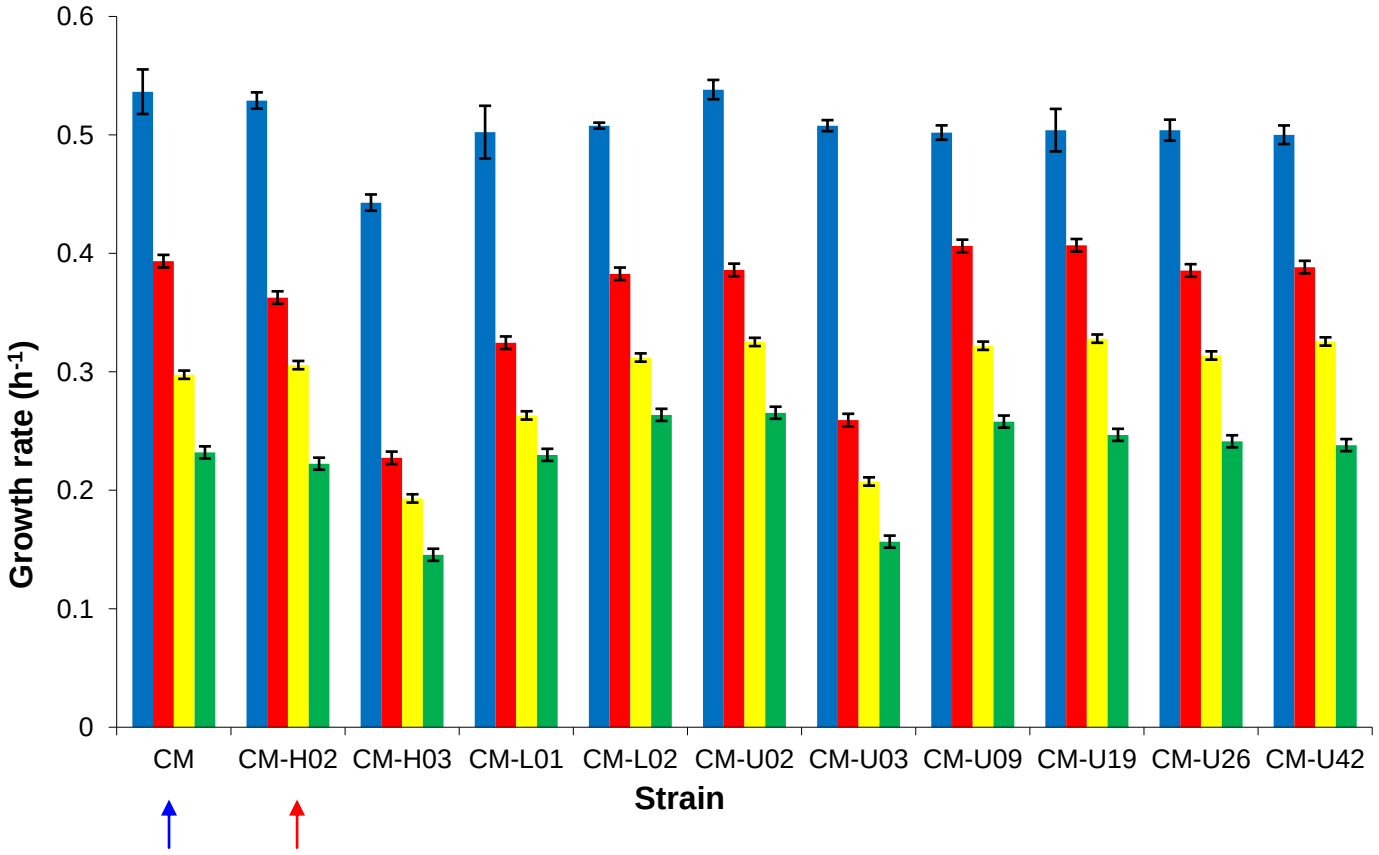


Figure 4.4: Growth rates of CM (↑)and transformants thereof. Ethanol tolerance was determined using RETA with ethanol concentrations of 0 (■), 6 (■), 8 (■), and 10 (■) % (v/v). Letters following hyphens represent the marker rescued; H (*HIS3*), L (*LEU2*) and U (*URA3*). Numbers identify the particular transformant. ↑ indicates the strain chosen for subsequent backcrossing experiments.

a)

Strain	Uracil requirement	Sequence
WB	Auxotroph	GATCTGACATTATTATTGTTG**A**AAGAGGACTATTTGCAA
WBU	Prototroph	GATCTGACATTATTATTGTTG**G**AAGAGGACTATTTGCAA

Co-ordinate of sequence shown IV: 116846-116884

b)

Strain	Leucine requirement	Sequence
SM	Auxotroph	GTTATCCCAGGTTCCTTGGG**G**TTTGTTGCCATCTGCGTC
SML	Prototroph	GTTATCCCAGGTTCCTTGGG-TTTGTTGCCATCTGCGTC

Co-ordinate of sequence shown III: 92095-92132

c)

Strain	Histidine requirement	Sequence
CM	Auxotroph	CGCACGGCCCCTAGGGC-TCTTTAAAAGCTTGACCGCGA
CMH	Prototroph	CGCACGGCCCCTAGGGC**C**TCTTTAAAAGCTTGACCGCGA

Co-ordinate of sequence shown XV: 722243-722281

* refer to Appendix 4.2 for chromatograms

Figure 4.5: Partial sequences of rescued loci in WBU, SML and CMH. The sequences illustrate the alterations of the nucleotide mutation conferring the auxotrophic phenotype. **a**) Partial sequence of the *URA3* locus, highlighting the replacement of the auxotrophic point mutation A→G in WBU. **b**) Partial sequence of the *LEU2* locus, highlighting the deletion of G in SML. **c**) Partial sequence of the *HIS3* locus, highlighting the insertion of C in CMH.

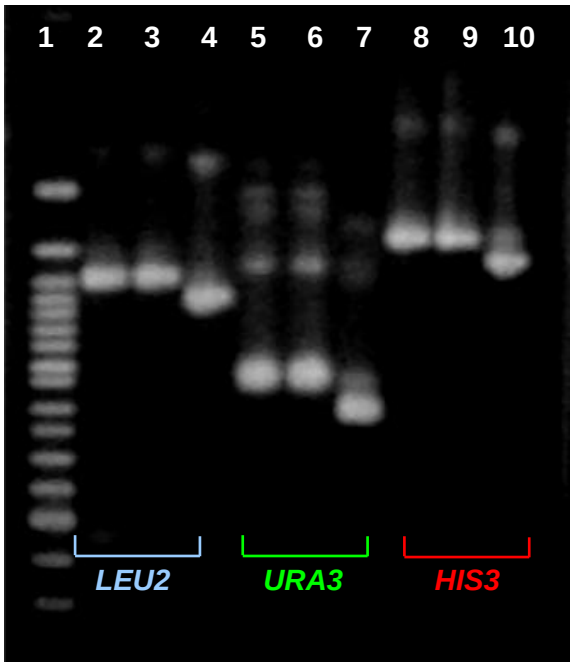
Probes targeting *HIS3*, *LEU2* and *URA3* markers were constructed using a PCR-based method (Section 2.7) that incorporates a digoxigenin (DIG) conjugated nucleotide into the PCR product. DIG-conjugated nucleotides have a higher molecular weight than non-labelled nucleotides thus a shift in the molecular weight of PCR products containing DIG is apparent (Figure 4.6). Probes carrying incorporated DIG are recognised by anti-DIG Alkaline Phosphatase-Horse Radish Peroxidase–antibody (anti-DIG-AP-HRP antibody). The conjugated alkaline phosphatase (AP) digests fluorescent or chemiluminescent substrates to produce a detectable signal.

To determine the sensitivity of the DIG-probes, each was spotted onto a nylon membrane and developed with a DIG-binding Horseradish Peroxidase (HRP) antibody. Both chemical precipitation substrate Nitro Blue Tetrazollium (NBT)/Bromo-Chlorol-Indolyl-Phosphate (BCIP) and Enhaced Chemiflourescent (ECF) substrates were used to develop the membrane (Figure 4.7). The probes were found to be highly sensitive, and was able to be detected when diluted by 100 times.

Genomic DNA was extracted (as described in Section 2.0) from transformants and host strains. Restriction enzymes were chosen on the basis that they cut at the target loci cut at moderate frequencies (~every 3 kb). If a marker had inserted in more than one site in the genome, then it is likely that multiple bands would appear on the Southern Blot.

Restriction digests were resolved on agarose gels, blotted overnight onto nylon membranes, and hybridised with the DIG-labelled probes described above. All probed blots yielded single bands of expected molecular weights for the insertion site of the auxotrophic rescue gene (Figure 4.8), except for the *HIS3* locus in the control (FY*) lane. However, this was likely due to carryover of DNA from the adjacent well. Southern probing for *HIS3* in the control FY was repeated several times and a single band was found for four different restriction endonucleases; an example is shown in Figure 4.9.

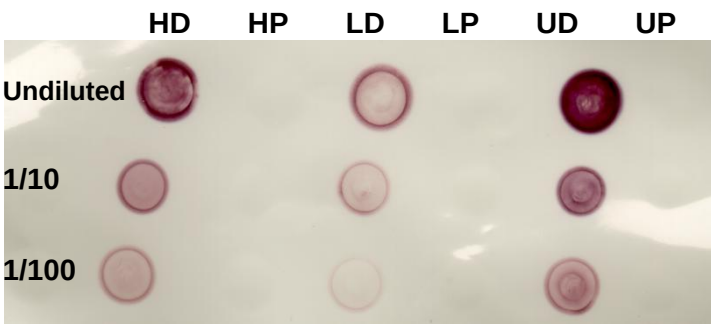
* FY (FY4) is a derivative of the commonly used lab strain S288C, but has nil auxotrophies.



Lane	Marker	DIG:dNTP
1	50 bp Ladder	-
2	*LEU2*	1:2
3	*LEU2*	1:4
4	*LEU2*	dNTP only
5	*URA3*	1:2
6	*URA3*	1:4
7	*URA3*	dNTP only
8	*HIS3*	1:2
9	*HIS3*	1:4
10	*HIS3*	dNTP only

Figure 4.6: Agarose (1%) gel of resolved PCR amplification of *HIS3*, *LEU2* and *URA3* probes using various ratios of DIG to dNTP.

a)



b)

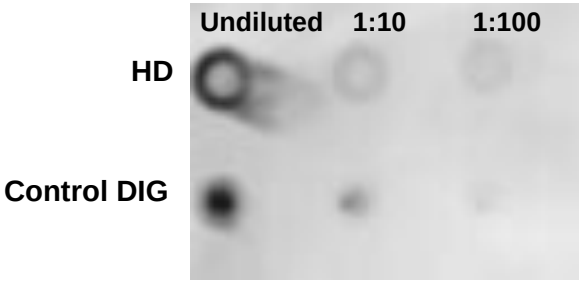


Figure 4.7: Detection of DIG incorporated into marker probes to be used in Southern Blot experiments:

- **a)** Detection of probes via NBT/BCIP precipitation: *HIS3* (H), *LEU2* (L) and *URA3* (U) probes were spotted undiluted and 1:10 serially diluted. D depicts DIG included in PCR reaction. P is a 'no DIG' control.
- **b)** Detection of probes via ECF chemiluminescence: The top row HD, contains the *HIS3* probe serially diluted out. The second row-Control DIG (5 μg/mL) has been serially diluted 1:10 etc. Bio-Rad VersaDoc was used to visualise.

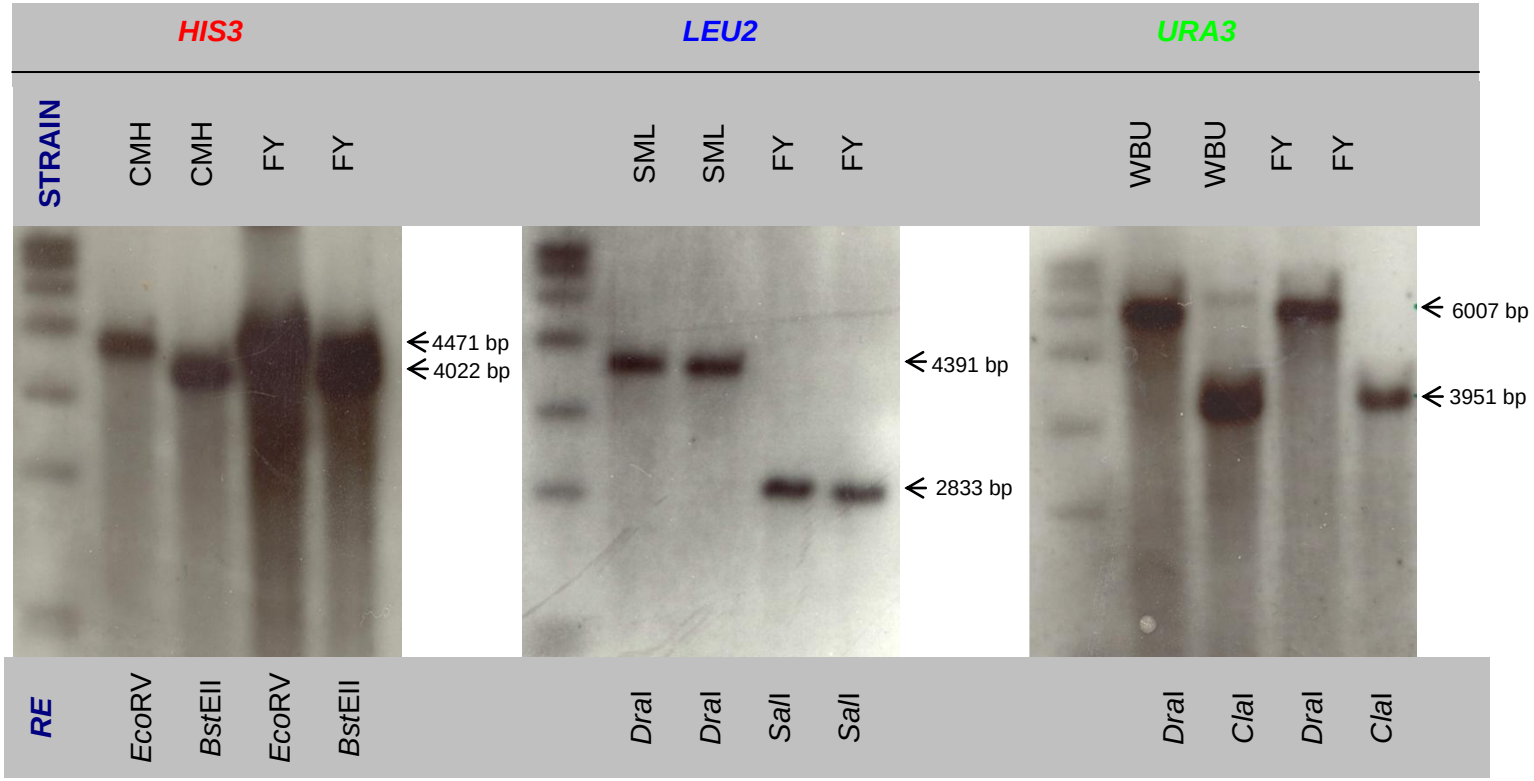


Figure 4.8: Genomic DNA preparations from strains CMH, SML, WBU and FY were digested with the restriction endonucleases indicated in the lower grey box. Digested DNA was blotted onto nylon membranes and probed using *HIS3-*, *LEU2-*, or *URA3-* DIG-labelled probes as indicated in the upper grey box. Bound probes were detected by exposure to x-ray film. The left hand lane in each blot contains molecular weight size standards. Arrows indicate expected sizes for bands generated from each of the targeted insertion sites.

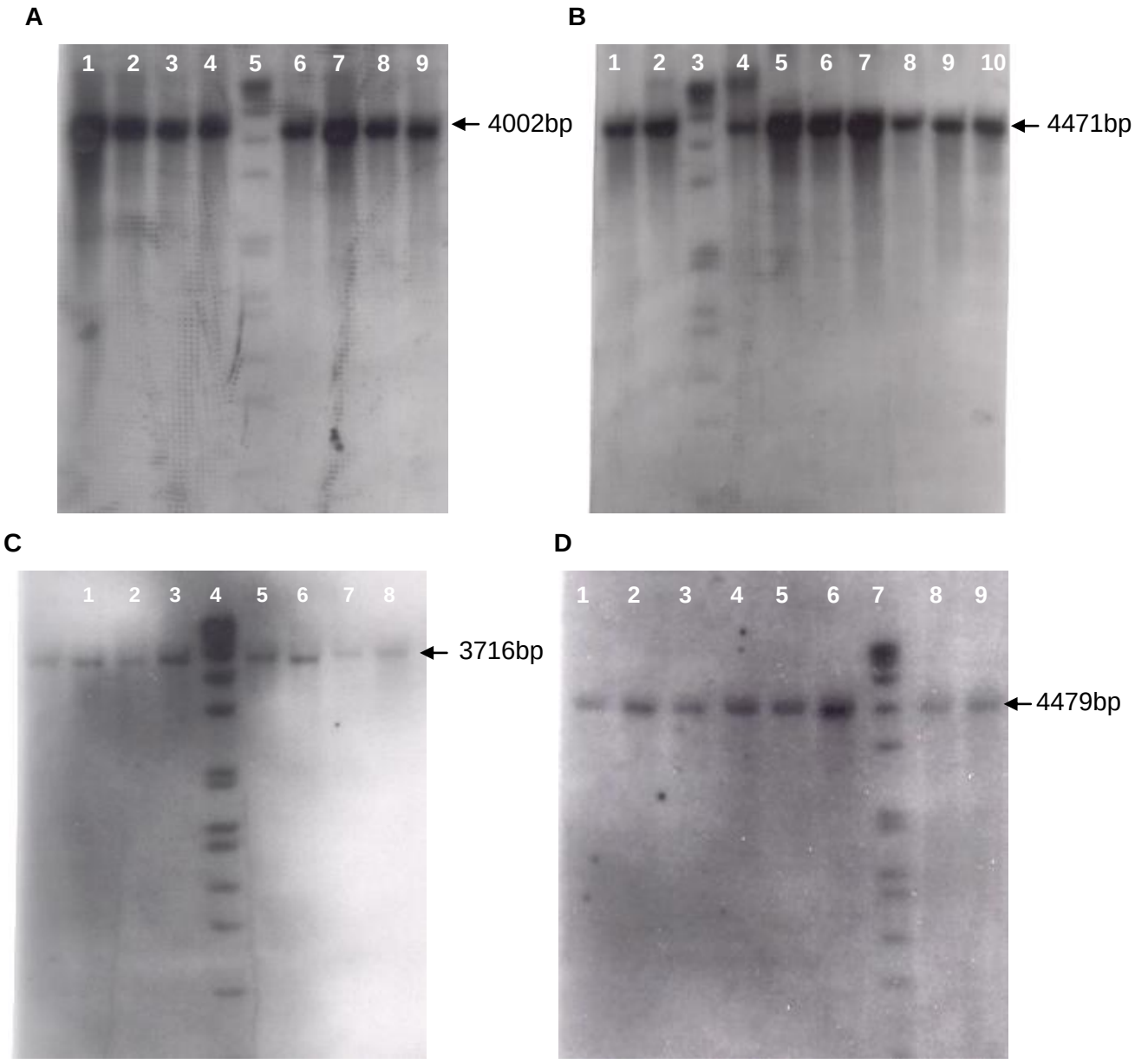
4.2.2.2 Rescuing auxotrophic markers in transformants alters ethanol tolerance levels in some transformants

As mentioned in Section 4.2.2.1, rescuing the auxotrophic markers impacted on the ethanol tolerance phenotype in some transformants. For example WB-H11, which had histidine prototrophy, had increased ethanol tolerance compared to its auxotrophic parent, WB (Figure 4.2). CM-H03 and CM-U03 appear to have decreased ET phenotype as shown in Figure 4.4. The growth rates of these transformants in the absence ethanol, however, also diminished which may indicate that decreased ET is a result of additional growth defects.

Interestingly, ethanol tolerance levels of SM were consistently diminished by transformation of the *HIS3* marker (refer to Figure 4.3). It was hypothesized that this may have been due to a mutation in the *his3* locus of SM that confers ethanol tolerance. Thus, the sequence of *HIS3* allele in WB, SM and CM were compared to one another (refer to appendix 4.1). However, no difference was found between the strains.

It was then hypothesised that the *HIS3* gene may have consistently inserted into an area of the genome of SM containing genes that contribute to ethanol tolerance. Southern blot analysis was used to test this. Parental and wild-type strains were included in the analysis as controls. Genomic DNA from FY4, SM, WA, WB, CM and *HIS3* transformants of each, were digested using four restriction endonucleases *Bst*EII, *Mfe*I, *Eco*RV and *Ssp*I. Each of these enzymes cut at sites that flank the *HIS3* locus, each yielding a different molecular weight fragment. Single bands of the expected size for *HIS3* were seen for all digests (Figure 4.9). Thus, it is unlikely that the hypothesis that the *HIS3* marker had inserted into other loci contributing to the ET phenotype.

Although the decline in ethanol tolerance phenotype was interesting, the main focus of work described in this thesis was to identify genes which confer ethanol tolerance in the ET mutants. Thus, transformants with altered ethanol tolerance were not investigated further in this project, but will be used in future research.


A
1 2 3 4 5 6 7 8 9
4002bp
B
1 2 3 4 5 6 7 8 9 10
4471bp
C
1 2 3 4 5 6 7 8
3716bp
D
1 2 3 4 5 6 7 8 9
4479bp

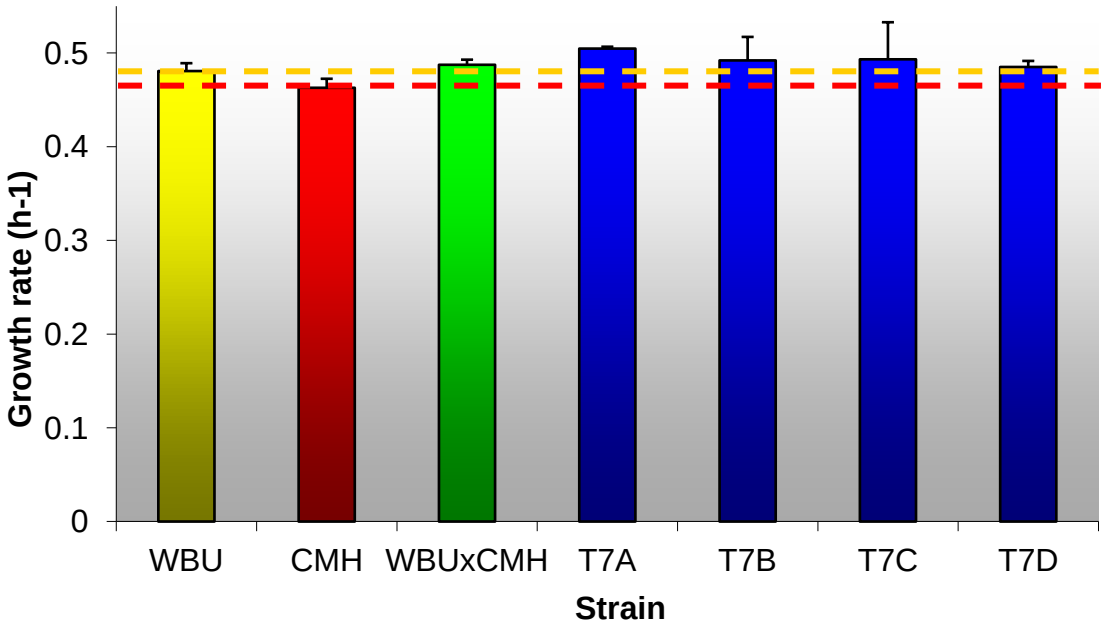
4.3 Backcrossing the Chemical ET mutant to its parent

WBU (an isogenic opposite mating-type of WA) and CMH were used for backcrossing of the Chemical ET mutant. The resulting diploid (WBUxCMH) was sporulated and the haploid spores were assayed for their ethanol tolerance phenotypes using the RETA. Cultures were exposed to 0, 8, 9, and 10 % v/v ethanol in YPD. In all cases only the 0 % and 8 % v/v ethanol are described in this chapter; results at higher ethanol concentrations were consistent with 8% v/v ethanol (results not shown). As seen in Figure 4.10, there is no significant difference in growth rates between ET and non-ET strains at 0% v/v ethanol. WBU was more inhibited by the presence of ethanol than CMH. Ethanol tolerance phenotypes for strains were scored as: non-ethanol tolerant (nET) or ethanol tolerant (ET). Strains with growth rates similar to CM were classified as ET and strains with growth rates similar to WB were classified as nET. Growth rates of the four meiotic haploids (tetrad set) in RETA are highlighted in different colours (Figure 4.11).

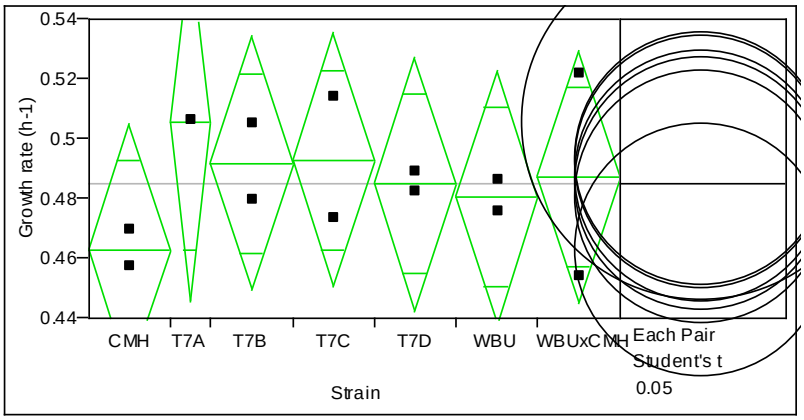
The diploid of WBU and CMH cross resulted in a diploid with equivalent ethanol tolerance levels to that of CMH. This indicated that the ethanol tolerance phenotype was dominant.

Nine WBUxCMH diploids were then sporulated to produce meiotic progeny. RETA of the meiotic progeny show that the ethanol tolerance phenotype clearly and consistently segregated in a 2:2 (ET : nET) fashion (Figure 4.11 shows a typical result for one of the tetrad dissections). The classic Mendelian 2:2 segregation of the ethanol tolerance phenotype, is indicative of a single gene conferring the ethanol tolerance in CM.

a)

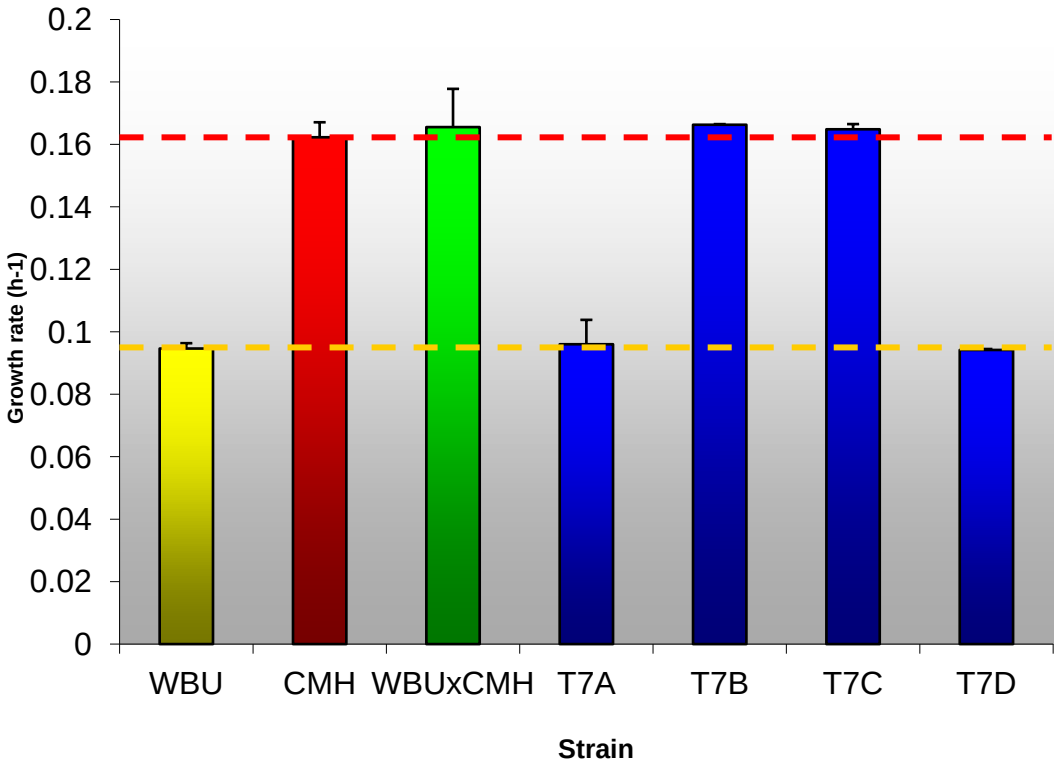


b)

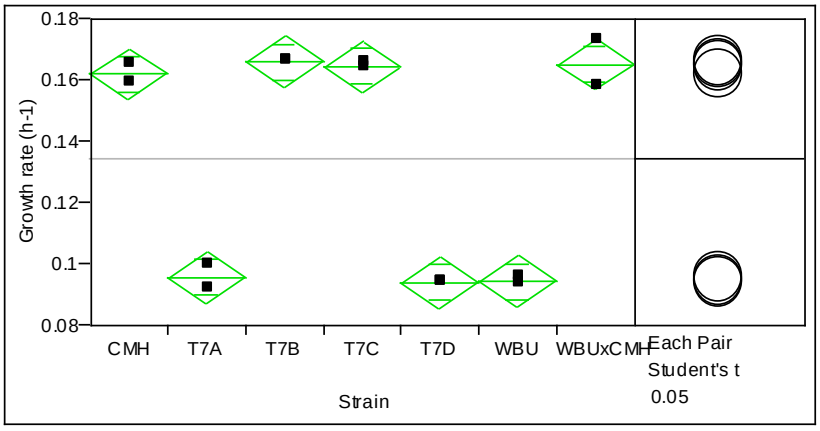


Level	Group	Mean
T7A	A	0.5057
T7C	A	0.4933
WBUxCMH	A	0.4875
T7D	A	0.4851
WBU	A	0.4808
CMH	A	0.4629

a)



b)



Level	Group	Mean
T7B	A	0.1663
WBUxCMH	A	0.1656
T7C	A	0.1649
CMH	A	0.1623
T7A	B	0.0960
WBU	B	0.0946
T7D	B	0.0942

4.4 Backcrossing the spontaneous ET mutant to its parent

An identical experimental strategy as that described in Section 4.3 for backcrossing of the chemical mutant, was used to backcross the spontaneous mutant (SML) to WBU, an isogenic (opposite mating-type) strain of the parental (WA) strain. RETA was used to quantify the ethanol tolerance levels of the diploid resulting from the cross between WBU and SMU.

Figure 4.13 shows that the ethanol tolerance phenotype is dominant. The diploid was then sporulated to produce meitotic progeny, which were also assayed for the ethanol tolerance phenotype. Growth rates of progeny in the absence and presence of ethanol are shown in Figure 4.12 and Figure 4.13 respectively.

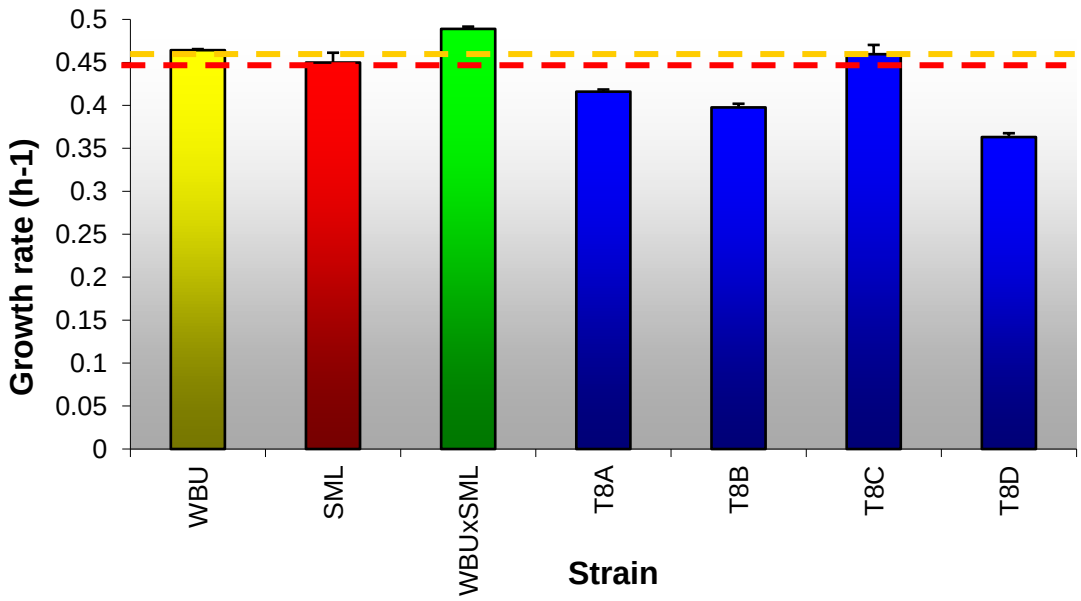
The meiotic progeny of the WBUxSML diploid, mostly segregated in a 2:2 ratio, however of the 27 randomly selected tetrads, 23 displayed 2:2 segregation of the ethanol tolerance phenotype while the other 4 displayed ambiguous segregation patterns (Figure 4.14), with intermediate levels of ethanol tolerance in the haploid spores.

4.5 Crossing ET mutants to DBY745

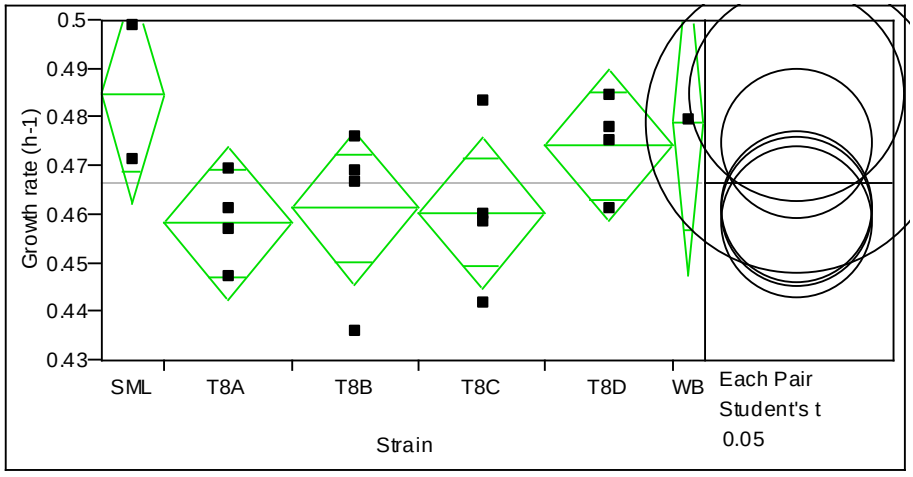
To confirm that the ethanol tolerance phenotype of SM and CM was dominant, each was crossed to an unrelated *S. cerevisiae* laboratory strain, DBY745 (abbreviated here to DB). DB has an *ade2* auxotrophic marker and also has a lower ethanol tolerance level than WA.

Figure 4.15 shows the growth rates of diploids (DBxWA), (DBxSM) and (DBxCM). under ethanol-stress conditions. Both (DB x SM) and (DB x CM) have higher growth rates than that of (DBxWA), confirming that the ethanol tolerance phenotype in SM and CM is dominant.

a)

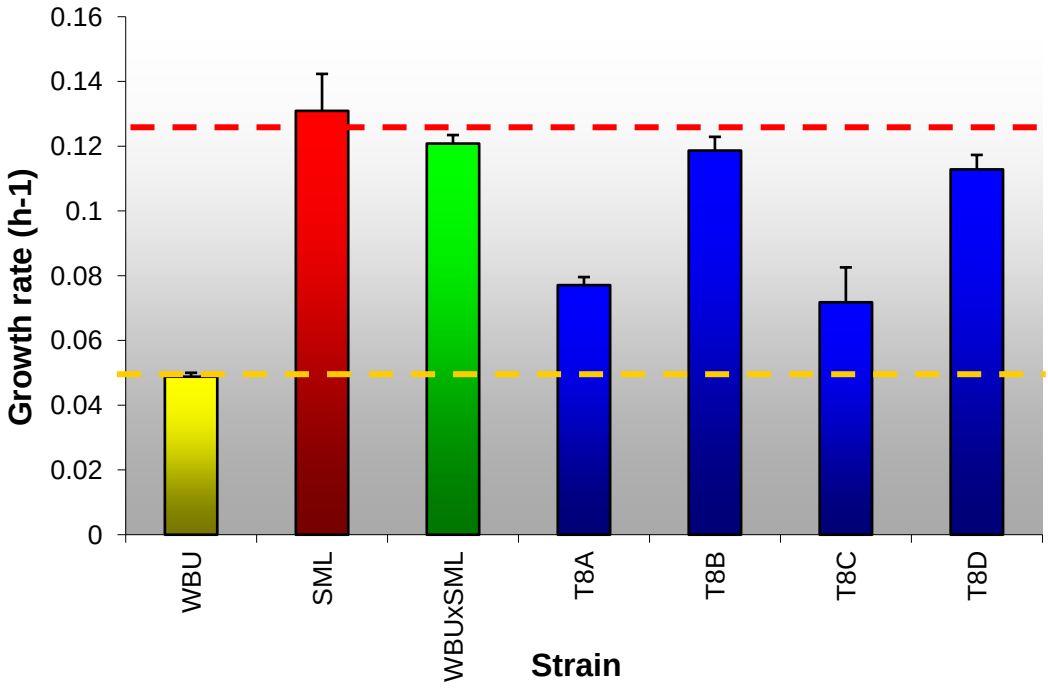


b)

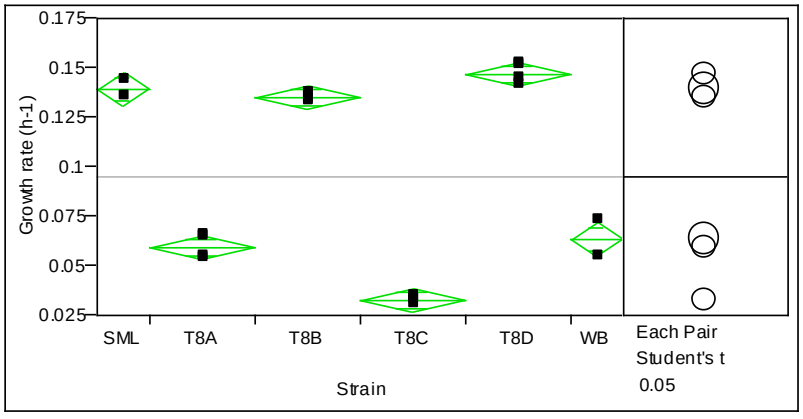


Level	Group	Mean
SML	A	0.4849
WB	A	0.4791
T8D	A	0.4745
T8B	A	0.4616
T8C	A	0.4606
T8A	A	0.4585

a)



b)



Level		Mean
T8D	A	0.14717500
SML	A B	0.13975000
T8B	B	0.13525000
WB	C	0.06376500
T8C	C	0.06272000
T8A	C	0.05962500

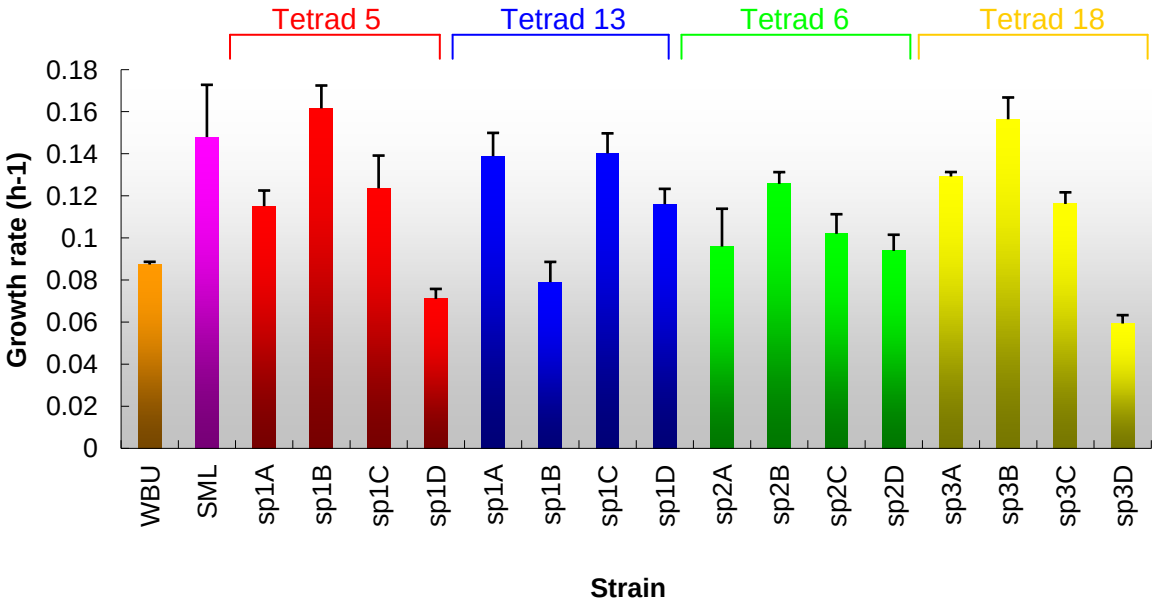


Figure 4.14

Results from a RETA showing the growth rate of SML meiotic haploid tetrad sets that deviated from 2:2 segregation in 8 %(v/v) ethanol. T followed by a number indicates the tetrad set and spores are designated A, B, C or D.

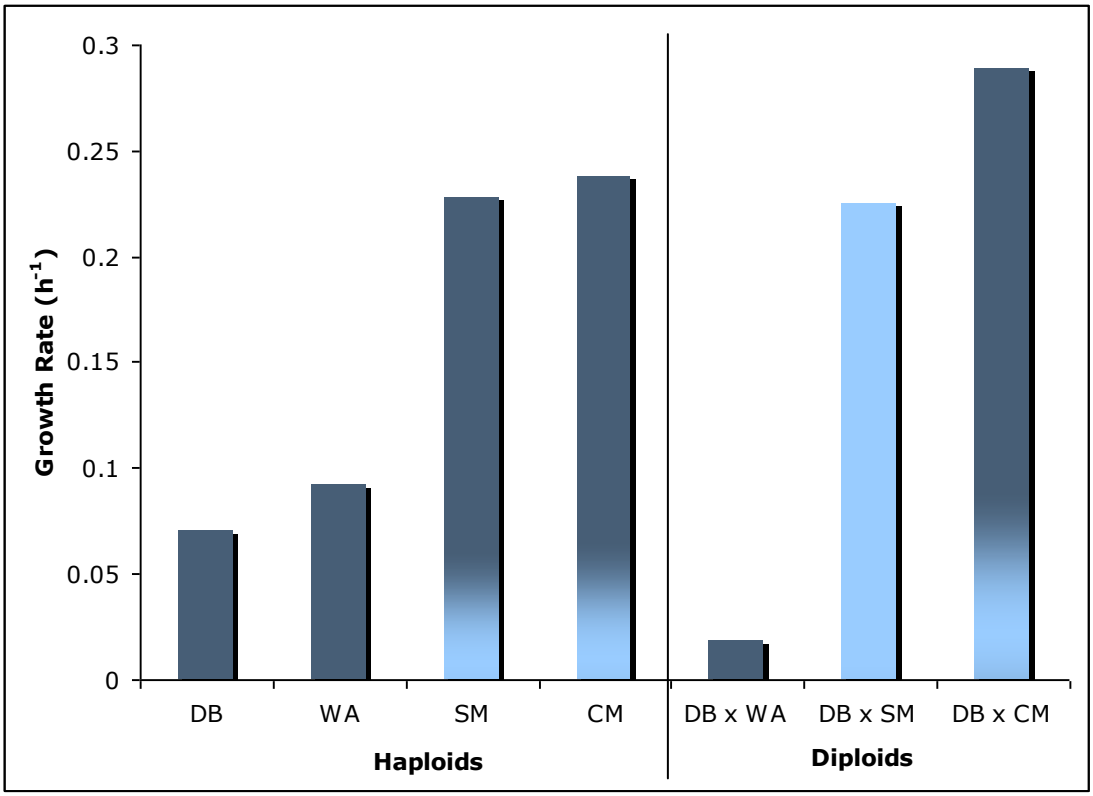


Figure 4.15

Growth rates of diploid progeny from crosses of DB with WA (DBxWA), SM (DBxSM) and CM (DBxCM). Cultures were grown in YPD in the presence of 6 % (v/v).

4.6 Discussion

This chapter describes the characterisation of genetic elements conferring increased ethanol tolerance phenotypes in *S. cerevisiae* W303-1A ET mutants SM and CM, created by Dr Dragana Stanley (PhD student, Victoria University).

Karyotypes of the ET mutants and the parental strain, WA, were compared to the parental strain using CHEF analysis. There were no observable chromosomal rearrangements in the ET mutants compared to the parental strain; at least to the resolution attainable using CHEF gels. Thus, the mutations conferring increased ethanol tolerance in CM and SM are not due to major chromosomal re-arrangements.

To characterise 'ethanol tolerance' genetic loci in the mutants, segregation patterns of the phenotype were determined in backcross experiments. To enable ease of selection of progeny from these matings, auxotrophic markers were changed to introduce complementarity. In most cases, changes in auxotrophy had no impact on ethanol tolerance however some transformants did not retain their ethanol tolerance levels, but they also showed diminished growth in the absence of ethanol and therefore were discarded. These changes in the phenotype may have been due to mutations introduced as a result of transformation; it is recognized in the yeast scientific community that lithium acetate transformation can induce mutations (e.g. http://www-sequence.stanford.edu/group/yeast_deletion_project/project_desc.html). Transformants chosen for further backcrossing experiments, retained ethanol-tolerance, and Southern Blot analysis indicated that all prototophic alleles inserted only once and in the correct location of the genome.

Interestingly, rescuing the *HIS3* auxotrophy in SM, consistently led to diminished ethanol tolerance. Sequencing of the *HIS3* locus in all three strains did not show anomalies. It was hypothesized that the prototrophic allele may have integrated into the genome such that it disrupted genes which contribute to ethanol tolerance. However, extensive Southern blot analysis revealed that this was probably not the case; an SM *HIS3* transformant (SH006), which exhibited a diminished ethanol tolerance phenotype, had only one insertion and this was at the *HIS3* locus. Even though this was an interesting, difficult to explain, observation, the aim of this project was to identify ethanol tolerance conferring loci in the ET mutants, thus the *HIS3* rescued SM strains with diminished ethanol tolerance phenotype were put in storage

for future work. A leucine prototroph of SM (SML) was used in backcrossing experiments, as rescue of *leu2* auxotrophy had no impact on ethanol tolerance.

Diploid progeny from backcrossing experiments showed that the ethanol tolerance phenotypes were dominant for both mutants. To confirm this observation, ET mutants were also crossed to an unrelated laboratory strain of *S. cerevisiae*, namely DBY745 (DB) that has ethanol tolerance levels similar to the parental strain. Again, the ethanol tolerance phenotype was dominant. This observation is consistent with several publications that have reported ethanol tolerance to be dominant (Jimenez J., and Benitez et al.,, 1987; Marullo et al., 2004).

Diploids from backcrosses were sporulated and the haploid progeny assayed for ethanol tolerance. The ET phenotype segregated 2:2 in spores from the CM backcross, suggesting a single locus is responsible for conferring increased ethanol tolerance. Backcrossing of SM to the parental strain showed that the genetics of ethanol tolerance in this strain is more complex than in CM. Of 27 asci that were assayed for the ethanol tolerance phenotype, 23 segregated 2:2 but 4 deviated from this. Rather than 2:2, segregation produced spores with intermediate ethanol tolerance (Figure 4.14).

The occasional deviations from a 2:2 ratio might be explained by segregation of a single locus with occasional gene conversion or by linkage of two (or more) loci, which co-segregate at a frequency determined by the distance between them. However, an intermediate phenotype could not be caused by gene conversion at a single locus; clearly there must be two or more genes contributing to the ethanol tolerance phenotype. Thus, it is likely that the ethanol tolerance phenotype in SM is determined by two or more closely linked loci.

In conclusion, genetic analysis of CM and SM mutants indicate that in both cases the phenotypes are conferred by dominant loci. In the case of CM, this is a single locus but in SM, it is probably 2 or more closely linked loci. The next chapter describes an attempt to further characterise ethanol tolerance conferring mutations using Comparative Genome Hybridization (CGH) Tiling Microarrays.

Chapter 5

Attempts to identify mutations conferring the Ethanol-Tolerance phenotype in SM and CM

5.1 Introduction

This chapter describes attempts to characterize, at a molecular level, mutations conferring ethanol-tolerance in SM and CM. Two approaches were trialed:

1. Isolating ethanol tolerance conferring genes by the generation of genomic libraries of SM and CM, transforming these into the nET parental strain, with the aim of screening these for ethanol tolerance.
2. Using Comparative Genome Hybridization (CGH) to detect ethanol tolerance conferring mutations.

5.2 Results

Chapter 4 concluded that the genes conferring ethanol tolerance in SM and CM were dominant. Thus, it was reasoned that it should be possible to isolate the ethanol tolerance genes (particularly for CM, in which ethanol tolerance is conferred by a single gene) by generating a genomic library, transforming the library into the parental strain and plating onto ethanol selection medium; previous work indicated that ET mutants are able to grow on 12% v/v ethanol plates while the parent cannot.

5.2.1 Attempts to isolate ET-conferring genes from SM and CM by generating genomic libraries

Genomic DNA from the ET mutants was isolated and digested with the restriction enzyme *Sau*3AI (as described in Section 2.3). The DNA was digested using various concentrations of *Sau*3AI in order to obtain fragments of a size that could capture complete ORFs.

Large fragments of 5-12 kB were isolated from an agarose gel and ligated into the plasmid p416 GPD (ATCC 87360- http://www.atcc.org), which was kindly donated by Dr. Ian Macreadie (CSIRO, Parkville). The ligation product was then transformed by electroporation (Section 2.18) into *E.coli* DH10B and plated onto ampicillin medium to select for

transformants containing plasmid with the genomic DNA fragments. The plasmids from the transformants were then isolated and cut with *Eco*RV, which cuts the plasmid only once, to produce a linear DNA molecule. The molecular weight of plasmids was expected to increase following successful ligation of 5-12 Kb fragments. However, little or no change in the molecular weight of the plasmid was observed. Various phosphatase concentrations, vector to insert ratios, buffers and transformation methods were tried but with no success. Thus, an alternative plasmid, pBC KS+ (Stratagene), was trialed. This plasmid contains the *E.coli* bluescript lacZ gene, which allows for blue/ white screening of ligation products. In this case ligation/transformations were successful but the majority of inserts were approximately ~1 Kb, and transformation frequency was low (the highest efficiency obtained was 50 transformants/μg DNA. Thus, the ligations captured very little of the CM genome. Library constructions were attempted numerous times trialing a range of conditions (e.g. vector to insert ratio and various phosphatase concentrations), and with the assistance of scientists with experience in library generation, but with little success. However, at the time of attempting to resolve problems encountered in constructing gene libraries, Affymetrix GeneChip *S. cerevisiae* Tiling Arrays became available, and attempts to generate gene libraries from ET mutants were put on hold (and not revisited for work described in this thesis).

5.2.2 Attempts to identify ethanol-tolerance conferring genes in SM and CM using Tiling Arrays

Tiling arrays consists of over 3.2 million, 25-mer oligos of the entire *S. cerevisiae* genome in a tiled format on a microchip. The oligos overlap by 20 base pairs and have about a 5 base pair resolution. Sample *S. cerevisiae* genomic DNA is sheared, labeled and hybridized to the tiling arrays. The tiling arrays used for this work were originally designed for Chip on CHIP analysis (http://www.affymetrix.com), however, Gresham (2006) developed a program called SNP (Single Nucleotide Polymorphisms) Scanner which was able to determine SNP locations in *S. cerevisiae* RM11-1a using data output from the tiling arrays. The authors found that SNP Scanner was able to identify 93.3 % of known SNPs with a minor false positive rate.

5.2.2.1 Minimizing background noise in SM and CM for CGH analysis

Because SM and CM were generated using adaptive evolution strategies (Stanley et al. 2009), it was reasoned that there was probably a number of non-ET conferring mutations in these mutants; particularly in CM, which was generated by EMS (ethylmethane sulphonate)

mutagenesis. Thus, iterative backcrossing of the ET mutants to the parental strain was performed to dilute incidental, non-ET conferring, mutations in CM and SM genetic backgrounds.

5.2.2.1.1 Iterative backcrossing of CM

The following describes an extension of the work described in Chapter 4, with 2 additional rounds of backcrossing. For each backcross, a spore that retained the ET phenotype was randomly chosen to backcross to the parental strain. Figure 5.1a shows the iterative backcrossing scheme used to produce the strains from which genomic DNA was hybridized to the Tiling Microarrays.

RETA was used with 0, 8, 9 and 10% v/v ethanol to assay ethanol tolerance levels of backcrossed progeny. A summary of progeny ethanol tolerance levels in 8% v/v ethanol is shown in Figure 5.1b. When the nET parental strain was crossed with an ET strain the diploid consistently displayed ethanol tolerance levels identical to the ET parent strain in all backcrosses (data not shown).

The graph shows that for each tetrad, a 2:2 segregation of the phenotype was observed, where half of the progeny inherit the ET phenotype. After three successive backcrosses, a single progeny (C3-7C), which retained the ethanol tolerance phenotype, was chosen to be used in the Tiling Microarray analysis in order to determine the mutation giving CM the ethanol tolerance phenotype.

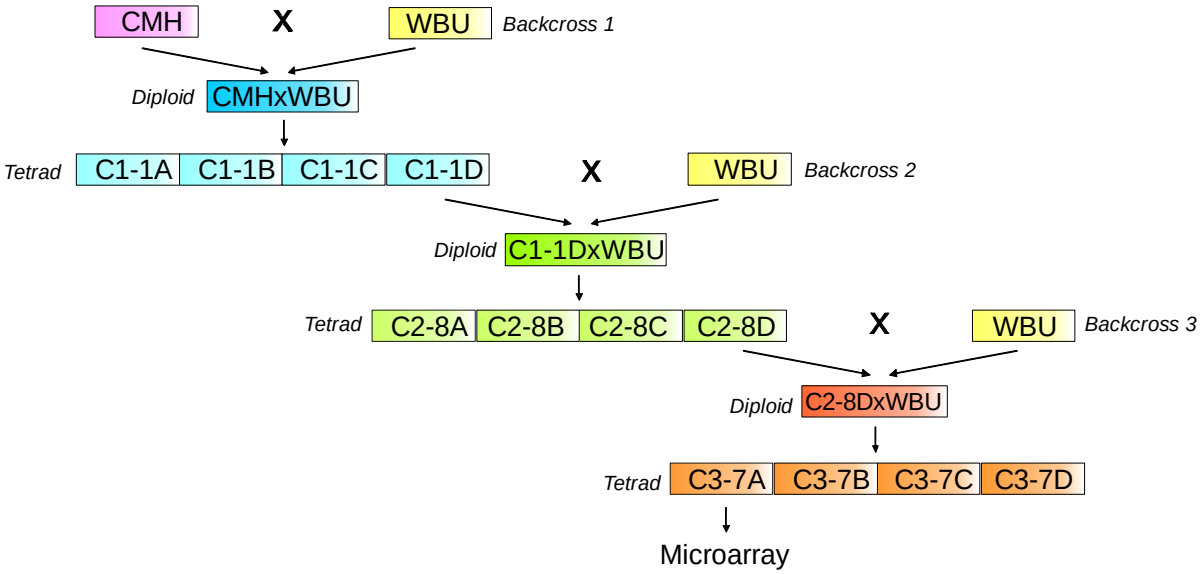


Figure 5.1a

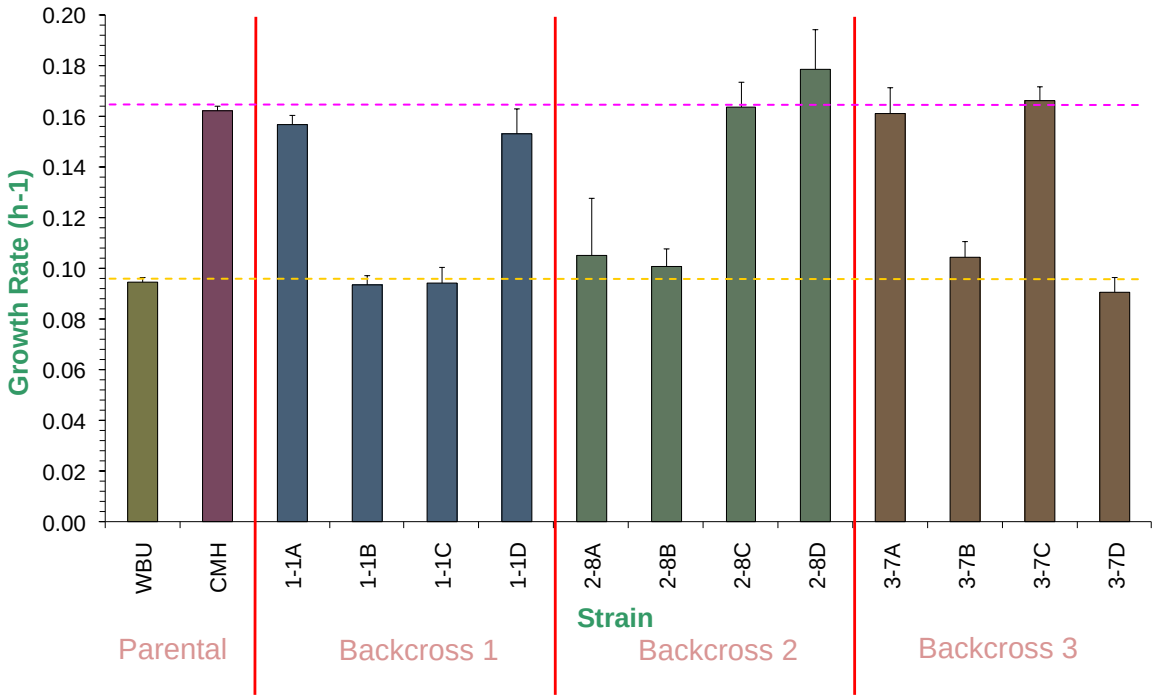


Figure 5.1b

5.2.2.1.2 Iterative backcrossing of SM

SM was put through three successive backcrosses as shown in Figure 5.2a, and associated ethanol tolerance levels of the progeny are shown in Figure 5.2b. All diploid progeny displayed equivalent ethanol tolerance levels to the SM parent. The majority of the tetrads displayed 2:2 segregation of the ethanol tolerance phenotype however, as was the case for results described in the previous chapter, there were some differences from this with occasional intermediate phenotypes (data not shown).

Thus, care was taken to choose a tetrad set that displayed 2:2 segregation. After three successive backcrosses, a single progeny (S3-2A), which had retained the ethanol tolerance phenotype, was chosen to be applied to the Tiling Microarray in order to determine the mutation giving SM the ethanol tolerance phenotype.

5.2.2.2 Application of Comparative Genome Hybridization (CGH) to identify Mutations

Affymetrix GeneChip® *S. cerevisiae* Tiling 1.0R Arrays were used to perform the CGH on the nET parental strain, C3-7V and S3-2A. Tiling Arrays carry 3.2 million overlapping 25-mer probes of the *S. cerevisiae* genome in a tiled format, enabling a 5 bp resolution, and have been successfully applied to identify SNPs between laboratory *S. cerevisiae strains* (Gresham 2006)

Genomic DNA was isolated from S3-2A and C3-7C (Section 2.14) and sent to Australian Genomic Research Facility (AGRF) where the wet-lab component of the Tiling Array analysis was performed. Genomic DNA was sheared, labeled with fluorescent markers and hybridized to the Tiling Arrays (Section 2.19). Signal intensities were read using GeneChip® Fluidics Station 450 and the output files were in .cel format.

Tiling Array data was returned from AGRF in .cel format and analysed using the SNP Scanner program with the assistance of Dr. Anthony Borneman (AWRI), Assistant Professor David Gresham (Princeton University) as well as Dr. Richard Harrison (Sanger Sequencing Centre). The .cel files were assembled using *S. cerevisiae* genome sequence library file Sc03b_MR_v03, in the SNP Scanner program. Unfortunately, SNP Scanner was unable to process the data.

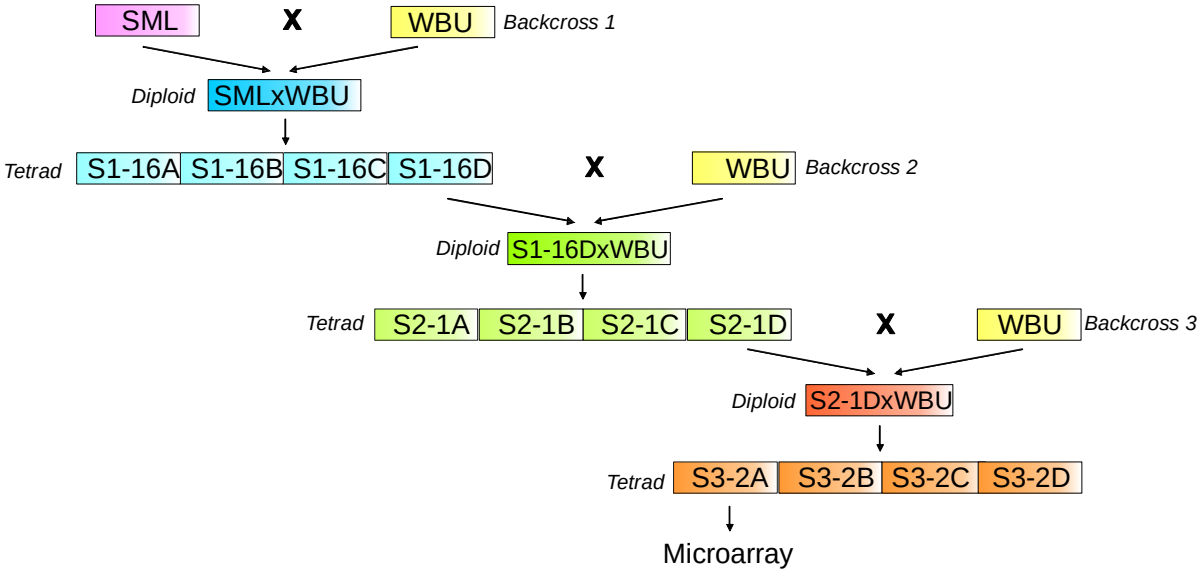


Figure 5.2 a

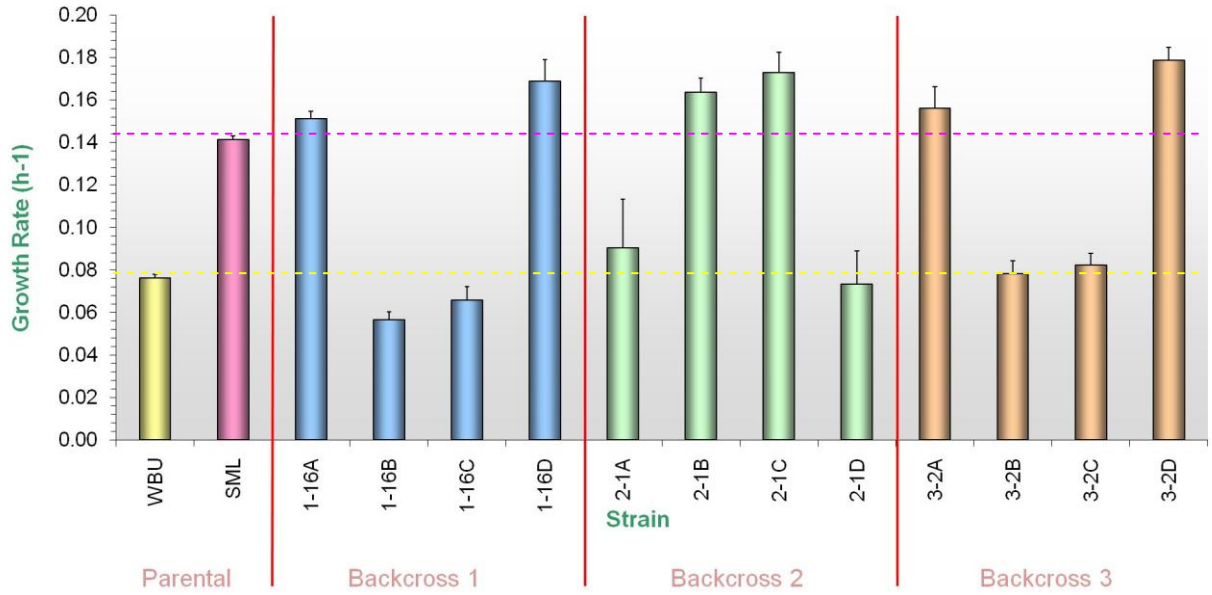


Figure 5.2 b

As an alternative, programs TAS (Tiling Analysis Software) and IGB (Integrate Genome Browser) were trialed. TAS takes the signal intensity for the control (parental) strain and divides it by the intensity for the same probe hybridized to the mutant strain. Theoretically, where probes bind to both strains, there should be a signal value of zero. Where the probe binds to the parental strain and not to the mutant the signal value would be positive, indicating sequence differences. A signal was considered 'different' when >20 probes gave continuous signal intensities with the threshold above ±0.3 at the same locus.

Initial observation of the data gave an unexpectedly large amount of noise. For example, Chromosome VIII (Figure 5.3) has extremely variable signal intensities throughout the entire chromosome. The majority of the intensities were expected to be close to the baseline, which was not the case. In addition, for some loci, there was no signal data for the parent or mutant, rendering these regions effectively 'invisible' to the analysis and inability of SNP Scanner to apply the algorithm and resolve the data (personal communication David Gresham-Princeton University and Richard Harrison-Wellcome Trust Sanger Institute). This may explain why the SNP Scanner program could not process the data.

Despite the immense amount of noise, repetitive sequences such as transposons and Autonomously Replicating Sequences (*ARSs*) were identified as 'different' in the array data. Since the repetitive regions are not unique the amount of labeled genomic day will bind to the Tiling arrays in a random amount. For example in Figure 5.4 a decline or increase in signal from the baseline indicates loci that differ significantly between the parental and mutant strains. Coding regions that gave a 'difference' signal were also largely repetitive sequences as can be seen for the *ASP* genes (Figure 5.4). However, these repetitive regions were unlikely to give insight into the locations on the ethanol tolerance conferring regions in the genome and were excluded from further consideration.

As the large amount of background noise made it difficult to process the data, it was decided to concentrate initially on ORFs rather than intergenic regions. More than 200 differences in ORFs were identified (Appendix 5.2). Ten mutations from C3-7C and ten from S3-2A, were chosen for verification experiments. Primers were designed to target these and PCR amplified products were sent to The AGRF for Sanger sequencing (Refer to Appendix 5.1 for tables and data). Due to the sequence similarity of W303-1A to S288C (Schacherer et al. 2007), the sequences of the PCR products from backcrossed ET-mutants, were compared to the sequences of the same loci in S288C (obtained from *Saccharomyces* Genome Database: SGD - http://www.yeastgenome. org/).

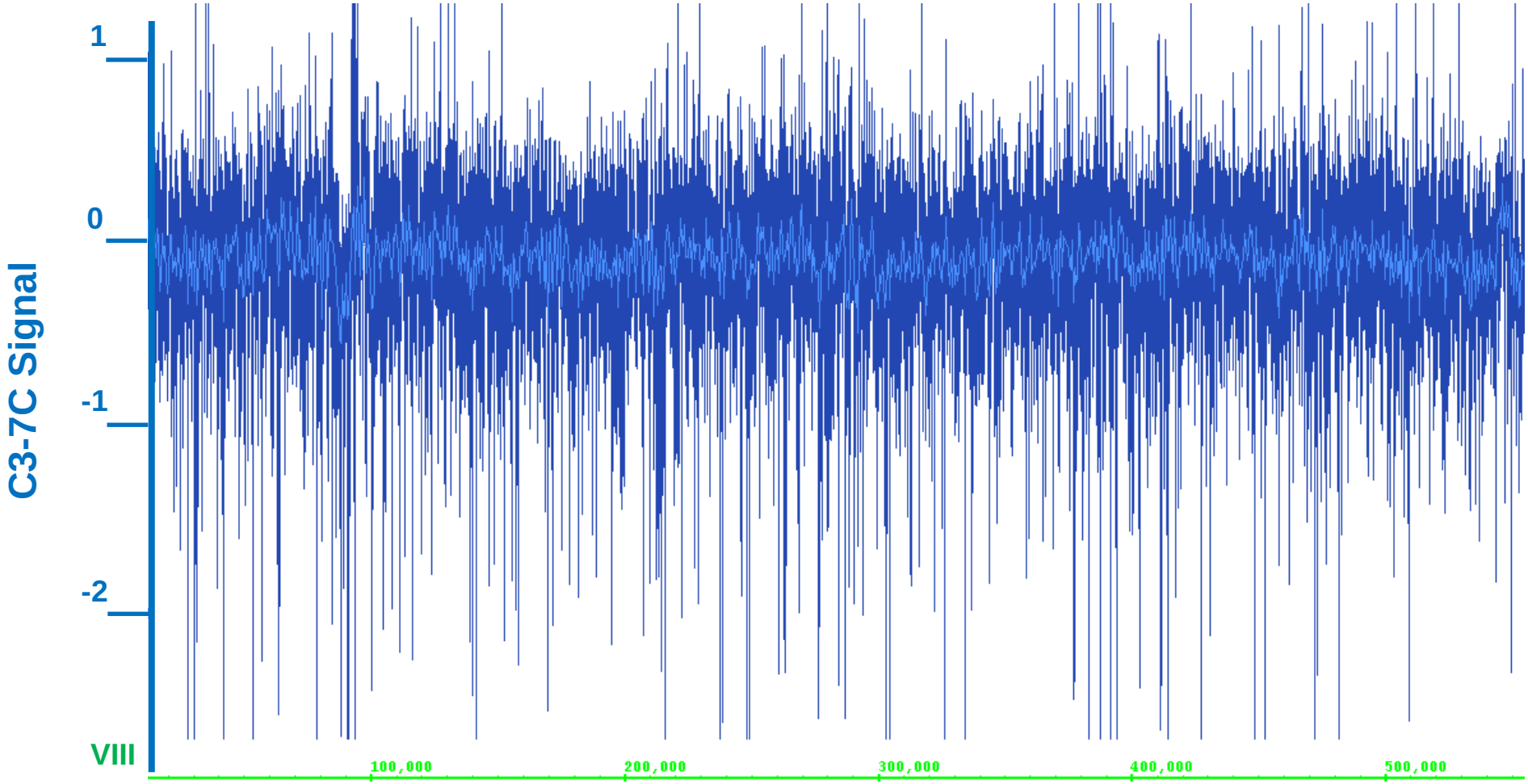


Figure 5.3: An example of the signal data output from the Tiling microarray analysis of Chromosome VIII, when the signals of the parental strain are compared to the backcrossed chemical mutant.

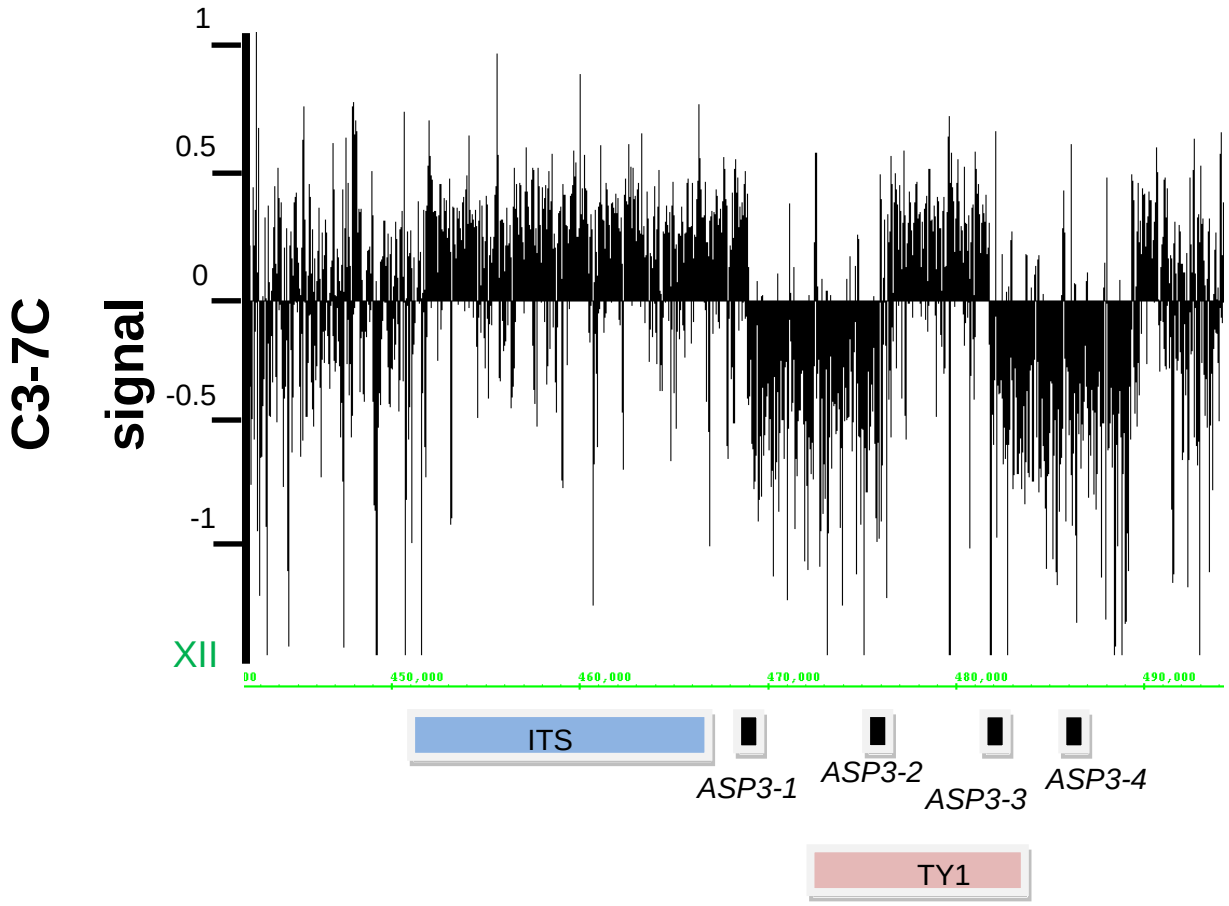


Figure 5.4: Example of repetitive regions with CGH signals that are greater than the assigned cut-off for experimental variation in Tiling Microarrays. Multiple probes bind to Chromosome XII: 450,000- 490,000 since the locus contains highly repetitive regions in the genome including transposons (TY1), inter-transcribed spacers (ITS) and asparginase (ASP) encoding regions.

It was found that there was a large degree of noise in the data and that in all twenty cases the regions with significant difference, were false positives.

Nevertheless, in addition to the above twenty putative mutations that turned out to be false positives there was one additional large mutation on Chromosome IV of C3-7C (Figure 5.5). At this locus there was ~6 Kb decline in the signal, between coordinates IV: 1,154,000 – 1,164,000 bp, which includes ORFS *HXT3*, *HXT7* and *HXT6*. The major deviation in signal intensity can be seen by comparing the signal intensities of the S3-2A against the C3-7C on the same y-axis scale, and is indicative of a large deletion.

Primers were designed to target regions flanking *HXT3 to HXT7*, such that a single 3 Kb product was produced following PCR amplification of the deletion locus. As shown in Figure 5.6, the parental strain and an additional wild type *S. cerevisiae* laboratory strain FY4, produced multiple PCR fragments, whereas C3-7C produced a single 3 Kb product, which is diagnostic for the deletion. To further characterize the genomic mutation, the 3 Kb product was then sequenced and the predicted amino acid sequences of protein encoded by the mutation region were compared to the S288C wild type Hxt protein sequences by CLUSTALW alignments. The comparison was performed by aligning the predicted protein sequence of *HXT3*, *HXT6*, *HXT7* and the *HXT3-7* mutation. The amino acid sequences of *HXT6* and *HXT7* are almost identical apart from an Alanine to Threonine (indicated in Figure 5.7 by ⬇) at position 559. Figure 5.7 shows that the amino acid sequence of *HXT3-7* mutation begins in *HXT3* at position 0-91 (highlighted in yellow), followed by the sequence of *HXT7* which is distinguished from *HXT6* by the A →T at position 559. There are no stop codons throughout the sequence, indicating the *HXT3-7* mutation in C3-7C, is a result of a fusion between *HXT3* and *HXT7.*

To determine whether or not this mutation segregated with the ET phenotype, 24 progeny from the backcrossing experiments were analysed, using PCR, for the *HXT3-7* mutation and for ethanol tolerance. Data indicated that the mutation and phenotype did not co-segregate (Figure 5.8). Thus, the *HXT3-7* mutation does not contribute to the ET phenotype in C3-7C. No mutations indicated in SM and CM by the Tiling microarrays; could be confirmed by Sanger sequencing.

There were no further mutations identified and confirmed from the CGH datasets for either C3-7C or S3-2A.

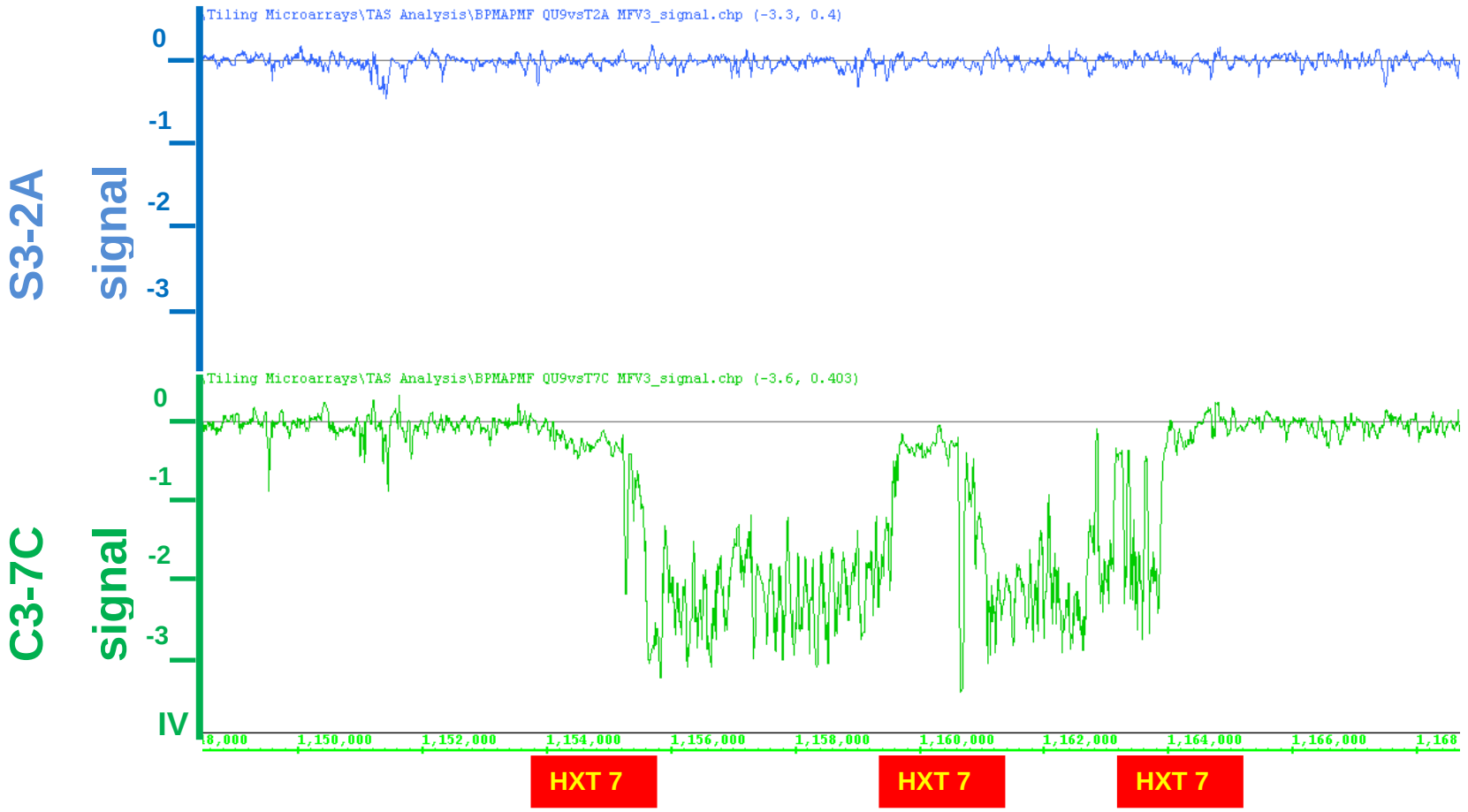
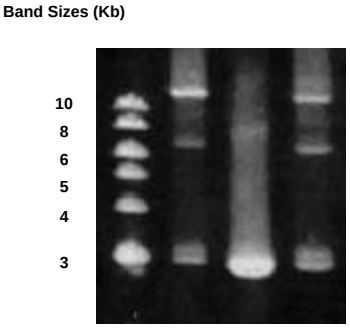


Figure 5.5: Output from TAS and visualization on IGB comparing signal outputs from tiling array analysis of S3-2A and C3-7C at Chromosome IV: 1,140,000 – 1,160,000. The signal intensities are produced by dividing the individual probe intensities for the ET mutants by the equivalent probes in the parental strain. Refer to Appendix 5.6 for simplified diagram which demobstrated portion deleted from genome.



Lane	Contents	RETA
1	1kb Marker (NEB)	-
3	FY4	nET
4	C3-7C	ET
4	WBU	nET

Figure 5.6: PCR amplification of Chromosome IV *HXT3-7* mutation of C3-7C, the nET parent (WBU) and an additional commonly used laboratory nET strain FY4. The parental strain and FY4 gave multiple PCR products whereas the ET 3C-7C gave a single product which was indicative of a large deletion in Chromosome IV.

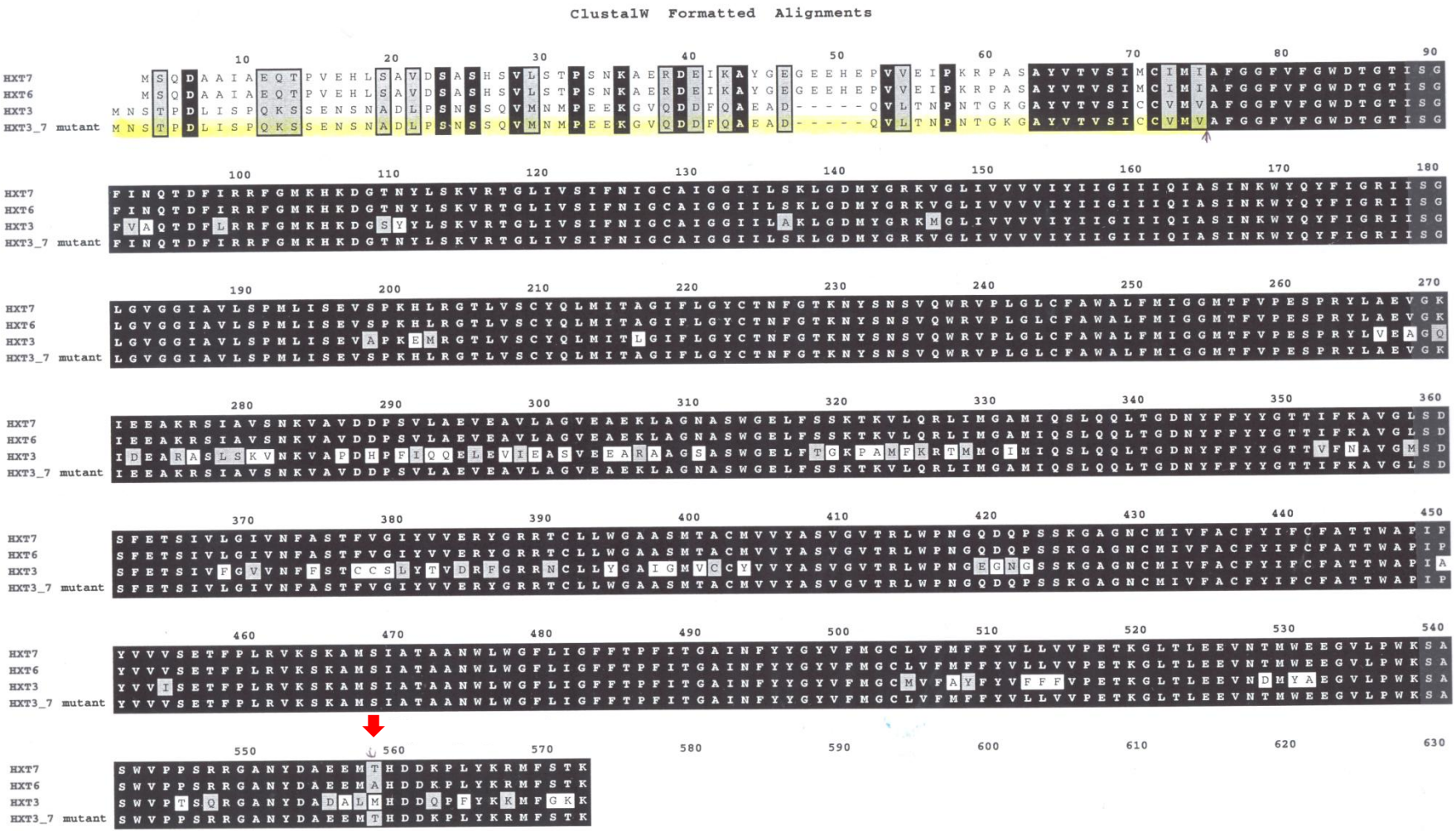


Figure 5.7: ClustalW analysis of the *HXT3-7* deletion from C3-7C showing the amino acid sequence of the Hxt3-7 fusion protein (bottom line). The region highlighted in yellow is the amino terminus of Hxt3. The remainder of the sequence is that of Hxt7; at position 559 (⬇) there is a single amino acid difference that distinguishes *Hxt6* from Hxt7.

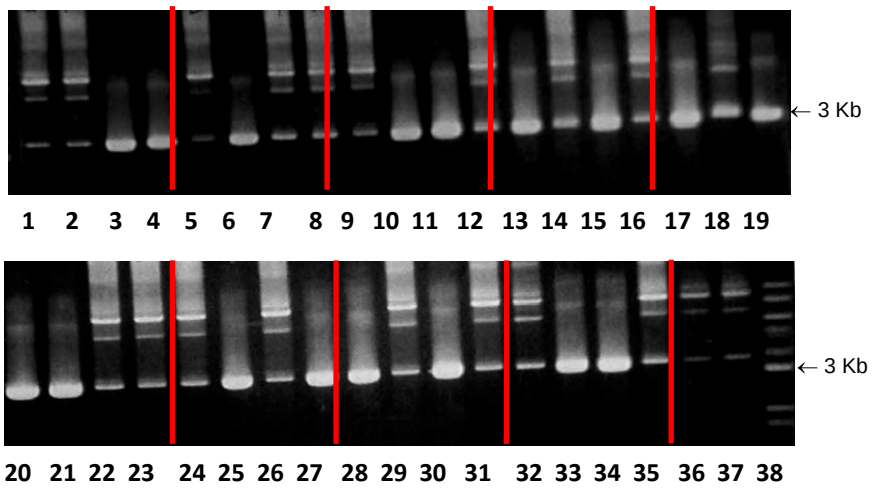


Figure 5.8b: PCR amplification of the *HXT* mutation region in various tetrad sets from CM backcross. The presence of the *HXT* mutation, indicated by a single 3 kb PCR product (+) and the absence by multiple products (-).

5.4 Discussion

The aim of work described in this chapter was to identify the genetic mutations which confer ethanol tolerance in the ET mutants CM and SM. Attempts were made to generate genomic libraries of the ET mutants, which were to be transformed into the nET parental strain and selected for the ethanol tolerance phenotype. Numerous attempts over several months to generate genomic libraries to isolate ethanol-tolerance conferring genes from SM and CM were unsuccessful and attempts to modify vector to insert ratio, phosphatase treatment and enzyme sources. Various vectors, and digested genomic preparations were trialed, however the vectors were unable to ligate large inserts and with high efficiency. Attempts by other scientists in the laboratory, also failed to generate suitable genomic libraries for this work.

Other vectors and genomic fragment insert isolation methods could have been trialed but, because of time constraints, this was not an option. Instead, it was decided to adopt a different approach using Affymetrix Tiling Microarrays, which had previously been shown to identify SNPs (Gresham et al. 2006). Tiling Arrays are gene chips which have an organisms genome arrayed in the form of overlapping oligonucleotides; Affymetrix GeneChip® *S. cerevisiae* Tiling Arrays have a 25-mer oligonucleotides with 5 bp overlaps.

Before array analysis was performed the ET mutants, CM and SM, were iteratively backcrossed to dilute incidental mutations that would have arisen during the adaptive evolution procedures that were used to generate them (Stanley et al. 2010). This was particularly important for CM which was generated using EMS mutagenesis, and therefore would be predicted to carry numerous mutations. Segregation of the ethanol tolerance phenotypes in CM and SM confirmed the findings of the previous chapter where CM ethanol tolerance phenotype segregates in a 2:2, whereas SM mostly segregated in 2:2 with the occasional 1:2:1 and 1:3.

Once backcrosses were performed genomic DNA of the backcrossed ET mutants and parental strains were hybridized to Affymetrix Tiling Microarrays by AGRF. Attempts to process the data from this using SNP Scanner software were unsuccessful. The SNP Scanner program was unable to process the data due to a high level of background noise and because the datasets for the parent and CM were incomplete, where the signal intensities of some probes were missing). Alternative programs TAS and IGB were used to analyse and visualize the data.

Data generated, showed that CGH generated a large amount of noise, resulting in false positives. In consultation with experts in the field it was reasoned that this was likely due to problems with wet-lab procedures, which was out-sourced and therefore could not be easily rectified.

Nonetheless, a large deletion spanning Chromosome IV: 1155265-1164666 subsequently called *HXT3-7* mutation in CM was identified and confirmed. Further analysis of the amino acid sequence of the *HXT3-7* mutation, found that the large deletion resulted in a fusion between Hxt3 (low affinity) and Hxt7 (high affinity) hexose transporter proteins (Figure 5.7). This was an extremely exciting result as hexose transporters are central to a yeast's fermentative capacity (Salmon et al. 1989, Pretorius et al. 2003, and it has been hypothesized that when yeast are exposed to ethanol stress, their ability to uptake sugars is compromised and may lead to stuck ferments (Ansanay-Galeote 2001, Chandler 2004, Santos 2008).

Karpel et.al. (2008) performed an analysis comparing hexose transporter protein encoding ORFs of different *S. cerevisiae* strains including wine strains. The authors suggested that *HXT3* may have a role in ethanol tolerance since deletion of the gene resulted in lower fermentation rate. Snowdon et al. (2009) found that the Ethanol Tolerance Protein gene (*ETP1)* is required for the transcriptional activation of Exitus NAtru (*ENA1,* a plasma membrane Na^+-ATPase exporter) that plays a role in Hxt3p activation. *ETP1* has been found by Snowdon et al. (2009) to be required both in ethanol stress adaptation as well as when ethanol is a sole carbon source. Thus, there are clear links between *HXT3* and tolerance to ethanol stress.

Other indications that hexose transporters are important in ethanol stress tolerance came from the work of Chandler et al. (2004), in which the authors found that high affinity transporters such as *HXT6* and *HXT7* were up-regulated under ethanol stress with a simultaneous decrease in expression of low affinity hexose transporters (e.g. *HXT1 and HXT3*). Results by Stanley et al (2009b) confirm these findings. Stanley et al. (2010b) found that *HXT7* was up-regulated in CM specifically under ethanol stress conditions and that the sugar utilization rate was more efficient than for the parental strain under ethanol-stress conditions. Thus, considering the role important roles of *HXT3* and *HXT7* during ethanol-stress conditions, it was hypothesized that the Hxt3-7 fusion protein may play a role in conferring the ethanol tolerance phenotype in CM. However, results in Figure 5.8 illustrate that the *HXT3-7* mutation was, disappointingly, not associated with ethanol tolerance phenotype.

Since the mutation did not segregate with the ET trait, it can be concluded that Hxt3-7 fusion protein does not confer the ethanol tolerance phenotype in CM.

At the time of processing the above CGH data, an opportunity arose to sequence the entire genome of one of the ET mutants, using Solexa (Illumina) sequencing. Thus, it was decided not to further pursue the Tiling array strategy but rather focus efforts on sequencing.

Chapter 6

Genome Sequencing of Ethanol-tolerant Chemical Mutant

6.1 Introduction

As mentioned in the previous chapter, comparative genome analysis using Tiled Microarrays did not enable the identification of mutations that confer ethanol-tolerance in CM or SM. However, at the time of conducting the array experiments, an opportunity arose to sequence the genome of one of the ET strains. It was decided to sequence CM since the ethanol-tolerance phenotype in this strain clearly segregated as a single gene. SM, on the other hand, displayed a more complex segregation pattern, suggesting that more than one genetic locus was responsible for the ethanol tolerance phenotype, making identification of ET conferring mutations more difficult.

To minimize the amount of background noise, in the form of incidental mutations in CM that do not contribute to ethanol tolerance, the same backcrossed strain, C3-7C, described in Chapter 5, was used here.

6.2 Results

Genomic DNA was isolated from C3-7C (Methods 2.10). Genome sequencing, using the Illumina Genome Analyzer sequencing platform, was outsourced to GeneWorks Pty Ltd. Sequence data was then assembled using CLC Genomics Workbench and W303-1A genomic sequence as a reference (kindly provided by Dr. Chris Harris from Prof. E. Louis laboratory, University of Nottingham).

Genome sequence data comprised 25-bp reads with approximately five-fold coverage of the whole genome. However, there was lower coverage than this in some locations, with approximately 5% gaps in the overall sequence. Single Nucleotide Polymorphisms (SNPs) identified using CLC Genomics Workbench are shown in Appendix 6.1.

An example of the output when viewed in CLC Genomics Workbench is shown in Figure 6.1. A threshold of at least three-fold coverage of mutations was used as a cut-off to minimize false positives.

A total of 240 SNPs were detected (data not shown), however many of these were discarded because they resided in repetitive sequences (e.g. telomeres and transposons), leaving 147 candidates (Appendix 6.1). Other regions that were then discarded included: large repetitive sequences (because they cannot be resolved in short-read genomic sequencing data), regions that were >500 bp from ORFs, and multiple copy ORFs (e.g. Autonomously Replicating Sequences-ARSs). This left 104 potential ethanol-tolerance conferring mutations. Of these, 45 were chosen for further analysis, choice being based on location; SNPs that were near (<500 bp upstream or downstream) or inside ORFs previously associated with the ethanol tolerance phenotype or ethanol metabolism were considered good candidates. (Refer to Table 6.1).

Using Primer3 software, PCR primers were designed to target and amplify the 45 potential ethanol-tolerance conferring loci in C3-7C. Following amplification, PCR products were transformed into the parental strain WB, and ET transformants were selected on 12% v/v ethanol YPD plates (which is inhibitory to growth of WB but not C3-7C). One PCR fragment (SNP # 23, shown in Table 6.1), when transformed into WB, generated three ethanol-tolerant colonies. Ethanol tolerance of this transformant was verified using RETA (Figure 6.2). The sequence that was transformed is referred to as **E**thanol **T**olerance **C**onferring **S**equence (ETCS) for the remainder of the thesis and the ET-transformant was named WB-ETCS,

ETCS is located in an intergenic region ~ 500 bp upstream of *YBR238C* and ~500 bp downstream of *ERT1* (Figure 6.3) and from the original genomic sequence data it was found to carry one SNP, C→A at Chromosome II: 697850 (Refer to Figure 6.1). To validate the putative ethanol tolerance conferring SNP, Sanger sequencing technology was used to re-sequence ETCS and an additional three SNPs were found (Figure 6.4); thus, the locus conferring ethanol-tolerance in CM is on Chromosome II and has four SNPs at positions: 697850 (C→A), 697895 (T→G), 697907 (C→T) and 697928 (C→A).

Figure 6.1: Example of comparison of C3-7C sequence data aligned with data from the parent strain W303-1A. This figure shows a SNP (C→A) (↑) on Chromosome II: 697850 (that corresponds to position 704,076 on the diagram above).

SNP #	W303 Ref position	Name	Function	5' primer	3' primer
1	W303.chr14_217922	ATG2	autophagy	CTTCCAGGTTCGTTTACAAG	CGATAGGATTTTTCAACTGC
2	W303.chr10_271575	SIP4	Zn transcription factor	ATACGAAAGTGGTCAAAACC	ATCATCATCTTCCGCATATC
3	W303.chr11_327023	MNR2	Mg transporter	GTTTATGAGATTGAGGACGC	TTGGCAAATCCTTTTATCAC
4	W303.chr11_416671	PUT3	transcriptional activator	AAATACGACACTGGTCCAAC	ATATGCCTCTCGTCATCATC
5	W303.chr11_423974	URB1	Accumulation of rRNA	AGGGTCATAGTTACCATTCG	TTAAATGAACTAGCGGCTTC
6	W303.chr12_596437	ECM22	sterol ERG	GTCTCCGAGTTGGATAACAG	AATCCAAGCGTTATTGACAC
7	W303.chr12_630626	IRC20	helicase	GCGATTTTTGAATTGTTCTC	CTACATTCTTCTTGGGTTGG
8	W303.chr12_871637	CSR1	lipid & fatty acid metals	AACAAGATCATAAACGGTGG	GTGATTAGAAACTTGACGGG
9	W303.chr12_998640	CNA1	calineurin regulates stress	TCTTAGCACCGAAAACATTC	TAATAACCATGTTCAGTGCG
10	W303.chr13_187170	PRP39	pre-mRNA processing	ATTGAGTGCAATTCAGGAAC	CCTGTCATTTCGATTTACG
11	W303.chr13_544716	REC114	recombination	TAAATACGAAGCCCATTTTG	GCGAATATTTTCGAGAAGTG
12	W303.chr13_546572	ERG29	ergosterol biosynthesis	ACAGGAGTTGCCATATGTTC	CGCAAGGTAGTACCAAACTC
13	W303.chr14_49217	HXT14	hexose transporter	CAATTACCATGCTATCCGTC	AAGATCAAAATGCTTTAGCG
14	W303.chr14_411082	FAR11	cell cycle arrest	TTAAACGCTATTATCGGCTC	GTTTAACAGATCTTGGCTGG
15	W303.chr15_45919	GRE2	stress response	CATCGGTTCTGCTAGAAGTC	GCTGCATAAGAAGAGGTGAG
16	W303.chr15_450878	MSA1	factor	CACTGCTTGTAAACAACGTC	ACGAAAGAGAAGGTGGATG
17	W303.chr16_190637	GUP2	glycerol uptake	ACGACTGGTAGCAGAACATC	AGATGTAACGGATTACCCAC
18	W303.chr16_395425	BRO1	shock	ATTCAAAACGTGGTTAGTGG	TTTCCAAATCACTCAAGAGC
19	W303.chr16_864183	TIF3	translation initiation	CTTACCAAACTGAGCACCTC	TGATATTGACTGGACTGCTG
20	W303.chr02_15098	PKC1	Kinase	TTTGAACATGCCACATTAAG	GTTTGTCTTAGAACCATCCG
21	W303.chr02_266804	GRX7	oxidative stress response	GAAATGCCTAAAACAGATGG	CCCTGTCCATGAAGTAAGAC
22	W303.chr02_626722	MCM7	DNA replication	AAAATTGTGCTCGTCGTTAC	CGATAAAGATCTTACCAGCG
23	**W303.chr02_704067**	**ERT1**	**ethanol regulator of translation**	**GAAATTGCCTCTCTTGTACG**	**AACATCAGGCGTATACCATC**

Table 6.1: Table of SNP locations identified from Illumina sequencing of C3-7C genome and primers designed to amplify SNP regions.

SNP #	W303 Ref position	Name	Function	5' primer	3' primer
24	W303.chr04_646788	GIS1	supressor	AACACAGTGGAAGAACTTGG	AAAAATTGGTCTGTGTTTGC
25	W303.chr05_33512	CAN1	plasma membrane	GAGGTGTGGATAAACCAATG	ACAAATTCAAAAGAAGACGC
26	W303.chr07_664623	NNF2	RNA polymerase subunit	GGTTTTTCGCAAGTATTCAC	CAGGGCCATAGTATTTGAAG
27	W303.chr07_835914	TIF4631	transcription initiation factor	GGTGTTAACCTTTGCTGAAC	TATGTGAGAAACGGTGTTTG
28	W303.chr08_91266	STE20	signal translating kinase	AATGTGTTGTCAGCAGAATG	TTACGATTACCAAGGACCAC
29	W303.chr08_222530	RRP3	rRNA processing	CGAGAAACAAATAGGAAACG	AACTACCGAAGGAAAGTGTG
30	W303.chr09_300777	SSM4	DNA ligase	TATTATTATCTGCTGCTGCG	CAGCAACTCCAGAATTAACC
31	W303.chr16_371634	NOG1	nucleolar GTPase	ATACACAGGTGAAGGTTTCG	CTCCACCTTAACAATTTTGG
32	W303.chr13_916152	YMR317W	unknown	TAAGGCGAATTTTCTGGACCA	TCACATCCACATTCGAGGAA
33	W303.chr14_776115	YNR065C	Unknown	ATGTTTTACCAAAATCGGTG	TTCCTTTGGTTCAAGGTATG
34	W303.chr15_362644	ERP4	ER & golgi transport	TAAGAGCTTGAAAAGCAACC	TATGTACCAAGGGACTCGAC
35	W303.chr15_535179	CEX1	cytoplasmic export	GCAAAAACACAGGTTGATTC	TTCATTAAAGCACGAAACG
36	W303.chr01_27580	YAL064W	unknown	TGCAACAATTTGAAAGTCAG	TGTGAATGATGTTGTTACGG
37	W303.chr02_758639	RIF1	telomere length control	TCACTACGCAAGTCATCAAC	ATCCTCTAAAACGACCTTCC
38	W303.chr06_78836	AGX1	glycoxylase	AGAATTCTTGATGTATGCCC	GAACTGATTCGCCTATCTTG
39	W303.chr06_234958	IRC5	ATPase	TGGATAAATTGGCAAAAGAC	AACCAAGTTAAAGACCGGAG
40	W303.chr06_238624	YFR039C	unknown	AAAAGCGGTAAAAATATCCC	CACTCAGGTAGAGAATTGGC
41	W303.chr07_815908	NSR1	binds pre-rRNA	CGTCCTCAGATTTACGTTTC	GTTATCGTTGGCTTAGATGG
42	W303.chr07_842846	KRE11	ER & golgi transport	ACAATAAACCCAATCCCTTC	TCCGCTTACTGGATTAACAC
43	W303.chr08_385176	CRP1	cruciform DNA structure	CTTCTTCAATTGACCCTAGC	TCATTCAATTCCCTAGCATC
44	W303.chr09_264018	NEO1	Amino phospholipid	GGCGAGTCATTTATCAAAAC	GGTTGCTAAGAGACGACTTG
45	W303.chr13_310508	SOK2	supressor of kinase	CGAGATATTGTTGTTGTTGC	AGTTTTTGAAGTTTTTCCCC

Table 6.1 continued: Table of SNP locations identified from Illumina sequencing of C3-7C genome and primers designed to amplify SNP regions.

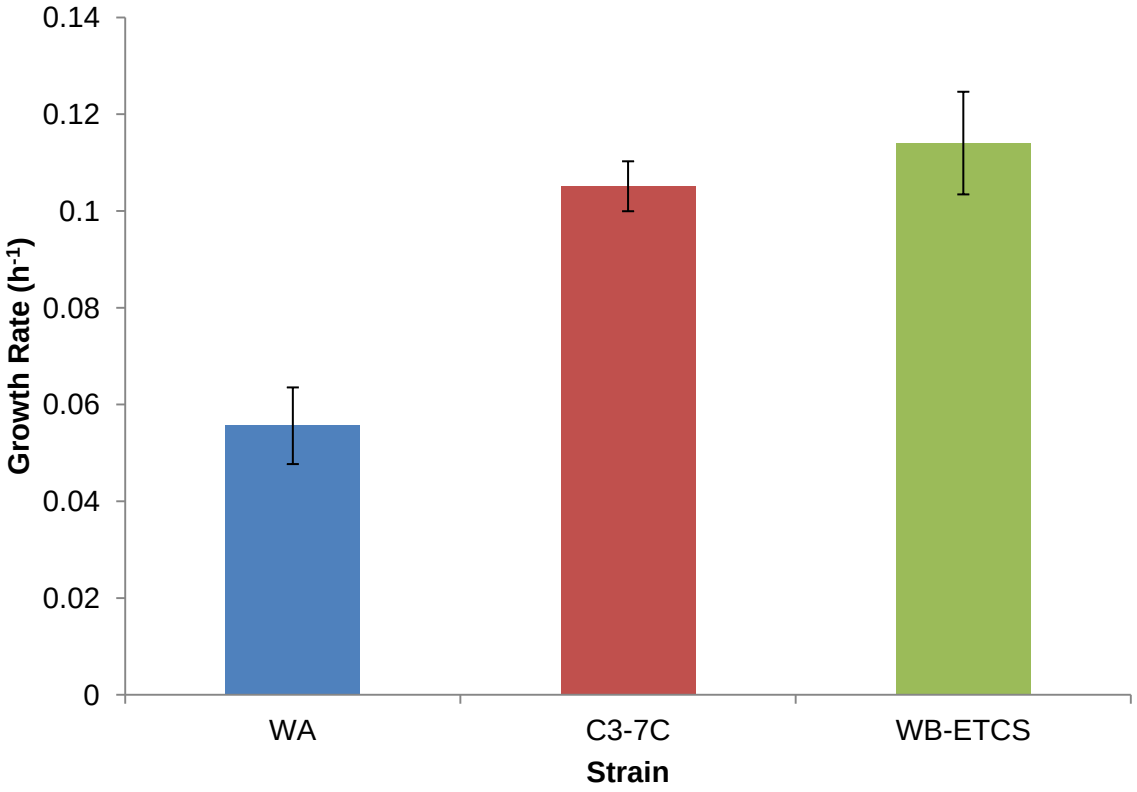


Figure 6.2: RETA results showing growth rates in 8% v/v ethanol of the parental strain WA (■), ethanol-tolerant C3-7C (■) and WB-ETCS (■).

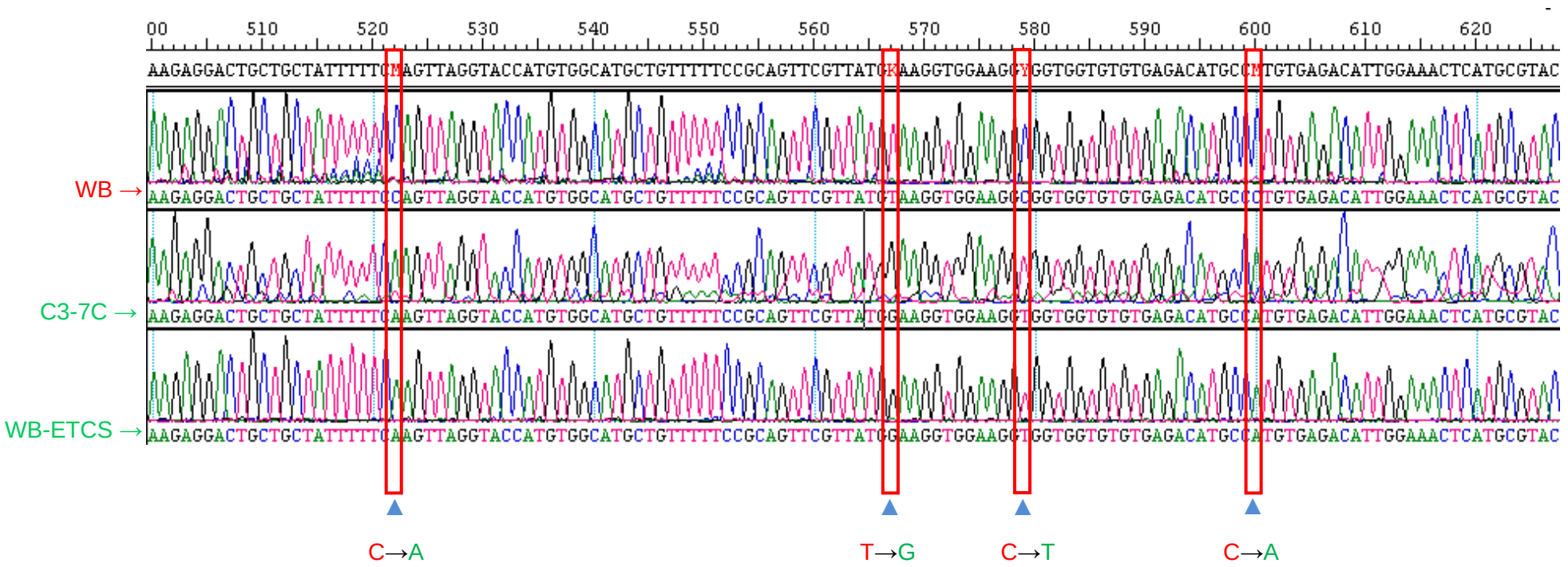


Figure 6.3: Validation of SNPs putative ethanol tolerance conferring in the ETCS of C3-7C and WB-ETCS. The above sequences compare the ETCS of C3-7C and WB-ETCS with the allele in WB. SNPs are indicated by (▲). Note: SM contained identical ETCS sequence to the parental WB.

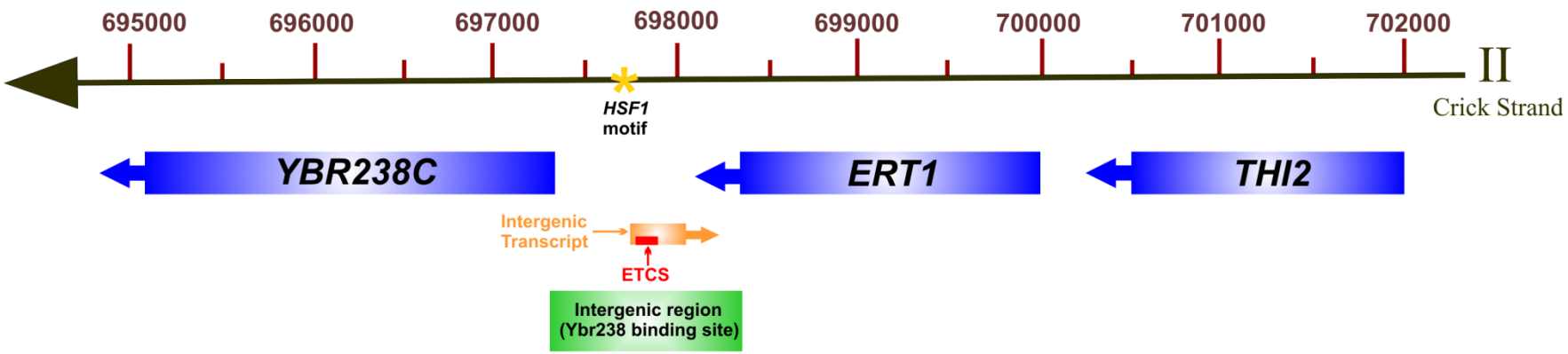


Figure 6.4: Schematic diagram showing the ETCS locus (■) on Chromosome II in the *S. cerevisiae* genome. ETCS resides in an intergenic transcribed region (■)(Miura et al. 2006) between ORFs *YBR238C* and *ERT1.* Upstream of ETCS is a *HSF1* binding motif (✱). The protein product of *YBR238C* binds to the entire intergenic region (■) between *YBR238C* and *ERT1* (Harbison et al. 2006).

6.3 Discussion

The genome sequence of C3-7C, which carries the ethanol-tolerance locus of CM, was compared to that of *S. cerevisiae* W303-1A, revealing a total of 147 credible SNPs. Given this large number of mutations, it would have been helpful to perform further rounds of backcrossing to reduce background noise before sequencing one of the progeny from a later backcross. However, due to time and budgetary constraints this was not possible. Instead, a sample of 'most likely' candidate ethanol-tolerance loci in C3-7C was screened; 45 mutations at loci previously associated with ethanol-tolerance or ethanol metabolism were PCR amplified from CM and transformed into WB. One of these, which carried a SNP C→A at Chromosome II: 697850, produced ethanol-tolerant transformants. Verification of the SNP revealed three additional SNPs; 697895 (T→G), 697907 (C→T) and 697928 (C→A) (Figure 6.3). Finding additional SNPs, highlights the importance of verifying 'whole genome' datasets, particularly when derived at relatively low coverage; there was only five-fold coverage of the genome in the original sequencing data. Given that the ethanol tolerance phenotype segregated in a single-gene fashion and that the Ethanol Tolerance Conferring Sequence (ETCS) was the only locus to generate an ethanol tolerance phenotype when transformed into WB, this was a very exciting result.

The ETCS resides in an intergenic region located between *YBR238C* (unknown function) and *ERT1* (Ethanol Regulator of Translation) (Figure 6.4). Immediately upstream of *ERT1* is *THI2* (THIamine metabolism), which is involved in thiamine biosynthesis. These three genes have not previously been identified as having a role in ethanol tolerance by functional genomic screens (van Voorst et al 2006, Kubota et al 2004, Kumar et al 2008). This may be due to the limited discriminatory power of these screens, unable to resolve all genes involved in ethanol tolerance.

ERT1 was recently identified as a potential transcriptional regulator involved in the utilization of non-fermentable carbons, in particular ethanol (Turcotte et al 2010). *THI2* (THIamine metabolism), has been identified as a transcriptional activator of thiamine biosynthetic genes. Thus, both *ERT1* and *THI2* have been identified as potential transcriptional regulators. These two genes are of interest not only because of their proximity to ETCS, but also because transcription data from earlier experiments on CM (Stanley et al. 2008), indicated that their expression is up-regulated during ethanol stress. Also, *ERT1* and *THI2* are candidate transcriptional

regulators (supplemental data in Segal et al. 2003); therefore their increased expression may influence the expression of other genes, perhaps including genes associated with ethanol tolerance. It will be of interest in future studies to determine whether the ETCS impacts on expression of *ERT1* and *THI2*.

YBR238C is about 500 bp upstream of the ETCS (Figure 6.4). Little is known about this gene and it has not previously been linked to ethanol tolerance. There is direct physical evidence from a fractionation experiment that Ybr238 localises to the inner mitochondrial membrane (Nouet et al 2007), and, from global protein localisation analysis, it may also reside in the cytoplasm (Huh et al 2004). The significance of Ybr238 locating to the mitochondrion or cytoplasm, in the context of ethanol tolerance, is not known.

There is also evidence in supplemental ChIP-chip data (Harbison et al 2004), that Ybr238 binds to the entire intergenic region between *YBR238C* and *ERT1* (Figure 6.4). This latter observation is suggestive of *YBR238C* being autoregulatory; its protein product binds to its own promoter thereby regulating its expression (other examples of autoregulation in *S. cerevisiae* can be found in Wang 1998 and Schwank et al 1997). If this is the case, presumably mutations in this region will impact on the affinity of Ybr238 for the intergenic region thereby perturbing any regulatory function.

Interestingly, the ETCS resides in a transcribed intergenic region (Miura 2006) (Figure 6.4). Intergenic transcribed regions have, in recent years, been the focus of a considerable amount of research. Advances in intergenic transcript analysis using techniques such as RNA-seq, are beginning to unravel the complex matrix of interactions between non-coding and coding regions of the genome. Schmitt and Paro (2004), for example discuss how "*…it is the very act of reading the DNA, not the message produced, that carries out the regulatory job*", and Martens et al. (2004 and 2005) found that when there is an abundance of the amino acid serine, in yeast growth medium the cell no longer requires increased expression of an intergenic transcript, *SGR1,* which suppresses the expression of adjacent *SER1,* a gene involved in the biosynthesis of the amino acid serine. In a similar vein to the Martens et al (2004 and 2005) finding, it is possible that the ETCS transcript somehow interferes with binding of Ybr238c to the intergenic region between *YBR238C* and *ERT1*. For example, the mutated transcript may outcompete Ybr238c for binding to

the intergenic region. However, this is all very speculative, requiring further work to test these ideas.

Another interesting feature of the region surrounding ETCS in CM is the presence 58 bp up-stream of ETCS resides a Hsf1p (Heat Shock Factor) binding motif, AAAGAAGAGAAAT (http://genome.ucsc.edu). This is interesting because Hsf1p is a heat shock transcription factor regulating the expression of hundreds of genes associated with stress responses (Hahn et al 2004). Of particular importance here, *HSF1* has previously been shown to be induced under ethanol stress conditions (Morimoto 1998, Yamamoto 2008, Ding 2009, Takemouri 2005, Ma 2005). If this motif is indeed a Hsf1 binding site and it regulates the expression of *YBR238C*, it is possible that one or more of the ethanol-tolerance conferring SNPs (in the transcribed intergenic region) impacts on Hsf1 affinity for this region and thereby changes expression of *YBR3238C*. However, as mentioned previously the role of *YBR238C*, in ethanol tolerance are unknown, and thus it is difficult to speculate further on the significance of a potential role for Hsf1p binding in this region.

There are many outstanding questions regarding the ETCS, one of which concerns the SNPs in this locus: are all four required for the ethanol-tolerance phenotype? If not, which are needed? Are these SNPs found in highly ethanol-tolerant industrial yeast strains of *S. cerevisiae*? These questions will be addressed in the following chapter.

Update on intergenic region between YBR238C and ERT1

Immediately prior to submission of this thesis, *S. cerevisiae* S288C transcriptome data from Yassour et al (2009) and Xu et al (2009) were incorporated into Gbrowse of SGD. This data identified additional and differing transcript regions in the intergenic region between *YBR238C* and *ERT1*, giving further insight into the ETCS locus. Data from Yassour et al 2009 identified the *ERT1* transcript beginning at II: 697,850 compared to Nagalakshmi et al (2008) and Xu et al (2009), which indicated that the transcript began at II: 698,877 (Appendix 6.2). Therefore, according to transcript data from Yassour et al (2009), the ETCS may reside in the 3'-UTR of *ERT1*. Thus, the ETCS may impact on the regulation of *ERT1* expression. Further analysis of the raw data from Yassour et al (2009), Nagalakshmi et al (2008) and Xu et al (2009) will be required to test this however, this data is not currently available.

Chapter 7

Identification of SNPs in ETCS that confer ethanol-tolerance in *Saccharomyces cerevisiae* and transformation of ETCS into wine yeast

7.1 Introduction

In the previous chapter four SNPs were identified in an intergenic region that confers ethanol-tolerance when introduced into *S. cerevisiae* WB. However it was not known which of the four SNPs are required for this phenotype, and it was of interest to determine whether or not the same SNPs are found in industrial strains of *S. cerevisiae* and play a part in conferring ethanol tolerance.

In the work described in this chapter;

I. The specific SNPs in ETCS involved in conferring the ethanol-tolerance phenotype were determined.
II. The nucleotide sequences of ETCS loci of various industrial *S. cerevisiae* strains were compared.
III. The effect on ethanol tolerance of introducing the ETCS of CM into wine strains of *S. cerveisiae* was determined.

7.2 Results

7.2.1 Determining which of the SNPs in the ETCS of CM confer ethanol tolerance

A COunterselectable REporter (CORE) approach, originally described by Storici and Resnick (2006), was used to systematically introduce each of the four SNPs, and all possible combinations thereof, into WB. The CORE approach has two transformation steps. The first step utilizes a cassette carrying a selectable marker and a lethal gene, the latter which is normally switched off or inactive. The cassette is flanked by sequences that target the insertion site to be modified. Transformants containing the CORE cassette will grow when plated onto appropriate selective

medium; non-transformants are non-viable. In the second transformation step a cassette carrying the desired modifications replaces the CORE cassette from the first step. The lethal marker is then activated to kill cells still containing the first CORE cassette, leaving only transformants containing the desired modification. In this case, the CORE cassette contained *KanMX4* as the selectable marker and *URA3*, which is lethal for strains grown on medium containing with 5'-Fluoroorotic Acid (5'-FOA) the parent strain is *ura3*$^{-}$ and therefore is not sensitive to 5'-FOA.

There are fifteen possible combinations of the four SNPs in ETCS (Appendix 7.1). Eleven of these were generated and tested for ethanol tolerance; the remaining four SNP combinations (SNP 2, SNP 1_2, SNP 2_3 and SNP 3_4) proved to be difficult to produce but were deemed redundant as sufficient information was gleaned from the eleven successful transformations to determine which of the SNPs are required for ethanol tolerance.

Transformations were validated by sequencing and transformants were tested for ethanol tolerance using RETA at 8% v/v ethanol. Figure 7.1 shows growth rates in 8% v/v ethanol of the eleven strains containing various SNP combinations. The parental strains and WB-ET SNPs (containing SNP1_2_3_4) were included as controls and indicators of ethanol tolerance levels. It is clear that SNPs 1 and 3 in combination always gave rise to ethanol tolerance, neither was sufficient on its own, and SNPs 2 and 4 do not contribute to the ethanol tolerance phenotype; any combination containing both SNP1 and SNP3 gave rise to ethanol tolerance levels equivalent to C3-7C (Figure 7.1).

7.2.2 Comparison of nucleotide sequences of ETCS alleles of wine, sake, bio-ethanol and laboratory S. cerevisiae strains

With advances in genome sequencing technology, many yeast genome sequences are available in The National Center for Biotechnology Information database (NCBI-http://www.ncbi.nlm.nih.gov/sites/gquery). This database enables the comparison of sequences across a wide range of yeast strains, from laboratory to wine to biofuel strains (Table 7.1). Industrial yeast strains have varied ethanol tolerance levels but are generally greater than for laboratory yeast strains. Thus, it was of interest to determine whether or not industrial strains have the same SNPs as the ETCS in CM.

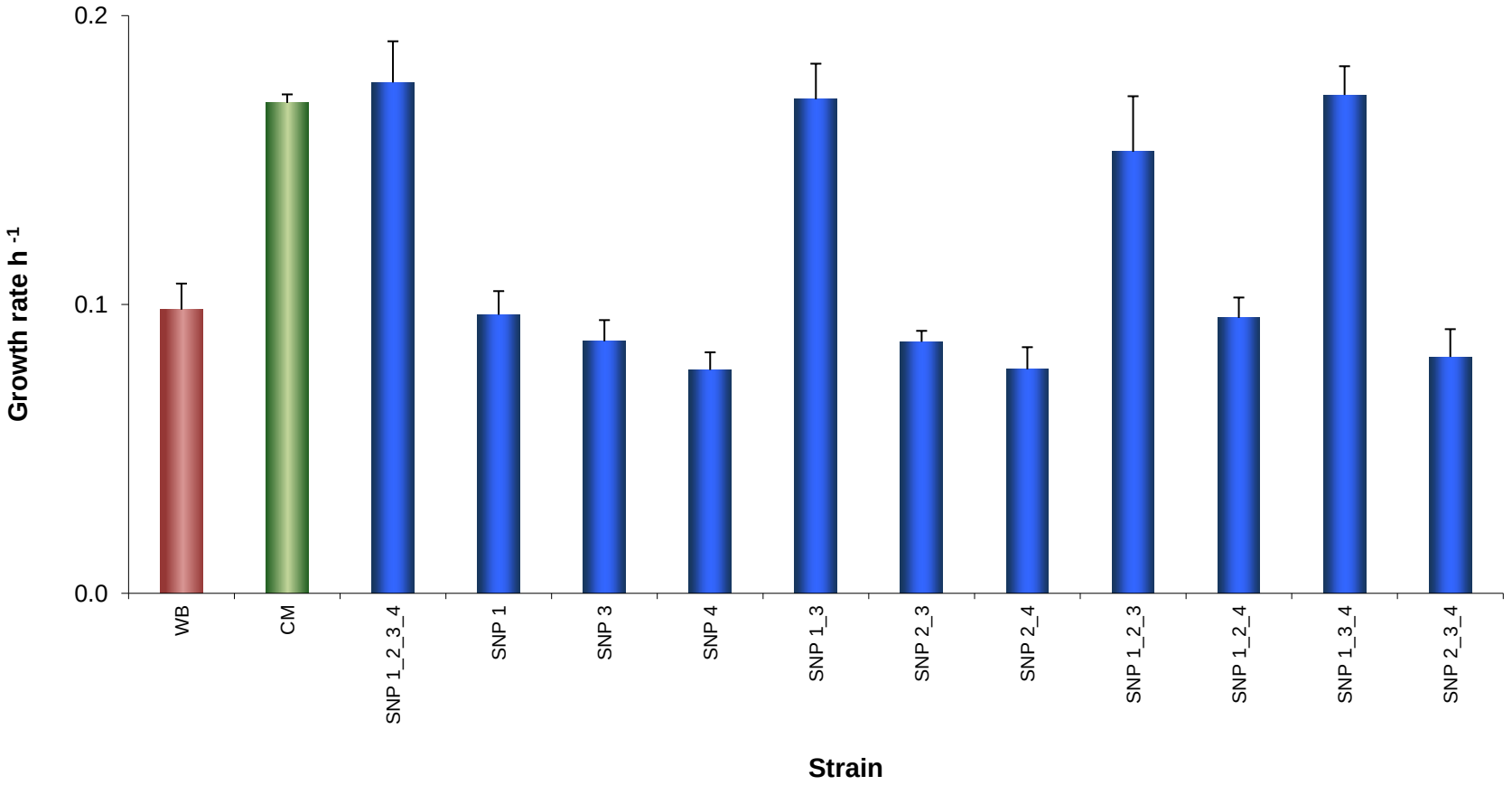


Figure 7.1: Determining which SNPs in ETCS are required for ethanol tolerance. Only SNP1 and SNP3 in combination gave rise to ET transformants. The non-ethanol tolerant parental strain WB is shown in red and ethanol-tolerant CM is shown in green. Transformants with various combinations of the ETCS SNPs are shown in blue. Strains were assayed in quadruplicate and error bars are based on $p<0.05$.

Chr II: 697,836 SNP 1 SNP 2 SNP 3 SNP 4 697,958

STRAIN	Application	TCCTCCTATTTTTTCCACTTACCTACCATCTCCCATCCTCTTTTTTCCCCACTTCCTTATCTAACCTCCAAGCCCCTCCTCTCTCACACATCCCCCTCTCACACATTCCAAACTCATCCCTACACA
CM	Lab	A G T A
WB	Lab	
S288c	Lab	
Y55	Lab	G A
SK1	Lab	
L-1528	Wine	G A
L-1374	Wine	G A
AWRI 796	Wine	G A
AWRI 1493	Wine	M K
RM11-1a	Wine	G Y A
M22	Wine	G A
BC187	Wine	G A
DBVPG1106	Wine	G A
DBVPG6044	Wine	
YIIc17_E5	Wine	
AWRI 1631	Wine	A G T A
AWRI 1537	Wine	M G A
AWRI 1688	Wine	M G T A
AWRI 1620	Wine	M K Y M
AWRI 1498	Wine	A
AWRI 1684	Ale	Y
YPS163	Ale	Y
NCYC110	Ale	
NCYC361	Ale	
K11	Sake	
Y9	Sake	
JAY291	Biofeul	G A
YS2	Baker	G A
YS4	Baker	G A
YS9	Baker	G A

Table 7.1: Table showing SNPs (■) present in alleles of ETCS of various laboratory and industrial *Saccharomyces cerevisiae* strains. Sequences were obtained from SGRP (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_cerevisiae_sgrp) and NCBI (http://www.ncbi.nlm.nih.gov/genome/). In diploid strains the ETCS regions may be heterozygous, that is, containing both the ET conferring and nET SNPs. These are indicated by IUPAC codes: M = C/A, K= T/G and Y= C/T (http://www.bioinformatics.org/sms/iupac.html). The reference sequence along the top of the table is for S288C obtained from (http://www.yeastgenome.org/) which is equivalent to the parental WB strain.

A clear and interesting observation is that the intergenic region is highly conserved across the various *S. cerevisiae* strains except at the CM ETCS SNP loci. However, very few industrial strains contain SNPs 1 and 3, whilst over half of those analysed have SNP 2 and SNP 4, which were found not to confer ethanol tolerance when transformed into WB. It is possible that these latter SNPs are functionally important but presumably do not impact ethanol tolerance as measured in RETA.

Some wine strains including AWRI 1537, AWRI 1620 and AWRI 1688, were found to be heterozygous for at least some of the SNPs in ETCS.

7.2.4 Introduction of ETCS into industrial wine strains AWRI 1620, AWRI 1493 and AWRI 1498

Given that the ETCS from CM was able to confer increased ethanol tolerance levels in the laboratory strain WB, it was reasoned that introducing this sequence into industrial wine strains might increase their ethanol tolerance. The ETCS was PCR amplified from C3-7C and transformed into industrial wine strains (as described in Methods 2.18). However, there was not a selectable marker for these transformations, thus, it was decided simply to attempt direct transformation (not using the CORE cassette method) and screen colonies by PCR amplification and sequencing for the ETCS locus. Fortunately, transformants that were homozygous for ETCS were obtained for each of the three industrial strains (for sequencing data refer to Appendix 7). Transformants were then plated onto YPD medium and random colonies were selected for verification of transformation using Sanger sequencing.

The three wine strains used for this study were AWRI 1493, AWRI 1498 and AWRI 1620. AWRI 1620 is heterozygous for all four SNPs (Table 7.1), and genome sequencing recently performed at AWRI on various industrial wine strains including AWRI 1620, allowed the determination of haplotypes at the ETCS allele (personal communication Dr. Anthony Borneman). As shown in Table 7.2, AWRI 1620 ETCS locus contains one allele with the recessive nET genotype and the other allele with the ET genotype. A transformant of AWRI 1620 was isolated and Sanger sequencing showed it to be homozygous for the ETCS allele.

	SNP Location on Chromosome II of AWRI 1620			
Allele	**II: 697850**	**II: 697895**	**II: 697907**	**II: 697928**
1	A	G	T	A
1’	C	T	C	C

Table 7.2: Table of haplotypes of ETCS locus (personal communication Dr. Anthony Borneman). Allele 1 contains nucleotide sequences equivalent to the ETCS SNPs as seen in CM. Allele 1’ contains nucleotide sequences equivalent to the parental WB strain.

RETA at 14% v/v ethanol stress (Figure 7.2) indicated that the transformed AWRI 1620, homozygous for ETCS, had a small but statistically significant increase in growth compared to the heterozygous AWRI 1620.

AWRI 1493 is heterozygous at SNP1 and SNP2 loci of the ETCS allele (haplotypes unknown), and homozygous wild-type (i.e. nET) for SNP3 and SNP4. An AWRI 1493 transformant was generated which was homozygous for the four ETCS SNPs. RETA at 14% v/v indicated that there were no significant differences between AWRI 1493 and its ETCS transformant in ethanol tolerance (Figure 7.3).

AWRI 1498 is homozygous for ETCS SNP 1 but at the loci of SNP2, SNP3 and SNP4, it had the nET genotype. A transformant of AWRI 1498 was made homozygous for the four ETCS SNPs. RETA showed a decrease in ethanol tolerance levels of the transformant compared to the parent AWRI 1498 strain (Figure 7.4).

7.2.5 Conservation of the intergenic region between YBR238C *and* ERT1 *across* Saccharomyces *sensu stricto species*

With a high level of conservation in the *YBR238C-ERT1* intergenic regions across strains of *S. cerevisiae*, it was of interest to compare the same region across different, but closely related, species of the *Saccharomyces* sensu stricto group (http://genome.ucsc.edu/). It is evident, at least for *S. kudriavzevii*, *S. paradoxus*, *S. mikatae* and *S. bayanus*, that there are blocks of conserved sequence interspersed by highly divergent sequences (Figure 7.5). For example, between II: 697,400 and 697,600, there is high level of conservation compared to II: 698,100-698,200 where there is almost none.

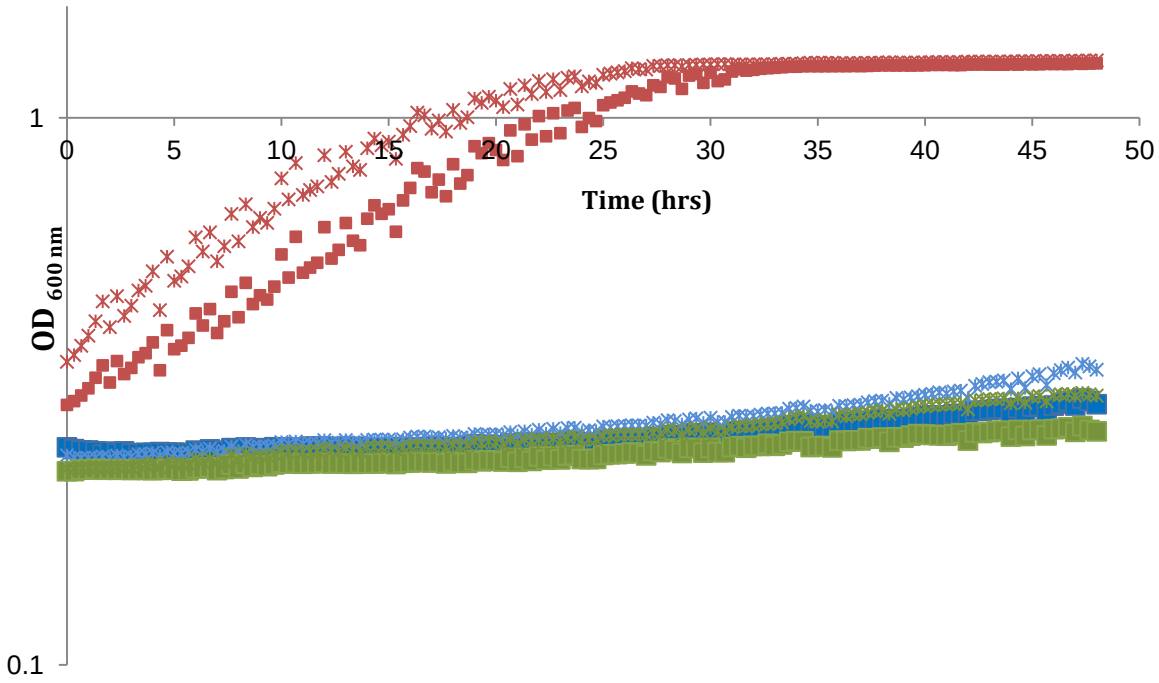


Figure 7.2: RETA growth curves of AWRI1620 and AWRI 1620 transformed with ETCS, making the locus homozygous for this allele. Growth of transformed AWRI 1620 is shown as stars and the original heterozygous strains are shown in squares. The colour of the symbols correspond to the ethanol levels in RETA: 14% v/v ethanol in red, 16% v/v ethanol in blue and 18% v/v ethanol in green. Strains were assayed in quadruplicate, and differences seen between the transformant and original AWRI 1620 strain at 14% v/v ethanol are statistically significant (α=0.05).

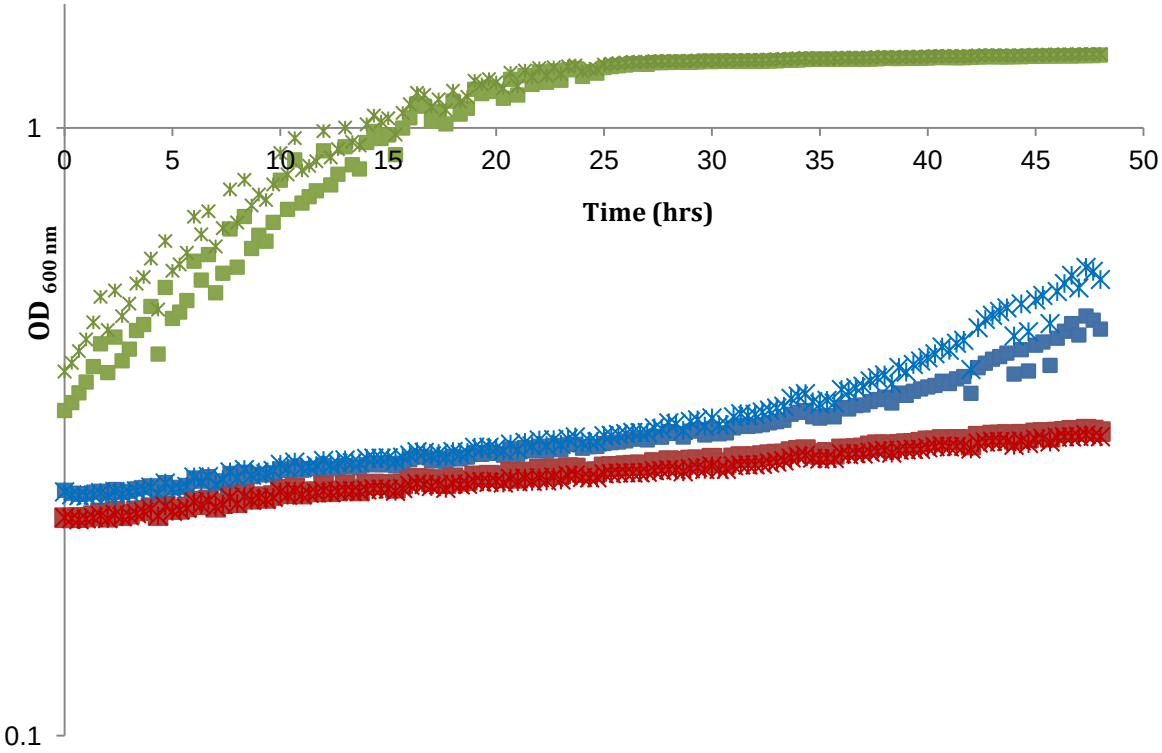


Figure 7.3: RETA growth curves of AWRI 1493 and AWRI 1493 transformed with ETCS making the locus homozygous for this allele. Transformed AWRI 1493 is shown as stars and the original heterozygous strains are shown in squares. The colour of the symbols correspond to the ethanol levels in RETA: 14% v/v ethanol in green, 16% v/v ethanol in blue and 18% v/v ethanol in red. Strains were assayed in quadruplicate (α=0.05). No differences can be seen between the transformed and original AWRI 1493.

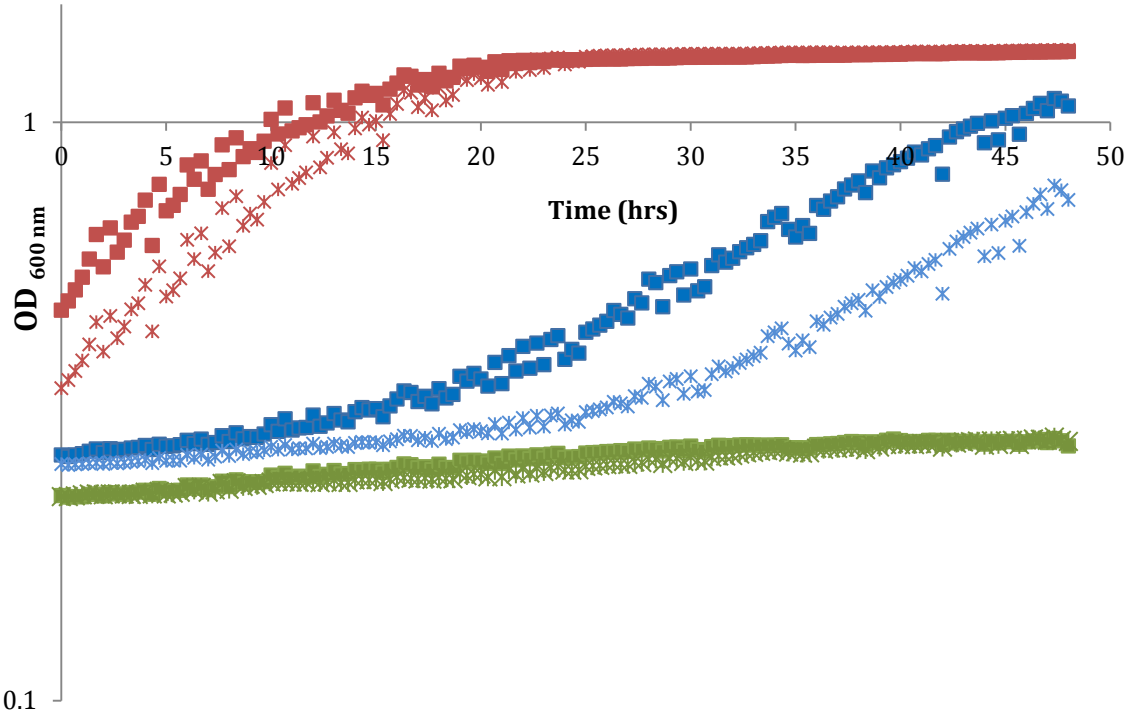


Figure 7.4: RETA growth curves of AWRI 1498 and AWRI 1498 transformed with ETCS making the locus homozygous for this allele. Transformed AWRI 1498 is shown as stars and the original heterozygous strains are shown in squares. The colour of the symbols correspond to the ethanol levels in RETA: 14% v/v ethanol in red, 16% v/v ethanol in blue and 18% v/v ethanol in green. Strains were assayed in quadruplicate; the slight decrease in growth at 16% v/v ethanol was statistically significant (α=0.05).

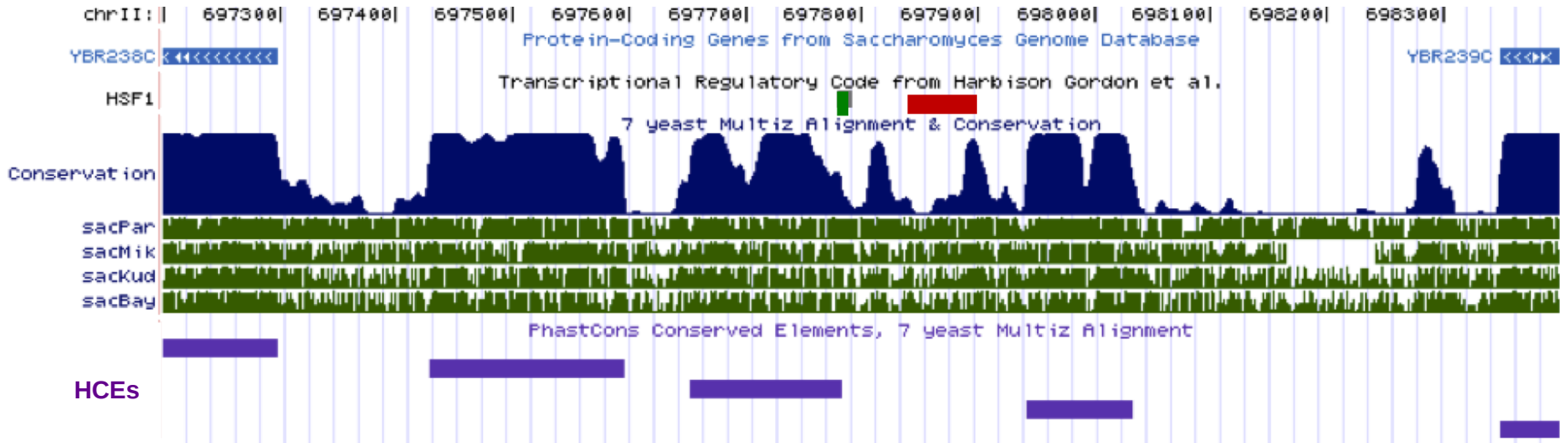


Figure 7.5: Sequence alignment of Chromosome II in intergenic region between *YBR238C* and *ERT1* for various *Saccharomyces* species. The peaks shown in navy blue (■) indicate regions of sequence conservation across species and areas of evolutionary significant Highly Conserved Elements (HCEs) are shown in purple (■). The transcriptional motif for *HSF1* site is shown in green (■). ETCS is shown in red (■). Data obtained from http://genome.ucsc.edu/.

7.3 Discussion

Chapter 6 described the application of genomic sequencing to identify the ethanol-tolerance conferring locus in C3-7C. The locus was found to carry four SNPs (relative to the wild-type allele in the nET parent, WB) in an intergenic region on chromosome II, and was subsequently named the Ethanol Tolerance Conferring Sequence (ETCS). This chapter determined which of the four SNPs were required for ethanol tolerance. This was achieved by introducing different combinations of the ETCS SNPs into WB. Of the fifteen possible combinations, eleven were generated (Figure 7.1) which were enough to conclude that SNPs 1 and 3 together are required for ethanol tolerance; neither SNPs 1 or 3 alone confer ethanol tolerance and SNPs 2 and 4 have no impact on the phenotype.

Given that the ETCS was able to confer increased ethanol tolerance in a laboratory strain, it was of interest to determine if the ETCS SNPs were present in industrial strains such as those used in biofuel, wine and sake, which are typically highly ethanol tolerant (Pretorius et al 2000). It was hypothesized that these strains would contain the ET SNPs (SNPs1 and 3), whereas less ethanol tolerant industrial strains such as baking yeasts might contain the nET alleles. Nucleotide sequences of the ETCS locus for twenty-five *S. cerevisiae* industrial strains were compared and most were found not to carry the ET SNPs. The most likely explanation for this is that SNPs 1 and 3 confer a selective disadvantage or are selectively neutral in an industrial yeast genetic background. Therefore, the mechanisms conferring ethanol tolerance in industrial strains might differ to those in CM.

However, sequence comparison of the entire intergenic region, revealed a high le vel of conservation; interestingly, only the four ETCS SNP loci were heterozygous and the remainder of the region was identical in all industrial *S. cerevisiae* strains analyzed. This is consistent with intergenic region being functionally important, since conservation across strains is usually an indication of adaptive significance.

A comparison of nucleotide sequences of the intergenic region between *YBR238C* and *ERT1* orthologs, across *Saccharomyces* sensu stricto species was performed to determine if the conservation seen across *S. cerevisiae* industrial strains is also conserved across species. In general, the high level of conservation was not observed and there was no conservation observed at or near the ETCS locus.

However, some areas throughout the intergenic region evolutionarily conserved; for example between II: 697450-697600 (Figure 7.5). These highly conserved regions were identified by Siepel et al (2005), who referred to them as Highly Conserved Elements (HCEs). The authors performed genome sequence comparison between representative strains of the sensu stricto species and developed a program called PhastCons to identify regions of evolutionary conservation. The authors found only ~13.7% of intergenic regions show conservation between sensu stricto species. Thus, the HCEs found in the intergenic region that ETCS resides in, suggests that the intergenic region may be functionally important.

Since, most industrial strains did not contain the ET conferring ETCS, it was of interest to introduce it into wine yeast genetic backgrounds to determine its effect on ethanol tolerance. Three wine yeast strains: AWRI 1620, AWRI 1493 and AWRI 1498, were made homozygous for the ETCS from CM (Figure 7.2-7.4).

AWRI 1620 is heterozygous for all four SNPs of ETCS (Table 7.2) and recent genome sequencing work by Dr. Anthony Borneman (AWRI) found that these SNPs are on the same chromosome. Thus, AWRI 1620 is heterozygous for ETCS (Table 7.1). Given that the ethanol tolerance phenotype conferred by ETCS was found to be dominant in CM (see Chapter 4), replacing the recessive allele, making AWRI 1620 homozygous for ETCS, was not expected to have an effect on ethanol tolerance phenotype. However, as seen in Figure 7.2, the AWRI 1620 transformant had slightly increased ethanol tolerance.

AWRI 1493 is an industrial wine strain prone to stuck fermentations and is less ethanol tolerant then AWRI 1620 and AWRI 1498 (Schmidt et al 2005). AWRI1493 is heterozygous for SNPs 1 and 2, whereas SNPs 3 and 4 loci are identical to the nET WB strain; thus it does not contain the ETCS. A comparison of AWRI 1493 and its homozygous ETCS transformant showed that the allele does not impact measurably on ethanol tolerance (Figure 7.3).

In contrast, when the ETCS locus was introduced into AWRI 1498 (an industrial wine strain known for its robustness) to produce a homozygous diploid, a slight decrease in ethanol tolerance was observed (Figure 7.4). Thus, the presence of ETCS appears to interfere with the ethanol tolerance mechanisms in AWRI 1498. AWRI 1498 has one copy of SNP1 and the remainder ETCS SNP loci are identical to the nET parent.

One possible explanation for the three different results described above is that there is an interaction between Ybr238 and ETCS (as discussed in Section 6.3). Previous studies have found that the Ybr238 protein putatively binds to the entire intergenic region that ETCS resides in. Genome sequencing data (Borneman et al 2011) indicates that there are 9 and 10 SNPs respectively, in the *YBR238C* nucleotide sequence of AWRI 1620 and AWRI 1498. It is possible that the variation in Ybr238 nucleotide sequence impacts on the binding affinity of the putative transcription factor to the ETCS locus. Whether the binding of Ybr238 influences ethanol tolerance in a positive, negative or neutral effect, ETCS in ethanol tolerance is unknown, and will be the subject of future work.

Clearly the impact of ETCS on ethanol tolerance is dependent on the genetic background of the strain it resides in: the laboratory strain W303, the ETCS allele from CM confers a substantial increase in ethanol tolerance, in wine yeasts the effects are more variable. As discussed above, the fact that ethanol-tolerance conferring SNPs found in CM is absent from most industrial strains suggests they may confer a selective disadvantage (or are at least selectively neutral). The result obtained for transformed AWRI 1498 is consistent with this; its ethanol-tolerance is decreased when ETCS SNPs were introduced. AWRI 1620 already contains an allele with the ETCS SNPs, suggesting that this genetic background is at least not detrimentally affected by the ETCS allele. The additional copy of ETCS in the homozygous transformant strain gave slightly increased ethanol tolerance suggesting a possible gene dosage effect.

Thus, in the case of ETCS, genetic background impacts substantially on ethanol tolerance phenotype associated with this allele. This is an interesting observation and will be a good model to study how intergenic regions interact with their genetic background. It is well known that genetic background influences phenotypic effects of mutations, due to networks of gene interactions (Wagner et al 2011, Dworkin et al 2009, Glazier et al 2002).

Chapter 8

Outputs, Conclusions and Future Directions

8.1 Outputs and Conclusions

The major objective of this project was to identify genes that confer ethanol tolerance in ET mutants SM and CM; Dr. Dragana Stanley generated these strains by adaptive evolution using ethanol as a selective pressure (Stanley et al 2010). For one of the mutants, CM, the aim was substantially realised; an Ethanol Tolerance Conferring Sequence (ETCS) was identified. Specifically two SNPs in ETCS were shown to generate the ethanol tolerance phenotype when transformed into the parent WB. Importantly ETCS also conferred increased ethanol tolerance in an industrial wine yeast: this is the first report of an intergenic transcribed region having a role in conferring ethanol tolerance in *S. cerevisiae*. However, two other wine strains showed no significant increase in ethanol tolerance when transformed with ETCS; in fact, for one of these strains, there was a minor decrease. Thus the impact and role of ETCS in ethanol tolerance is not simple, being dependent on the genetic background of the host strain.

The mutations that confer increased ethanol tolerance in SM remain to be determined. Nonetheless, work described in this thesis demonstrated that the phenotype was dominant and mostly segregated in a 2:2 (nET:ET) ratio with an occasional deviation, producing progeny with intermediate ethanol tolerance, suggesting two or more genes are involved in this phenotype.

Other major outputs of the work described in this thesis include:

- A Rapid Ethanol Tolerance Assay (RETA) was developed and successfully applied to quantify ethanol tolerance levels for numerous strains of *S. cerevisiae*. This assay, and variants thereof, should find broad application in yeast research on stress tolerance, toxicity, and nutrient requirements. A manuscript describing this assay is being prepared for publication.

- Tiling Arrays proved inadequate for the identification of ethanol tolerance conferring mutations in CM and SM. Whilst a large *HXT3-7* deletion was identified on chromosome IV of CM, it did not segregate with the ethanol tolerance phenotype and therefore was not further investigated.

- Genome sequencing of the backcrossed CM (C3-7C) revealed a region carrying four SNPs, subsequently named ETCS, as the single locus conferring the ethanol tolerance phenotype. Equivalent levels of ethanol tolerance were conferred in WB when the strain was transformed with ETCS from CM. Further analysis found that two SNPs (1 and 3) were responsible for conferring the ethanol tolerance phenotype.

- The intergenic region carrying ETCS in CM was found to be highly conserved across various *S. cerevisiae* industrial strains suggesting functional importance. However, the ethanol-tolerance conferring SNPs identified in CM were not common to this region. The same region is much less highly conserved across *Saccharomyces* spp. than it is across species of *S. cerevisiae*.

8.2 Future Directions

8.2.1 Identification of genes that confer ethanol tolerance in SM

- To identify the ethanol tolerance conferring loci in SM, further rounds of backcrossing SM to dilute our incidental mutations not associated with ethanol tolerance would be performed, followed by application of a sequencing approach the same as that used for work on CM.

- An interesting observation from work in this thesis was that when SM was returned to histidine prototrophy there was a consistent decrease of ethanol tolerance. This should be further explored to determine its molecular basis.

8.2.2 Determining how the ETCS allele in CM contributes to ethanol tolerance

- It is not at all clear what mechanisms lead to increased ethanol tolerance in CM; we do not know how ETCS confers ethanol tolerance in this genetic background. There is however good reason to hypothesize that one or more of the neighboring genes (*YBR238C*, *ERT1* and *THI2*) may have a role in conferring ethanol tolerance. By inserting each of these three genes, individually, into expression plasmids and transforming them into a *S. cerevisiae* W303 strain, it should be possible to determine whether or not they play a role in ethanol tolerance. It is important to note that none of these genes were identified in functional genomic screens, to play a role in ethanol tolerance (van Voorst et al 2006, Kubota et al 2004, et al Kumar 2008). Nonetheless, it would be prudent to test each of the knockouts for *YBR238C*, *ERT1* and *THI2* using RETA.

- Experiments should be performed to verify that the region ETCS reside in is transcribed; this could be achieved by RNA-seq.

- As described in Chapter 6, there was some discrepancy between the transcription regions for *ERT1* identified by Nagalakshmi et al (2008) and Yassour et al (2009). Verification of the transcirption region for *ERT1* should be verified to determine if ETCS resides in the 3'-UTR of *ERT1* as suggested by Yassour et al (2009).

- Stanley et al (2010) performed transcriptome microarrays on CM and identified hundreds of genes associated with ethanol tolerance with altered expression, relative to its parent W303, when exposed to ethanol stress. The data from Stanley et al (2010) however was probably affected by the large number of incidental (non-ethanol tolerance conferring) mutations in CM, leading to many false positives and negatives. Transcriptome analysis should be repeated using W303+ETCS instead of CM, and comparing this to W303 when exposed to ethanol stress. This would give insight to the mechanisms by which ETCS confers ethanol tolerance.

8.2.3 ETCS and ethanol tolerance of industrial wine strains of S. cerevisiae

- The introduction of ETCS into industrial yeast strains gave differing results ranging from decreased to increased ethanol tolerance. However, these findings are based on the use of a simple assay, RETA, which is very limited in what it assesses of the phenotype. The ETCS may impact on fitness in ways other than ethanol tolerance. To determine if this is the case, competition growth curves, comparing each of AWRI 1620, AWRI 1493 and AWRI 1498 with respective homozygous ETCS transformants would be performed in a range of different media, including grape juice to compare fitness levels.

- Building on the experiment described in the above dot point, given that AWRI 1620 is a commonly used wine strain and it had an improved ethanol-tolerance phenotype when transformed to homozygosity for ETCS, it would be of interest to determine whether the improved ethanol tolerance levels, translates to reduced risk of stuck ferments in red wine ferments.

- As mentioned in Section 7.2.4, increased ethanol tolerance of AWRI 1620 homozygous for ETCS (relative to the heterozygous AWRI 1620 parent) may be due to gene dosage. This could be achieved by introducing an increasing series of ETCS copy numbers and observing the effects on ethanol tolerance levels of WB using RETA.

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GUIDE FOR APPENDIX DVD

The appendix data referred to throughout this thesis has been copied onto a DVD (attached to back cover of thesis). The following described appendices are in Word, Excel, .FASTA, .gbk and PDF file formats. The files have been labeled with the following listed titles (shown in bold) and descriptions, corresponding Appendix Chapter or Contents.

THESIS PDF: Contains an electronic copy of the thesis word document

Appendix 1:

Appendix 1.1 – Complete Table of genes identified in functional genomics studies as being required for survival under ethanol stress. The table shows which and how many publications identify genes associated with ethanol-stress tolerance.

Appendix 1.2 – Complete table of genes identified in microarray studies as having up-regulated expression under ethanol stress. The table shows which and how many publications identify genes as ethanol-stress responsive.

Appendix 3:

Appendix 3 – Is an Excel spreadsheet used as a template to process the vast amount of OD data from RETA. The folder also includes the DMfit Macro required to process the data.

Appendix 4:

Appendix 4 – Partial Sanger sequence chromatograms to verify return to protogrphy of nutrient trasnformants

Appendix 5:

Appendix 5.1 – Original .cel file outputs from Affymetrix Tiling Microarrays and associated library files corresponding to signal co-ordinates

Appendix 5.2 – Comparison of Signals via TAS program (Affymetrix) used for viewing in IGB.

Appendix 5.3 – Tables of signals from Tilling Array Analysis which appeared to be significantly different in mutants compared to the parental strain.

Appendix 5.4 – Listed of mutations identified by Tiling Microarrays chosen for further analysis due to their link with ethanol tolerance.

Appendix 5.5 – A list of genes verified to be false positives from Tiling Microarrays. Also contains an example of false positive verified by Sanger sequencing.

Appendix 5.6 – Simplified diagram in addition to Figure 5.5 to demonstrate portion of genome deleted on chromosome IV.

Appendix 6:

Appendix 6.1 – The genome sequence of CM is in fastA and .gbk format and arranged according to the relevant chromosome.

Appendix 6.2 – CLC Genome Browser (CLC Bio) was used to make preliminary identify mutations in C3-7C compared to an W303-1A strain. The locations of the mutations on the chromosome are shown.

Appendix 6.3 – SGD Gbrowse view of ETCS site showing transcripts, loci identified by various authors.

Appendix 7:

Appendix 7.1 – List of all possible combinations of ETCS SNPs

Appendix 7.2 – Sanger sequencing chromatograms to verify various combinations of SNPs introduced into WA the parental strain. RETA was used to determine the effect of SNPs on the ethanol tolerance phenotype.

Appendix 7.3 – Sanger sequencing chromatograms to verify homozygous ETCS industrial wine strains.

The following Figure legends appear on the reverse side of corresponding figures

Figure 4.9: Southern blot analyses of various SM *HIS3* transformants using restriction enzymes *Bst*EII, *Eco*RV, *Mfe*I and *Ssp*I. DIG-labelled marker was used to determine molecular weights of bands. Arrows indicate expected band sizes.

A		***B***		***C***		***D***	
*Bst*EII		*Eco*RV		*Mfe*I		*Ssp*I	
Lane	**Contents**	**Lane**	**Contents**	**Lane**	**Contents**	**Lane**	**Contents**
1	WA	1	WA	1	WA	1	WA
2	WA-H13	2	WA-H13	2	WA-H13	2	WA-H13
3	WB	3	Marker	3	WB	3	WB
4	WB-H11	4	WB	4	WB-H11	4	WB-H11
5	Marker	5	WB-H11	5	Marker	5	SM
6	SM	6	SM	6	SM	6	SM-H06
7	SM-H06	7	SM-H06	7	SM-H06	7	Marker
8	CM	8	CM	8	CM	8	CM
9	CM-H02	9	CM-H02	9	CM-H02	9	CM-H02
		10	FY				

Figure 4.10

a) RETA data showing growth rates of the chemical mutant CMH (red), the parental strain WBU (yellow) diploid progeny (green) in the presence of 0 % v/v ethanol. Tetrad set T7 A-D (blue).

b) Analysis of Variance (ANOVA) of triplicate growth rate values at 0 % v/v ethanol. Groups not connected by the same letter are significantly different.

Figure 4.11

a) RETA data showing growth rates of the chemical mutant CMH (red), the parental strain WBU (yellow) diploid progeny (green)in the presence of 8 % v/v ethanol. Tetrad set T7 A-D (blue).

b) Analysis of Variance (ANOVA) of triplicate growth rate values at 8 % v/v ethanol. Groups not connected by the same letter are significantly different.

Figure 4.12

a) RETA data showing growth rates for the chemical mutant SML (red), the parental strain WBU (yellow) diploid progeny (green) in the presence of 0 % v/v ethanol. Tetrad set T8 A-D (blue).

b) Analysis of Variance (ANOVA) of triplicate growth rate values at 0 % v/v ethanol. Groups not connected by the same letter are significantly different.

Figure 4.13

a) RETA data showing growth rates for the chemical mutant SML (red), the parental strain WBU (yellow) diploid progeny (green) in the presence of 8 % v/v ethanol. Tetrad set T8 A-D (blue).

b) Analysis of Variance (ANOVA) of triplicate growth rate values at 8 % v/v ethanol. Groups not connected by the same letter are significantly different.

Figure 5.1: a) Schematic of iterative backcrossing strategy for the Chemical Mutant. Three successive backcrosses of the chemical mutant (CMH) to its isogenic parent (WBU) were performed. Diploids were sporulated and ethanol-tolerant progeny from tetrads were chosen to be backcrossed to the parental strain. Tetrad progeny are named according to the backcross number e.g.C1 followed by '–' and then the specific progeny identification number e.g. 1A. **b**) RETA of progeny to determine the growth rates of the strains in 8% (v/v) ethanol.

Figure 5.2: a) Schematic of iterative backcrossing strategy for the Spontaneous Mutant. Three successive backcrosses of the spontaneous mutant (SML) to its isogenic parent (WBU) were performed. Diploids were sporulated and ethanol-tolerant progeny from tetrads were chosen to be backcrossed to the parental strain. Tetrad progeny are named according to the backcross number e.g.S1 followed by '–' and then the specific progeny identification number e.g. 16A. **b**) RETA of progeny to determine the growth rates of the strains in 8% (v/v) ethanol.

Figure 5.8 Determination if *HXT3-7* mutation is associated with the segregation of ethanol-tolerance phenotype in CM

Lane	Contents	HXT Mutation	RETA	Lane	Contents	HXT Mutation	RETA
1	WBU	-	nET	21	1A	+	nET
2	FY4	-	nET	22	1B	+	ET
3	CH2	+	ET	23	1C	-	nET
4	2A	+	ET	24	1D	-	ET
5	2B	-	ET	25	4A	-	ET
6	2C	+	nET	26	4B	+	ET
7	2D	-	nET	27	4C	-	nET
8	10A	-	ET	28	4D	+	nET
9	10B	-	nET	29	3A	+	ET
10	10C	+	ET	30	3B	-	nET
11	10D	+	nET	31	3C	+	ET
13	11A	-	ET	32	3D	-	nET
14	11B	+	nET	33	5A	-	nET
15	11C	-	ET	34	5B	+	ET
16	11D	+	nET	35	5C	+	nET
17	17A	-	nET	36	5D	-	ET
18	17B	+	ET	37	WBU	-	nET
19	17C	-	nET	38	FY4	-	nET
20	17D	+	ET	39	1 Kb Marker		

***Figure 5.8a*:** The above table lists the progeny used to PCR amplify *HXT* mutation regions shown in Figure 5.8b. Thee progeny tetrad set and associated ethanol tolerance levels are indicated.