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Analysis of the Genomic Organisation and gene expression of brewery strains of yeast.

A thesis submitted to the University of Dublin in fulfilment of the requirements for the degree Doctor of Philosophy by

Jane Usher

2007

The Moyne Institute of Preventive Medicine,
Department of Microbiology
Trinity College
University of Dublin

Dedicated in loving Memory of
Shelia Malone
1951 – 2007.

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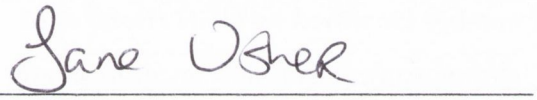

Jane Usher

Summary

The genomes of lager yeast arose from the fusion of two yeast strains most closely resembling *Saccharomyces cerevisiae* and *Saccharomyces bayanus*. The resultant strains have subsequently undergone genome duplications and rearrangements leading to the generation of a complex aneuploid genome. Genetic analysis has revealed that recombination between the two parent genomes has led to the presence of mosaic chromosomes.

The goal of this work is to contribute to the current knowledge of the genomic organisation of lager brewery strains of yeast. This work sets out to examine the nature of the mosaic chromosomes. To study these regions of interest, pulse field gel electrophoresis followed by Southern Blot analysis has been carried out, to identify any changes in chromosome structure in the lager strains. The analysis confirms the changes in gene copy number identified by CGH (Bond *et al.*, 2004) and reveals that the lager yeast chromosomes had not undergone any gross chromosomal rearrangements. To examine the chromosome organisation of chromosome XVI, at a nucleotide level, a genomic library was constructed. DNA sequencing of a large section of chromosome XVI revealed that the ORF YPR159W in the lager yeast is *S. cerevisiae* like. The intergenic region between YPR159W and YPR160W is highly conserved and shows 98% homology to the *S. cerevisiae* DNA sequence. The recombination between the parental strains occurs within the ORF YPR160W resulting in the generation of a unique hybrid gene that appears to be non-functional in the lager yeast.

The transcription profile of the lager yeast is important in understanding the performance of the lager yeast strains in the environment of an industrial fermentation. The polyploid lager yeast genomes, contain multiple copies of homeologous genes, present a complex problem for gene expression analysis and raise the question 'Are all alleles expressed in the lager yeasts?' To address this question, the expression of a number of *S. cerevisiae* genes having different copy number of alleles were examined during a standard fermentation. The expression profile and relative level of expression was compared to the expression in a haploid *S. cerevisiae* strain. The level of hybridisation for the genes examined on

chromosome III were significantly higher in CMBS-33 suggesting that the two *S. cerevisiae* alleles are being expressed, indicating that the expression pattern of the four genes is differential in the *S. cerevisiae* S150 strain and the lager yeast strain CMBS-33

To examine the effects of gene dosage on the expression patterns of lager yeasts, a yeast artificial chromosome containing over 100 Mb of *S. bayanus* chromosome XVI from a lager yeast was introduced into isogenic polyploid strains of *S. cerevisiae*. Analysis of the expression of the *S. bayanus* genes revealed that increasing the copy number of *S. cerevisiae* genes, caused a decrease in the expression of the *S. bayanus* homeologus genes, indicating the gene dosage effects may prevent overexpression of genes in polyploid strains of yeasts or that specific transcription factors contributed to the genome from *S. bayanus* genome are required for gene expression in the lager yeasts.

The hybrid genomes have undergone rearrangements and amplifications generating a series of mosaic chromosomes. Genome analysis of lager yeast strains, selected for their increased tolerance to stresses experienced during brewing, revealed that they have undergone additional chromosomal rearrangements. Analysis of lager yeasts exposed to stresses such as high gravity and increased oxygen concentrations, during a single round of fermentation, indicate that the genomes are highly dynamic and undergo rearrangements and gene amplification in response to stress.

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Abbreviations

A260	absorption at 260 nm
A280	absorption at 280 nm
Bp	base pair
cDNA	Complimentary DNA
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
dATP	2' – deoxyadenosine 5' - triphosphate
dCTP	2' – deoxycytodine 5' - triphosphate
dGTP	2' – deoxyguanosine 5' - triphosphate
dNTP	dideoxynucleoside
DTT	dithiothreitol
dUTP	2' – deoxyuracil 5' - triphosphate
EDTA	
e.g.	for example
et al.	*and others*
g	gram
HSP	Heat Shock Protein
IPTG	Isopropyl-ß-thiogalactoside
i.e.	that is
Kb	Kilobase pair
KV	kilovolt
l	Litre(s)
L agar	Luria-Bertani agar
L broth	Luria-Bertani Broth
M	Molar
Mg	milligram
µg	microgram
mM	millimolar
min	minute(s)
ml	millilitre
µl	microlitre
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction

PFGE	Pulsed-field gel electrophoresis
RNA	Ribonucleic acid
Rnase	Ribonuclease
r.p.m.	revolutions per minute
RT	Reverse Transcription
s	second(s)
SDS	Sodium dodecyl sulphate
SGD	*Saccharomyces* Genome Database
SSC	Saline sodium citrate
STRE	Stress response element
TAE	Tris-acetate/EDTA
TBE	Tris-borate/EDTA
Tris	tris(hydroxymethyl)aminomethane
U	Unit
UV	Ultraviolet
v/v	% volume in volume: expresses the number of millilitres of an active constituent in 100 millilitres of solution
w/v	% weight in volume: expresses the number of grams of an active constituent in 100 millilitres of solution
X-gal	5-bromo-4-chloro-indoyl-ß-D-galactoside
YEPD agar	Yeast extract Peptone agar
YEPD broth	Yeast extract peptone broth
YEPM agar	Yeast extract peptone agar with maltose agar
YEPM broth	Yeast extract peptone agar with maltose broth

Chapter 1
Introduction.

1.1 Saccharomyces sensu stricto.

The thesis presented here sets out to characterise the genomes of lager yeasts at a molecular level. The lager yeast belong to the kingdom Fungi belonging to the *Saccharomyces sensu stricto* group. The first lager brewing yeast that was pure-cultured was originally called 'Bottom Fermenting Strain #I', but was later named *Saccharomyces carlsbergenesis*. It is kept as the *S. carlsbergenesis* type strain CBS1513 and most lager brewing yeasts used today are closely related to this strain. Modern lager brewing yeast strains and the *S. carlsbergenesis* type strain are recognised as part of the *S. pastorianus* group (Yoshimoto *et al.,* 1999, Tamai *et al.,* 1998). The *Saccharomyces* genus includes two groups of species; *Saccharomyces sensu stricto* originally designated as the *Saccharomyces* species and were associated with the fermentation industry and *Saccharomyces sensu lato* comprising of species that are more distantly related to *S. cerevisiae*.

There are at least 1000 known strains of *Saccharomyces cerevisiae* currently used in the baking, brewing, winemaking and distilling industries. There are a number of industrial archives, from which yeast strains can be obtained such as The Euroscarf Collection, Collection deLevures d'Interet Biotechnology and the Culture Collection of Yeasts to name a few. Due to the high fermentative capacity of yeast, along with the ability of these organisms to survive extreme environmental conditions such as those experienced during an industrial fermentation, has lead to their exploitation in the brewing and baking industries and to the selection of a number of strains with unique characteristics.

Species differentiation in *Saccharomyces sensu stricto* has been a difficult task. This group of yeast consists of very closely related species, thus creating difficulties in applying the strict biological concept of species as a means of distinction. *S. cerevisiae* and *S. bayanus* have been considered the most distantly related species within *Saccharomyces sensu stricto,* (Vaughan-Martini & Kurtzman, 1985). Limitations in using conventional taxonomy have lead to the increasing use of molecular methods. This approach has been referred to as 'molecular taxonomy'.

The *Saccharomyces sensu stricto* yeast group contains the closely related species *S. bayanus, S. cariocanus, S. cerevisiae, S. kudruadzerii, S. mitakae, S. pastorianus* and *S. paradoxus* (Kellis *et al.,* 2003, Lopes *et al.,* 2002, Vaughan-Martini & Martini, 1987). The yeast in this group is considered to be degenerate tetraploids that arose from a whole genome duplication approximately 100 million years ago (Wolfe & Shields, 1997), (Fig. 1.1). Genomic duplication has been proposed to be an advantageous path to evolution as the duplicated genes supply raw genetic material for the emergence of new functions through mutation and natural selection. Polyploidy comes at the cost of genomic instability which will persist until the genome returns to a functionally normal ploidy through mutation, gene loss and genomic rearrangement.

Kellis *et al.,* (2004), have provided direct evidence of a whole genome duplication (WGD) in yeast through the sequencing and analysis of the related species, *Kluyveromyces walti,* whose divergence preceded the duplication event. It was shown that *S. cerevisiae* arose from the complete duplication of eight ancestral chromosomes and returned to functionally normal ploidy through massive loss of nearly 90% of the duplicated genes in small deletions. The WGD event occurred in the *Saccharomyces* lineage after the divergence from *K. waltii.* It has also been proposed (Kellis *et al.,* 2004), that the WGD may have occurred at the haploid or diploid stage either by endo-duplication (auto-polyploidy) or the fusion of two close relatives (allo-polyploidy). To understand the molecular composition of the organism, it is essential to have the complete DNA sequence of its genome.

The DNA sequence of *S. cerevisiae* genome was completed and made publicly available in 1996. (Goffeau *et al.,* 1996). It was achieved through the sequencing of DNA from random YAC or cosmid clones. From this information the organisation of the *Saccharomyces* genome was deduced, it was shown to be composed of approximately 12 million basepairs arranged over sixteen chromosomes, comprising of 6275 open reading frames (Mewes *et al,* 1997). Following further analysis of the initial data the number of ORFs was revised to 5500 of which 3,965 were ORFs that had been already identified by genetic analysis. Along with the identification of ORFs, the sequence for non-protein

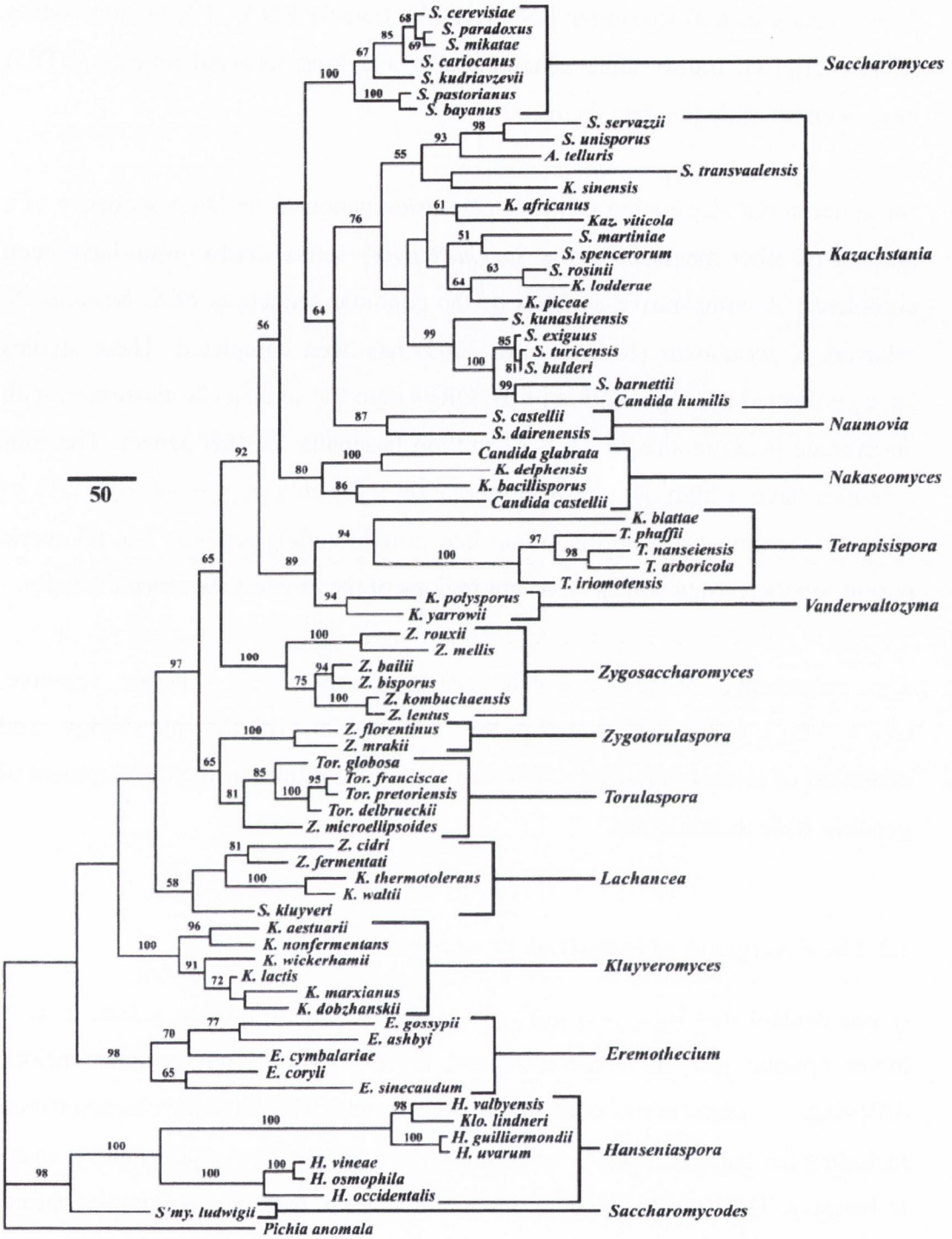


Figure 1.1: Phylogenetic tree resolving species of the '*Saccharomyces* complex' into clades], which are proposed as phylogentically circumscribed genera. (Kurtzmann, 2003).

coding genes such as ribosomal RNA (rRNA), transfer RNA (tRNA), non-coding RNA (ncRNA), transposable elements (Ty) and long terminal repeats (LTRs) have been identified in the genome.

Since the initial sequencing of the *S. cerevisiae* genome, the DNA sequence of a number of other members of the *Saccharomyces* sensu stricto group have been completed. A comparative analysis of the genomic sequences of *S. bayanus, S. mitakae, S. paradoxus* (Kellis *et al.,* 2003) has been completed. These studies have confirmed the organisation of the ORFs onto the sixteen chromosomes, with an average genome size of 11 – 12 million basepairs for this genera. The four genomes have a high degree of conservation with only approximately 1.3% of sites of insertions or deletions falling into protein coding regions. The telomeric regions on the chromosomes represent regions of the greatest sequence diversity.

The sequencing of the complete yeast genome is a valuable resource, (www.yeastgenome.org) allowing the examination of the physiology and evolution of related organisms. It is also a stepping-stone for the development of genome-wide technologies.

1.2 The Emergence of Industrial Yeast Strains.

It was evident that lager brewing yeast was different from other yeasts. It has a lower optimal growth temperature and lager yeasts do not produce meiotic offspring. *S. pastorianus* contains hybrid strains with diverse characteristics, including the lager brewing yeast strains, which are the most studied and relevant to industry. The lager brewing strains are believed to have arisen from the natural fusion event that occurred between an *S. cerevisiae* strain and a non – *S. cerevisiae* strain, most likely *S. bayanus*. The existence of two genome types in lager yeast strains has been confirmed by chromosome transfer experiments.

It is believed that the lager yeast genome underwent a genome duplication following the natural fusion of *S. cerevisiae* and a closely related *Saccharomyces* species, possibly *S. bayanus* (Tamai *et al.,* 1998, Hansen & Kielland-Brandt,

1995). This resulted in an allotetraploid strain that contains varying numbers of *S. cerevisiae*-like and non-*S. cerevisiae*-like chromosomes. Lager yeast strains are generally considered aneuploid in nature possessing an unequal number of chromosomes.

S. bayanus and *S. pastorianus*, contain diverse strains with different genetic and metabolic characteristics, revealing a hybrid origin. On the basis of molecular and genetic data, *S. bayanus* has been subdivided into two groups, (Nguyen *et al.*, 2000, Rainieri *et al.*, 1999), the first *S. bayanus* var *bayanus*, containing a miscellany of hybrid cultures and the second *S. bayanus* var *uvarum*, more commonly referred to as *S. uvarum* and contains strains of non-hybrid origin. This second group are usually isolated from grapes or wine fermentations, (Namova *et al.*, 2002). *S. uvarum* is the only group found in the species in which all strains have equal and consistent characteristics, (Rainieri *et al.*, 2003, Nguyen *et al.*, 2000). A schematic shown in Figure 1.2 shows the species of *Saccharomyces sensu stricto* that are currently acknowledged and the contribution of non-hybrid species to the genetic composition of hybrid strains. Genetic variation in the *Saccharomyces sensu stricto* is high at a strain level both phenotypically and at a molecular level.

1.3 Approaches for the Analysis of Industrial Yeast Genomes.

1.3.1 The Single-Chromosome Transfer Technique

Prior to the development of molecular genome analysis, approaches using the techniques of single chromosome transfer from lager yeast strains to the haploid laboratory strains of *S. cerevisiae* were used to elucidate the nature of the lager yeast genomes. Nuclear fusion (karyogamy) takes place following gamete fusion and is the event that instates the diploid phase in all organisms endowed with sexual reproduction. Nilsson – Tillgren *et al* (1981), described that when a haploid *Saccharomyces* strain mates with a *kar1* mutant, transfer of genetic information occurs at a low frequency between the nuclei. Nilsson – Tillgren *et al.*, (1981), used *kar1* – mediated chromosome transfer to obtain a *S. cerevisiae* strain that carried an extra copy of chromosome III from *S. pastorianus (S.*

carlsbergenesis). Since the brewing strain does not mate normally, the strain used in the *kar* crosses was a meiotic derivative of the brewing strain with mating capability. When disomic strains for chromosome III were crossed to haploid *S. cerevisiae* strains, normal spore viability was obtained allowing for tetrad analysis. Through this process, one of the two copies of chromosome III can be lost. If the original *S. cerevisiae* copy is lost, the result is a 'chromosome substitution strain' that carries a complete *S. cerevisiae* chromosome set, except for chromosome III, which comes from *S. pastorianus*. Meiotic analysis of crosses between 'chromosome III addition strains' and laboratory strains of *S. cerevisiae* revealed two important facts. Firstly, there is a functional equivalence of chromosome III from the brewing strain and *S. cerevisiae*, since ascospore viability and chromosome segregation were normal. Secondly, that in spite of the functional equivalence, the two copies of chromosome III were different since the overall frequency of recombination between them was much lower than that expected for perfect homologues. The new procedure allowed for the analysis of entire chromosomes from the brewing strains placed into a laboratory strain that could easily be manipulated genetically.

Casey (1986) performed a detailed study of chromosome X. The results were analogous with those obtained with chromosome III. Through using the chromosome transfer method it was possible to identify at least two different chromosomes X from *S. pastorianus*. Both types of chromosome had the wild-type alleles of ARG3, MET3, ILV3 CDC11 and HOM6 but only one had a functional copy of the RAD7 gene. Recombination with chromosome X of *S. cerevisiae* was limited to certain regions: the type I chromosome X recombined with the left arm between the centromere and *arg3*, while type II chromosome X recombined in the right arm between *ilv3* and *hom6*. Using probes from ILV3 and CYC1 genes, a restriction fragment length analysis was performed on types I and II chromosomes X. These experiments confirmed the observation that the genes in the recombining region are closely homologous to those of *S. cerevisiae*, while genes in the non-recombining regions are divergent in sequence and are specific to *S. pastorianus*. Using the ILV3 alleles from *S. pastorianus*, it was also observed that the lager strain is trisomic for chromosome X.

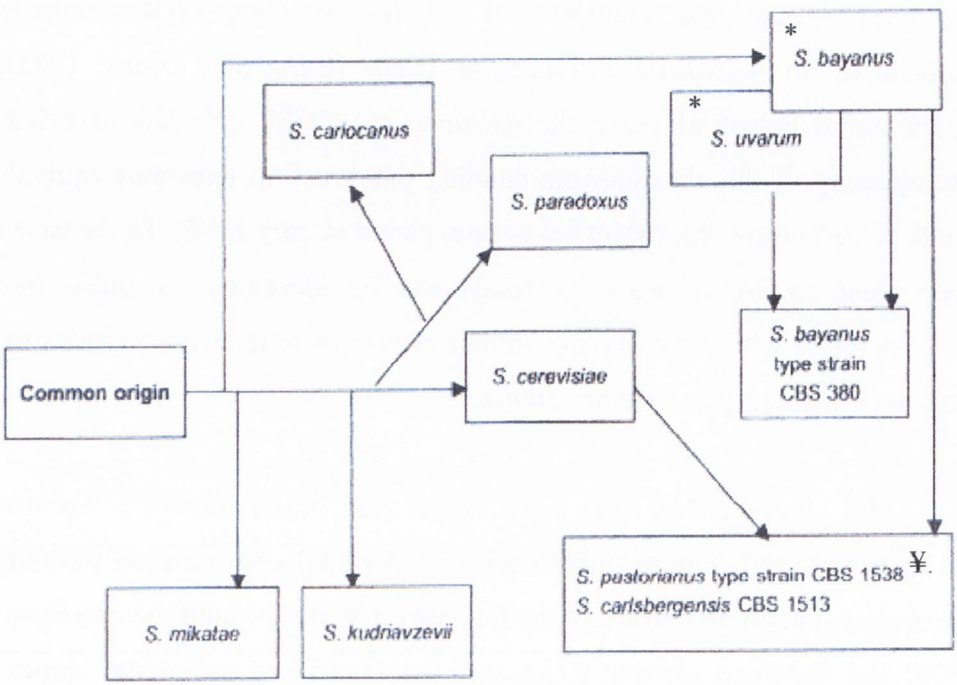


Figure 1.2: Schematic representation of *Saccharomyces sensu stricto* species and the contribution of pure species to hybrid groups. (Rainieri *et al.*, 2003).
The two lineages *S. bayanus** and *S. uvarum** have given rise to a variety of *S. bayanus* type strains. and together with *S. cerevisiae* form the basis for the hybrid strains contained with in the *S. pastorianus* group ¥.

1.3.2 Electrophoretic Analysis of Lager Yeast Strains.

The development of Pulse Field Gel Electrophoresis (PFGE) has greatly benefited the characterisation of structure and organisation of industrial strains of yeast. The first yeast chromosome fingerprint of *S. cerevisiae* using FIGE confirmed the presence of 16 individual chromosome bands (Carle and Olson, 1985). The separation of industrial yeast chromosomes on CHEF gels has revealed wide heterogeneity in the chromosome banding pattern. Chromosomes equivalent in size to *S. cerevisiae* chromosomes were apparent (Casey 1986). In the case of the lager yeast strains, at least 25 bands can be identified by pulse field gel electrophoresis with many having similar electrophoretic characterisitics to those in *S. cerevisiae* (Jespersen *et al.*, 2000).

It was also observed that lager yeast strains vary in the number of chromosome bands present and minute differences in individual chromosome mobility was noted most notably in chromosome X (Casey 1986). Through the combination of PFGE and Southern blotting techniques the identity of individual chromosome bands was deduced. In a groundbreaking paper by Casey (Casey 1986), the presence of three types of chromosome X in *S. pastorianus* was identified using a specific chromosome X DNA probe, thus confirming the earlier allele analysis.

1.3.3 Comparative Genomic Microarray Analysis of Lager Strains of Yeast

The development of whole genome microarray technologies has allowed for the investigation of information relating to the actual gene make up of industrial yeast strains.

The technique of CCGH (Comparative Competitive Genomic Hybridisation) to *S. cerevisiae* genes on DNA chips is used to determine the relative copy number of *S. cerevisiae*-like genes in the industrial strains of yeasts. The genomic DNA populations from two different yeast strains are differentially labelled by random priming with Cy3 and Cy5-tagged nucleotides and hybridisations carried out, (method described in section 2.6 and 2.7). The differently labelled DNA samples compete for hybridisation to the complementary sequence on the DNA chip. As

the microarrays utilise two differently labelled samples, a ratio of hybridisation for each gene is reflected in the red to green fluorescence ratios. The ratio of hybridisation between two DNA samples depends on the degree of sequence homology between the labelled sample DNA and the *S. cerevisiae* DNA on the chip and the copy number of the DNA sequence in the sample DNA. In the case of polyploid and anueploid strains of yeast the gene copy number and the sequence homology will affect the ratio of hybridisation resulting in a red: green ratio greater or less than one. Such a deviation in the ratio of hybridisation is indicative of a higher or lower copy number. However one of the downfalls of microarray analysis for industrial yeast strains is that only genes with strong homology to *S. cerevisiae*-like genes are detected.

While there is a limited amount of data to show the lager brewing yeast strains are allopolyploids / anueploid and contain *S. cerevisiae* and non – *S. cerevisiae* genomes, little is known about their organisation and the extent of recombination between the genomes or indeed the copy number of *S. cerevisiae* and non-*S. cerevisiae* genes.

Using the technique of CGH, Bond *et al.,* (2004), have characterised the genome organisation and gene copy number of a number of lager yeasts. In this study, it was observed that the CGH ratios of hybridisation for large regions of genes on any given chromosome was extremely similar with the average CGH ratios of hybridisation for the lager strains ranging between 0.5 and 1.8, when referenced against the haploid laboratory strain S-150. However, while there were many similarities in the CGH ratios, there were also some unique features observed within each strain. To determine the relationship between the CGH ratio of hybridisation and gene copy number, quantitative real-time PCR (qr-t PCR) technique was employed. Oligonucleotides to gene loci that represented specific CGH ratios were used to amplify DNA from the lager yeast strains. The degree of amplification was then compared to the amplification rate in a known haploid strain. Through using this technique it was possible to determine a minimum estimate of gene copy number of *S. cerevisiae* – like ORFs in the lager yeast strains. The minimum number of copies of each chromosome ranges from 2 – 4.

The conclusion reached through these numerous molecular studies is that two divergent types of the genes are present in lager yeast strains, one which showed a hybridisation pattern almost identical to that found in the corresponding *S. cerevisiae* gene. The other gene showed a divergent pattern, the former were referred to as the *S. cerevisiae* like gene (Sc-like) and the latter being referred to as the non-*S. cerevisiae*-like type (non-Sc). The identification of two types of genes is consistent with the possibility that lager brewing yeast contains two types of chromosomes a *S. cerevisiae*-like and non-*S. cerevisiae*-like chromosome.

Combining these molecular analysis with data from the single chromosome transfer experiments, it can be concluded that the lager brewing yeast contain three types of chromosomes:

1. Homologous chromosomes, recombining normally with their *S. cerevisiae* counterparts.
2. Homoeologous chromosomes, rarely recombining with their *S. cerevisiae* counterparts.
3. Mosaic chromosomes that were comprised of homologous and homoeologous segments.

This work showed that lager brewing yeast is a species hybrid but it did not reveal much about the ploidy of the lager yeast.

1.4 Aneuploidy in Lager Brewing Yeast.

1.4.1 Lager Yeast Specific Variability.

In addition to these unique CGH ratios associated with each chromosome, it was also observed that sub-telomeric ORFs in the majority of chromosomes showed a great variability in ratio of hybridisation compared to the rest of the chromosome in the lager yeast strains. Secondly, there were distinctive regions of up and down 'jumps' in CGH ratios occurring at specific loci on eight of the sixteen chromosomes, (Fig. 1.3). The specific locations of these 'jumps' and the ratios of hybridisation are listed in Table 1.1. Thirdly, sections of specific chromosomes, most notably chromosome XVI had an observed ratio of less than 0.2. A closer

examination of the loci associated with some of the 'jumps' in ratio revealed that in some cases *e.g.* Chromosome XV, they occurred in the proximity of transposable Ty elements or in the case of chromosome X at an ARS sequence. These locations are known to be hotspots for recombination events.

1.4.2 Chromosome III

One interesting 'jump' in the CGH ratio of hybridisation was observed on chromosome III. When the hybridisation ratios were plotted against the *S. cerevisiae* chromosome III gene order, a distinctive 'jump' in the ratio is observed at YCR039C, the MAT locus, (Fig. 1.4a). The lager brewing yeast strains showed two distinctive CGH ratios for ORFs to the left of the MAT locus but the ratios were the same for all lager strains for ORFs to the right. This result implies that the copy number of genes in a contiguous region to the right of the MAT locus on chromosome III in the lager strains is greater than that of the haploid strain of *S. cerevisiae*. It was also observed that there was a difference in the ratio of hybridisation between the two lager yeast strains, to the left of the MAT locus. 6701 and CMBS-33 had a ratio of hybridisation of 0.6 and 1.0 respectively. Therefore the copy number of *S. cerevisiae* – like genes, to the left of the MAT locus in the lager strain CMBS-33 is greater than that of similar genes in the other lager strain 6701. The genes to the right of the MAT locus in both strains were observed to be nearly identical. By combining the CGH data with real-time PCR analysis, it can be deduced that at a minimum, lager yeasts contain four copies of chromosome III (Fig. 1.4b). For the lager yeast strain, 6701, one of these is completely *S. cerevisiae* – like and the other are mosaic chromosomes containing non – *S. cerevisiae* like genes to the left of the MAT locus and *S. cerevisiae* genes to the right of the MAT locus.

1.4.3 Chromosome XVI.

The CGH analysis also revealed a number of other chromosomal regions where the hybridisation ratios between the lager brewing yeast strains and the haploid *S. cerevisiae* strain S-150 fell to less than 0.2. The most striking example was chromosome XVI, in the region from YPR159W to YPR190C encompassing 30

genes. This region lies directly preceding the telomere. With the exception of YPR165W, all of the other genes show a ratio of hybridisation of less than 0.2, (Fig. 1.5a). This lack of significant hybridisation represents a high level of sequence divergence in this region.

Using the relationship between the ratio of hybridisation and gene copy number it was possible to deduce the minimum chromosome XVI complement in the lager yeast strains. Chromosome XVI consists of three *S. cerevisiae* – like chromosomes containing the non – *S. cerevisiae* region YPR159W – YPR190C and one non – *S. cerevisiae* chromosome containing the telomeres and subtelomeric regions of a *S. cerevisiae* like chromosome, (Fig. 1.5b), (Bond & Blomberg, 2006). In another CGH study of lager yeast strains, (Kodama *et al.*, 2005), it was observed that the mosaic composition of chromosome XVI is conserved amongst the lager yeast strains, although some variations do exist. Chromosome XVI most likely arose from the recombination of homeologous chromosomes. In the case of chromosome III as discussed above, this recombination occurred at a site known for high genetic recombination, the MAT locus. In the case of Chromosome XVI the recombination occurs the ORF YPR159W where a Ty element is present. These sites of recombination may represent an example of adaptive evolution that confers a specific selective advantage to the particular lager yeast strain.

In summary, molecular approaches have revealed a considerable amount of information regarding the complicated genome of the lager yeasts. There is a greater knowledge of the chromosomal structure, specifically in relation translocations and recombination events that have taken place. The types and number of each chromosome are now known and that, on average, lager yeast have a tetrasomic chromosome content. While CGH can estimate the copy number, we still do not know the exact number of copies of each chromosome type, (Bond *et al.*, 2004, Kodama *et al.*, 2005).

The full characterisation of lager brewing yeast strains will require the complete genome sequence at the nucleotide level. This work however has been hampered

due to the polyploid nature of the lager yeast genome. Such analysis will require the sequencing and analysis of both the *S. cerevisiae* and non- *S. cerevisiae* components of the genome. Suntory Ltd. Japan, have reported the first description of sequencing the industrial lager strain Weihenstephan 34/70 (Nakao *et al.*, 2003). The strain was sequenced using a random shotgun approach, two types of contigs covering 23Mbp, a 95% coverage, were identified. The analysis revealed sequences with either 98% or 85% identity to the *S. cerevisiae* DNA sequence (Kodama *et al.*, 2005). The sequence data confirmed previous findings of the presence of three types of chromosomes in the lager yeast strains, *S. cerevisiae* – like, non – *S. cerevisiae* – like and mosaic chromosome arising from the recombination between homeologous chromosomes. The non – *S. cerevisiae* – like DNA sequence most closely resembles *S. bayanus (uvarum)*, however the sequences are not identical. This is possibly due to the difference in the strains of *S. bayanus* as only a single *S. bayanus* sequence is currently available.

1.5 The Ploidy Nature of Lager Yeast Genome.

The ploidy nature of lager yeast was investigated initially by looking at the ILV2 locus in a lager brewing strain (Kielland – Brandt *et al*, 1989). Part of the study looked at the consequences of eliminating the ILV2 gene function in the lager yeast. The two wild type alleles were deleted from the lager yeast strain. Using gene replacement techniques, lager yeast strains were obtained which carried one or the other deletion allele of ILV2. Southern hybridisation analysis showed that the lager yeast studied contained two copies of each of the two versions of the ILV2 gene. A generalisation of this result would imply that the lager brewing yeast is allotetraploid. However, further studies carried out on chromosomes III (Hoffman, 2000) and X (Casey, 1986), show that even though lager brewing yeast has a total DNA content corresponding to tetraploidy (Hoffmann, 2000) it appears to be irregular in chromosomal set-up and is probably aneuploid for some chromosomes or regions of chromosomes.

The majority of organisms with a sexual cycle double their ploidy at fertilisation and then reduce their ploidy by half at meiosis. In tumour cells changes in the

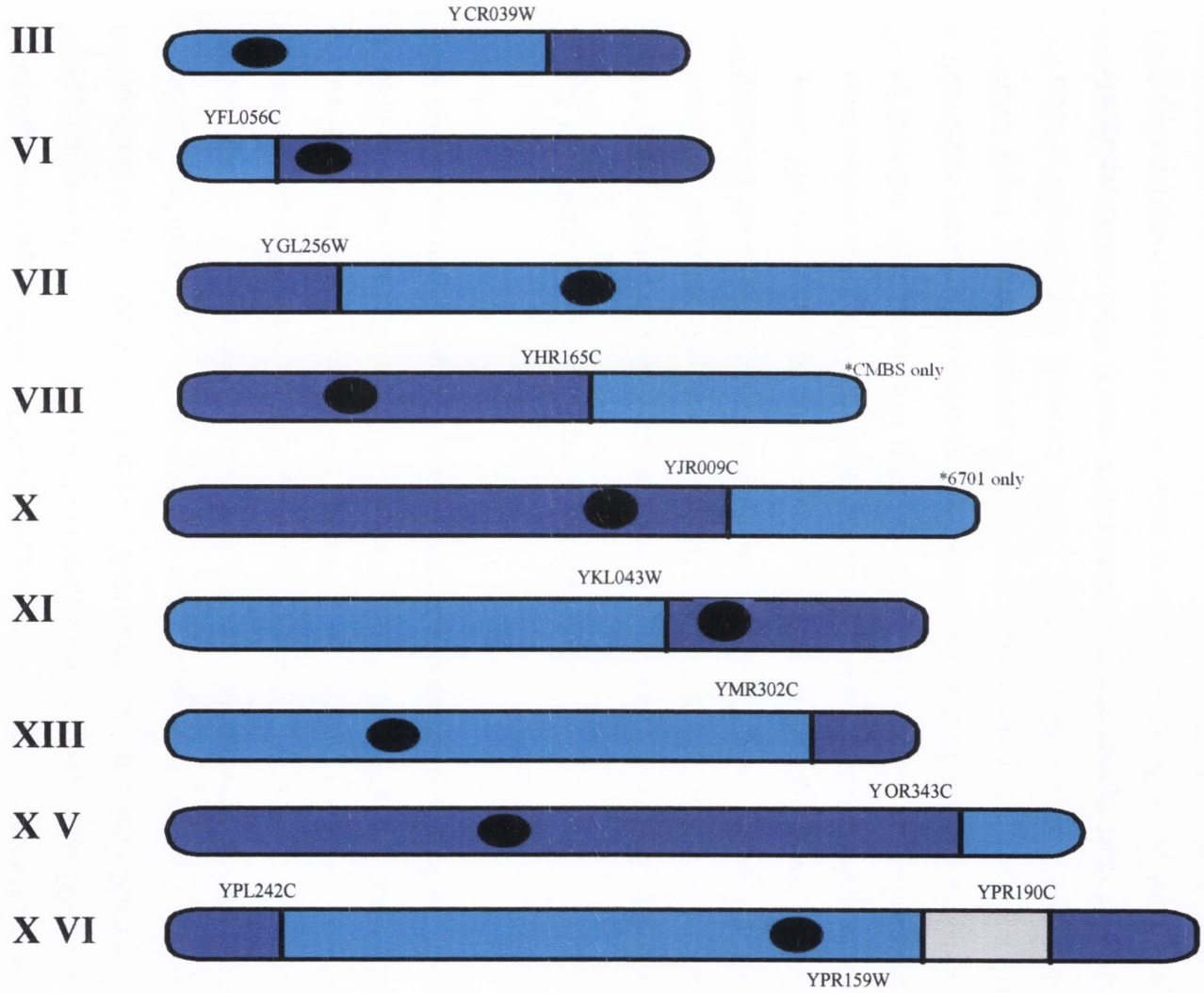


Figure 1.3 Schematic representation of the chromosomes where significant changes in the ratios of hybridisation were observed in the lager strains CMBS-33 and 6701.

Table 1.1 Locations of 'jumps' in hybridisation in lager strains.

Chromosome	Location of 'jumps' in hybridisation	Average Ratio of Hybridisation
III	YCR039C	1.0 →1.7
VI	YFL056C	0.5 →1.0
VII	YGL173C	1.0 →1.5
VIII	YHR165C	1.2 →0.6
X	YJR009C	1.1 →0.9
XI	YKL043W	1.5 →1.0
XIII	YMR302C	1.1 →1.8
XV	YOR343C	1.0 →0.6
XVI	YPL242C	1.0 →1.8
XVI	YPR159W	1.0 →0.2
XVI	YPR190C	0.2 →1.8

The average ratio of hybridisation for the lager strain CMBS-33.

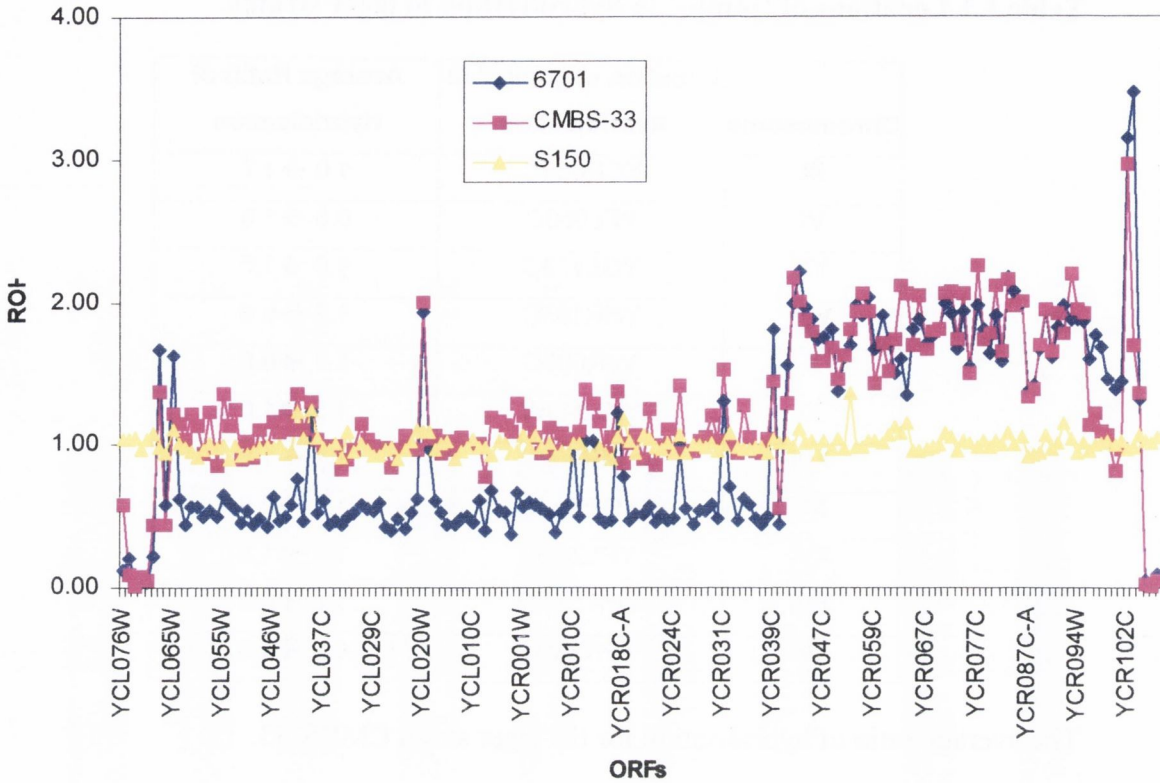


Figure 1.4a: DNA microarray analysis for chromosome III. The ratio of hybridisation of Cy5-labelled and Cy-3 labelled S150 DNA **Yellow line**. **Dark Blue** 6701 DNA, **pink line** CMBS-33. The ratios of hybridisation are plotted against the position of each ORF along chromosome III. The marked ORFs represent where the ratio 'jumps' (Bond *et al.*, 2004).

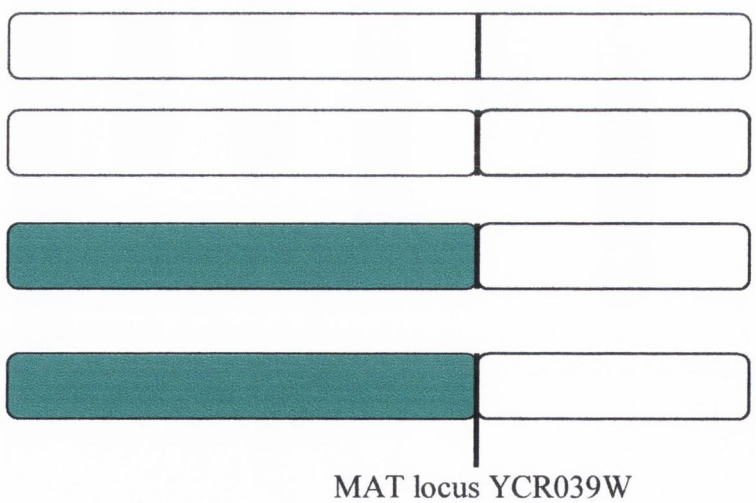


Figure 1.4b: Schematic representation of chromosome III in strain CMBS-33 as determined by CCGH. Based on this analysis chromosome III consists of one *S. cerevisiae* like chromosome and mosaic chromosomes, with the region to the left of the MAT locus non – *S. cerevisiae* like and the region to the right of the MAT locus *S. cerevisiae* like.

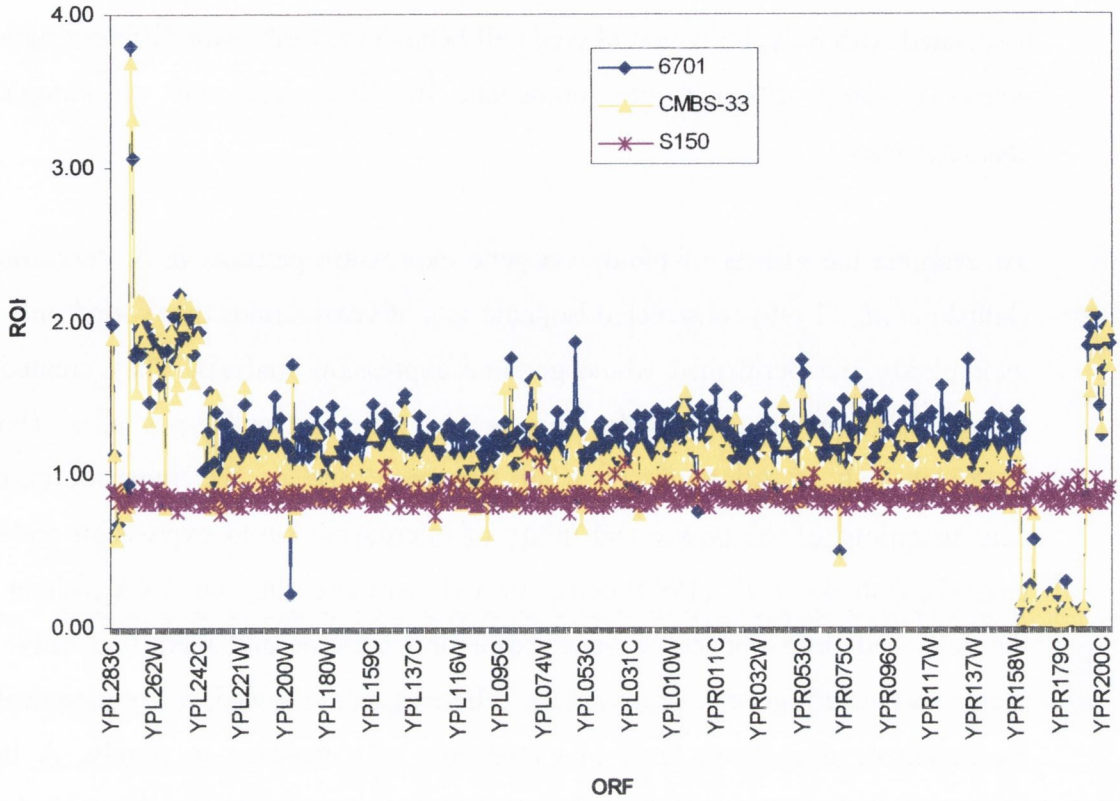


Figure 1.5a: DNA microarray analysis for chromosome XVI. The ratio of hybridisation of Cy5-labelled and Cy-3 labelled S150 DNA (**purple line**). **Dark Blue** 6701 DNA. **Yellow line** CMBS-33 DNA. The ratios of hybridisation are plotted against the position of each ORF along chromosome XVI. The marked ORFs represent where the ratio 'jumps' (Bond *et al.*, 2004).

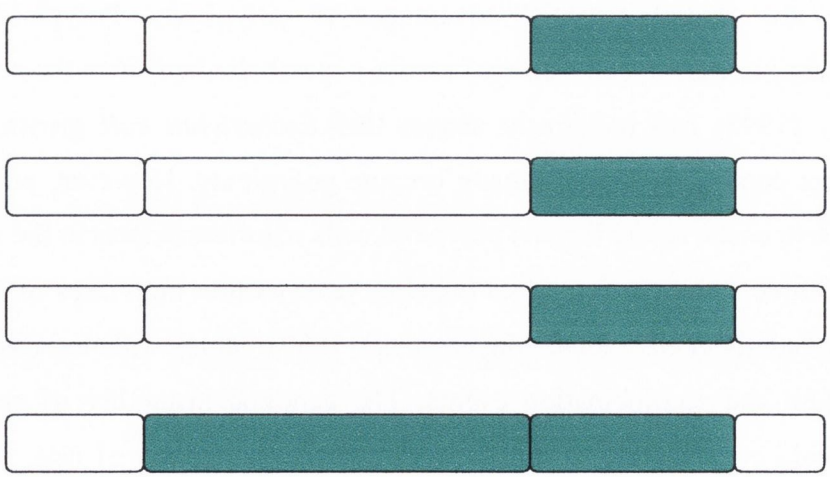


Figure 1.5b: Schematic representation of chromosome XVI in strain CMBS-33 as determined by CCGH. Based on this analysis chromosome XVI consists of three *S. cerevisiae* like chromosomes containing the non-*S. cerevisiae* region YPR159W- YPR190C and 1 non-*S. cerevisiae* chromosome with *S. cerevisiae* like telomeres.

number of chromosome sets can occur. Progression of a tumour is believed to be associated with polyploidy and altered cell behaviour. Cells with different ploidy generally show different developmental, morphological and physiological characteristics.

To examine the effects of ploidy on gene expression patterns in *S. cerevisiae*, Galitski *et al.,* (1999) constructed isogenic sets of yeast strains that varied only in their ploidy and performed whole genome expression analysis. They created a ploidy series (n, 2n, 3n and 4n) for each of the yeast cell types **a**, α (both haploids) and **a**α (diploid). In what has been one of the most definitive demonstrations of the power and utility of microarray based expression pattern analysis, Galitski *et al.,* (1999) demonstrated that increasing the DNA content of the cells had little impact on gene expression patterns and identified only 17 ploidy regulated genes, (Table 1.2). These genes showed a monotonically increasing or decreasing level of expression with increase in ploidy. A link between the expression patterns of some of these genes and altered morphology and/or the behaviour in polyploid cells was also demonstrated. The ploidy regulated genes had an unbiased distribution of locations in the yeast genome. This study also suggested that dosage compensation does not occur in *S. cerevisiae* when gene number increases.

There are several potential advantages to polyploidy. Polyploidy with the resulting increase in cell size may confer a metabolic benefit to the cell. Akerlund *et al.,* (1995) had previously shown that *Escherichia coli* grown in limiting nutrient conditions spontaneously became polyploidy. However, polyploidy can also have costs, newly formed polyploid cells manifest defects in the maintenance of genomic stability. Tetraploid budding yeast exhibit high rates of chromosome loss (Andalis *et al.,* 2004, Mayer *et al.,* 1990), along with increased levels of mutation and recombination events. The genomic instability of newly formed polyploid cells therefore should accelerate the generation of new variants, thus drawing parallels between the role of polyploidy in the evolution of organisms and the role of polyploidy in the development of tumours (Storchova *et al.,* 2004. Fujiwara *et al.,* 2005).

Recently, Storchova *et al.,* (2006) conducted a systematic approach to identify genes that are essential for the viability of polyploid yeast cells. Diploid strains created from a *S. cerevisiae* gene disruption library, containing defined single-gene deletions were mated together to form tetraploids. 39 genes in total were sensitive to tetraploidy and fell into three functional groups. First are genes that encode the mitotic spindle including components of the spindle pole body, (the yeast microtubule organising centre).The second group contains genes involved in chromosome cohesion and the last group contains genes that are essential for homologous recombination. It is interesting to note that such a small group of genes were identified, as more diverse pathways may have been expected to be identified from the screen. For example, Storchova *et al.,* (2004), noted that tetraploid cells have an altered cellular metabolism however no genes relevant to metabolic activity were identified. Also no transcription factors were found, which in itself is unusual for a genetic screen. This result suggests that transcriptional control is not a key regulatory mechanism for the maintenance of genomic stability in polyploid strains.

Tetraploid yeast are proportionally larger than diploid yeast and are genetically unstable with high levels of chromosome loss and interhomolog recombination. The tetraploid screen identified genes whose products are involved in mitotic spindle function, chromosome cohesion and DNA repair. Defects in spindle pole bodies are known to induce chromosome instability due to defects in chromosome segregation. Secondly, genes encoding cohesion are important for the accurate segregation of chromosomes therefore it would be expected that cohesion is essential in cells with increased ploidy. Thirdly, the genes essential for homologous recombination most notably RAD52 epistasis group were identified. These genes are normally only essential in the presence of DNA damage. Therefore these observations imply that tetraploidy leads to an increase in DNA lesions. The increased damage in the tetraploid cells may reach a maximum threshold level rendering the cells dependent upon homologous recombination.

Table 1.2 Ploidy Regulated Genes.

Systemic Name	Standard Name	Function
YNR067C	DES4	Cytokinesis
YLR286C	CTS1	Endochitinase activity
YHL048W	COS8	Nuclear membrane protein
YNR065C	YSN1	Uncharacterised
YML120C	NDI1	NADH:ubiquinone oxidoreductase
YLR121C	YPS3	Aspartic protease
YLR411W	CTR3	High affinity copper transporter
YKL218C	SRY1	3-hydroxyaspartate dehydratase
YER067C		ORF, Dubious
YOR100C	CRC1	Mitochondrial inner membrane carnitine transporter
YIR019C	FLO11	Cell surface flocculin
YNL289W	PCL1	G1 cyclin
YKR013W	PRY2	Unknown function
YOR387C		Uncharacterised
YGL258W	VEL1	Unknown function
YDR309C	GIC2	Control of actin cytoskeletal organisation
YMR199W	CLN1	G1 cyclin

1.6 Transcription Analysis of Lager Yeast

Yeast are subjected to many types of stress and metabolic challenges throughout the fermentation process. As brewing is an ancient process, years of exposure to these conditions have resulted in the strains evolving mechanisms that allow the adaptation to such challenges. However, the advent of new processes such a high gravity brewing have placed new demands on the yeast cells (Emandes *et al.,* 1993. Morrison & Suggett, 1983). A greater understanding of how yeast sense and responds to the changing environment during fermentation can be obtained by studies of the transcriptome (Causton *et al.,* 2001., Kuhn *et al.,* 2001., Gasch *et al.,* 200). Olesen *et al.,* (2002), performed a transcriptome analysis of a lager brewing yeast during the time course of a fermentation. Although the study obtained transcriptome data it was hindered by the ability to distinguish between the expression from two different subgenomes. The expected results were to observe a clear progression of transcriptional regulation during the fermentation this would have lead to the grouping of genes into distinct clusters. However, this result was not observed, as when comparing controlled laboratory experiments one growth variable can be altered at a time and the response compared to the reference. However the progress of a fermentation represents a multitude of gradual altering conditions which leads to a transcriptional response which is often difficult to discern.

A more comprehensive study of yeast genome expression was conducted by Higgins *et al.,* (2003) looking at the cells response during the initial stages of a fermentation. The mRNA level of over 100 genes was threefold higher in the first hours of fermentation compared to that of the 23rd hour. The observed genes were involved in many cellular processes ranging from lipid, fatty acid and sterol metabolism to amino acid metabolism, cell stress and protein modification. It was observed that genes involved in ergosterol biosynthesis were induced in the initial stages of fermentation. Ergosterol is an essential lipid component of yeast membrane. The up-regulation of genes involved in thioredoxin and GSH cell functions was an indication the cells experience an oxidative stress response at the initial stages of fermentation. The induction of ergosterol and oxidative stress

response genes points to an interaction between the two cell functions (Bammert & Fostel, 2000., Schmidt *et al.,* 1999).

Lager yeast strains have adapted to growth under extreme environmental conditions, whilst maintaining the ability to convert carbohydrate into ethanol and other by- products. James *et al.,* (2003, 2002), used standard brewing system to grow a brewery yeast strain and examine its genome wide response to brewing at a transcription level. As brewery strains are polyploid in nature the high expression of genes may be an indication of multiple copies. This was observed by James *et al.,* YCR005C was found to be highly expressed, however it had previously been shown to be responsive to the ploidy of the cell (Galitski *et al.,* 1999). The opposite may also be relevant as a low level of expression of a gene during fermentation may be indicative of the partial or complete deletion of the gene. James *et al.,* (2003) also observed that genes encoding glycolytic enzymes were repressed when the carbohydrate concentrations were high, however these genes were not activated as fermentation proceeded. This result implied that brewery yeast strains do not undergo a typical diauxic shift. This is due to the continued supply of glucose and maltose in the wort along with the lack of oxygen to sustain respiration. It is also interesting to note that in this study that the majority of genes involved in ethanol production did not show an increase in expression as fermentation progressed.

During the brewing process, yeast are exposed to sever environmental conditions, however it was observed that general stress response genes; Heat Shock Genes were all repressed as fermentation progressed. Heat shock genes are also induced in response to other stresses such as exposure to high ethanol concentrations (Ruis 1997). Stress response genes are also activated through the binding of general stress response transcription factors such as Msn2/4. As yeast cells in a fermentation do not undergo a typical diauxic shift the stress genes cannot be activated via STRE binding of transcription factors. Therefore the tolerance of stress conditions may be achieved by the synthesis of high levels of trehalose.

The microarray data in these studies expands the knowledge of metabolic processes of brewery yeast in fermentation. Thus making it possible to identify the reactions that lead to the production of important metabolites that add flavour and quality to beer as well as identifying pathways responsible for unfavourable metabolites.

1.7 The Brewing Process & Fermentation

Brewers yeast are divided into two major groups, the top fermenting ale yeasts and bottom fermenting lager yeasts. The fermentation of ale yeasts is performed at room temperature and results in beers with a distinctive fruity aroma. In the case of lager yeasts fermentation is performed at a lower temperature usually 8-15°C and therefore the fermentations take longer; approximately two weeks, where as ale fermentation takes four – six days.

The brewing process consists of four main stages, which will be discussed briefly herein. The initial stage involves the malting of the barley malt, a process that converts insoluble starch to soluble starch, generating essential nutrients for yeast development. The next stage is wort production. The mashing process subjects the malt to a heat treatment during which the starch grains are gelatinised and are more readily degraded by enzymes. As a result fermentable and non-fermentable sugars are formed, (Cvengroschova *et al.*, 2003). The released proteins are responsible for the foam in beer. The amino acids and small peptides are necessary for yeast activity further in the brewing process. After the mashing a sweet syrup (wort) is produced.

The wort is filtered to remove solids and then boiled. This process has many effects: the wort is sterilised, the heating inactivates all malt enzymes, much of the soluble proteins are coagulated and separated. Also during the boiling, browning reactions take place between sugars and amino acids in the wort, which results in the darkening in wort colour. Followed by a cooling period any precipitate that has formed is removed. Wort is oxygenated prior to the primary fermentation, yeast is added and the fermentation process begins. Oxygenation of

the wort is essential for the synthesis of sterols and unsaturated fatty acids during fermentation. Poor attenuation of worts, slow fermentation rate and poor yeast growth are observed when there has been inadequate oxygen added to the wort in the beginning of the subsequent fermentation. During fermentation, fermentable carbohydrates in wort are converted by the yeast into alcohol, carbon dioxide and numerous other by-products. The by-products have a considerable effect on the taste, aroma and other characteristic properties of the final beer.

At the end of the fermentation, yeast cells flocculate and form clumps, which may either, drop to the bottom of the vessel or rise to the top and float on the surface of the liquid. In general lager yeast strains are bottom – fermentors, while ale yeast strains are top – fermentors. However, in large cylindroconical fermenting vessels both yeast strains are collected at the bottom of the fermentor. When the fermentation is completed in such a vessel, it is cooled to 2 – 4°C, which causes both yeast types to sink. The yeast slurry is separated from the fresh beer. Typically the lower and upper part of the yeast sediment is discarded while the middle part is transferred to a storage vessel and repitched later on into a subsequent fermentation. It is not unusual for a yeast culture to be used between 7 –15 times. This process cannot be continued indefinitely as there is an increased risk of infection and of spontaneous mutations in the yeast.

The yeast strain used in specific breweries is strongly associated with the characteristic taste and aroma of the desired product. As a consequence a rather conservative attitude towards yeast strain improvement is maintained in the large brew houses across the world. However, high productivity of a brewery is required for a brewery to survive in a competitive economy. High gravity brewing (HGB) is a modern development in the brewing industry in which fermentations are performed at a much higher wort concentration than in a traditional brewing process.

1.8 Wort Composition

Wort contains fermentable and non-fermentable sugars. The fermentable sugars utilized by *S. cerevisiae* are glucose, fructose, sucrose, maltose and maltotriose. In addition to these sugars *S. pastorianus* can ferment melibiose. Maltose is the most abundant sugar in wort usually accounting for 60 – 65% of the total fermentable sugars. Glucose and maltotriose account for 10-15% and 15-20% respectively. 1 – 2% of wort is composed of sucrose and fructose (Verstrepen *et al.,* 2003, Ermandes *et al.,* 1993). α-glucans also called dextrins, β-glucans and pentose sugars such as ribose and xylose are non-fermentable by yeast. Approximately 15% of the carbohydrates in wort cannot be fermented.

Sugars are taken up into the yeast cells in a strict order, (Suihko *et al.,* 1993, Shimizu *et al.,* 2002). Sucrose is consumed first. It is hydrolysed outside the cell by the extracellular enzyme invertase to glucose and fructose. Meneses *et al.,* (2002) have observed that unlike laboratory strains, invertase activity in brewing yeast strains is poorly repressed by glucose and fructose. Glucose and fructose are transported into the cell. The uptake of glucose is faster than that of fructose. When half of the glucose present in the wort has been taken up into the cell, the yeast will start to transport maltose and maltotriose across the plasma membrane. (Dietvorst *et al.,* 2007, 2005, Day *et al.,* 2002).

1.9 Lagering – Maturing the beer.

Beer at the end of a primary fermentation contains little entrained carbon dioxide, it is hazy and the taste and aroma are inferior to that of a beer that is ready for sale. In order to refine the beer it is conditioned or matured. Traditionally, the beer is transferred to a maturation tank, what small amount of yeast that remains in the beer can utilise any remaining fermentable sugars. Any carbon dioxide that is produced dissolves in the beer as the vessel is closed. As well as carbonation, conditioning also involves the clarification of the beer by promoting the precipitation of proteins and polyphenols by cold storage. During maturation

flavour changes are also taking place. After conditioning, the beer is filtered to remove the remaining yeast and packaged in bottles, cans and kegs.

1.10 Measuring Fermentation Rate.

Brewers use two primary systems for measuring the amount of extract or dissolved alcohol in wort or beer – specific gravity or percent extract by weight. Specific gravity being the most commonly used will be discussed herein. Specific gravity is the density (weight per unit volume) of a substance divided by the density of water. The specific gravity of liquids is often measure with a hydrometer, whose weight displaces different volumes of liquid depending the density liquid.

Tables constructed in 1843 by Karl Balling and later improved upon by Plato correlate specific gravity with the percent by weight of extract of sucrose in solution. Sucrose produces the largest increase in specific gravity for a given percentage by weight in solution. Stating that a wort is 10 degrees Plato means that if the extract in solution were 100%, 10% of the weight of the solution is sucrose. However, in typical wort only a small fraction of the extract is actually sucrose. This is not a problem as sucrose was selected as the reference because it produces the largest increase in specific gravity for a given percent by weight in the solution.

1.11 High Gravity Fermentation

In traditional brewing, wort of 11-12%-dissolved solids is fermented to produce beer of usually 5% (volume/volume) ethanol. In high gravity brewing (14- 18% dissolved solids) and very high gravity brewing (> 18% solids) more concentrated wort is used for fermentation, which results in a beer with a higher alcohol content. There are two ways to increase the wort density, one is the addition of sugar syrups of adjuncts and secondly the amount of water can be reduced.

High gravity brewing was originally introduced in the 1950's in the US but has become more popular, especially in the production of pilsner- type lagers. The

current limit in high gravity brewing is 16-18% dissolved solids. More concentrated wort has a significant negative effect on the fermentation performance of yeast and is therefore seldom used in industry.

1.11.1 Advantages of High Gravity Brewing

Advantages of high gravity fermentation include an increased output of existing plant facilities by 20-30%, reduction in energy and labour cost per barrel produced and an increased ethanol yield. Beer produced through high gravity fermentation shows improved colloidal haze as an increased precipitation of polyphenol material at higher concentrations. There is also a greater microbiological stability due to the higher ethanol yield.

1.11.2 Disadvantages of High Gravity Brewing

Disadvantages associated with high gravity brewing include an increased investment is needed to modify brew houses or the process of wort production must be adapted. Attempts to carry out fermentations above 18°P have proven difficult due to the extreme stresses the yeast cells are exposed to, such as high osmolarity and high ethanol levels and nutrient starvation. As a result, poor yeast crop viability and stuck or sluggish fermentation rates have been experienced. Differences in flavour compound production, especially isoamyl acetate and ethyl acetate levels are noticed in high gravity brewing compared to beer brewed in the conventional process. The concentration of the acetate esters can be up to 75% higher in beers made with 20°P wort compared to normal gravity wort, (Meilgaard, 2001).

Another disadvantage of high gravity brewing is decreased foam stability. Hydrophobic proteins form the backbone of foam in high gravity brewing beers have less hydrophobic polypeptides compared to low gravity brewed beers when measured at the same alcohol concentration. (Cooper *et al.,* 1998). Polypeptide hydrophobicity is more important for foam stability than the size of the polypeptide. (Onishi and Proudlove, 1994).

1.12 Stresses during Lager Fermentation.

Stress effectors are defined as 'any chemical or physical parameter that has a negative effect on cellular growth', (Hohmann and Mager, 1997). An overview to cellular factors that have been correlated with stress resistance in *S. cerevisiae* is given is Figure 1.6.

Brewing yeast encounter several stresses during a fermentation process. The main aim of fermentation is to achieve maximum ethanol concentration possible from the available wort sugars. This is only possible if the optimal growth conditions are avoided. Stresses encountered during fermentation include: oxidative stress, cold and heat stress, osmotic stress, ethanol stress, nutrient limitation and hydrostatic pressure stress. These stresses will be discussed here in.

1.12.1 Oxidative Stress.

Fermentations are mainly anaerobic; however yeast cells do experience sudden changes in oxygen concentration when pitched into oxygenated wort after anaerobic storage. Yeast cells grown aerobically generate a number of chemically reactive highly unstable molecules such as – super anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH), referred to as reactive oxygen species, (ROS). Hydroxyl radicals are the most highly reactive ROS. Hydroxyl radicals are damaging towards cellular components such as DNA, lipids and proteins.

Yeast cells have several mechanisms, (enzymatic and non-enzymatic cell defences) to protect themselves from the damaging effects of ROS. Enzymatic cell defences utilise catalase and superoxide dismutase. Catalase catalyses the breakdown of hydrogen peroxide to dioxygen and water via to enzymes catalase A (CTA1) and catalase T (CTT1), which are found in the peroxisome and the cytosol respectively. Superoxide dismutase catalyses the conversion of superoxide anion to dioxygen and hydrogen peroxide, which is then further, degraded by catalase. Yeast cells have two superoxide dismutases, the cytoplasmic Cu/ZnSod (SOD1) and the mitochondrial MnSod (SOD2).

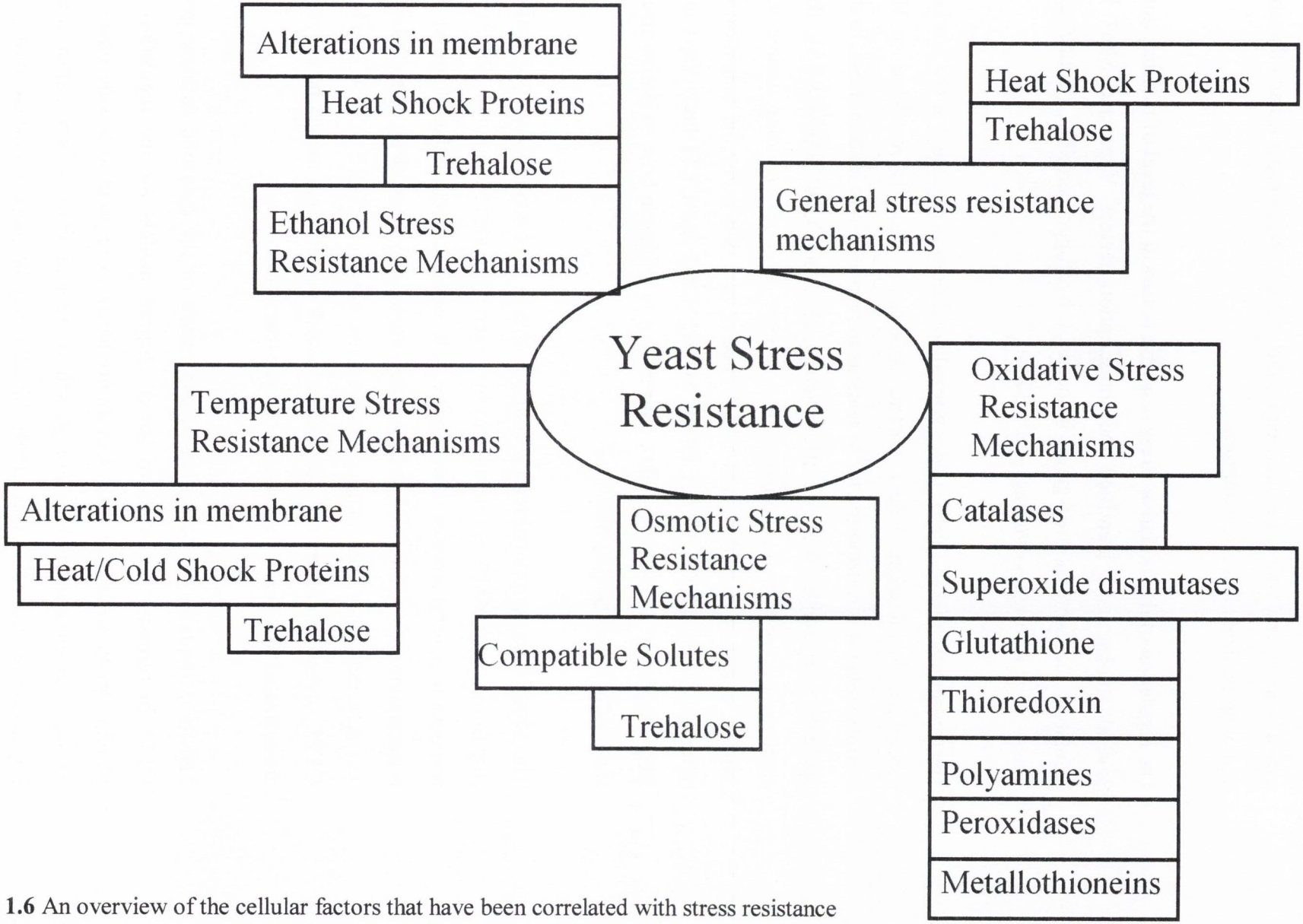


Figure 1.6 An overview of the cellular factors that have been correlated with stress resistance in *S. cerevisiae*.

Non-enzymatic defences to protect the cell against oxidative stress are provided by antioxidants e.g. glutathione and thioredoxin. Antioxidants generally act as radical scavengers, which are oxidised by the reactive oxygen species and thereby removing oxidants from the solutions.

The regulation of the oxidative stress response occurs at the level of transcription. Several studies have identified the transcription factors Yap1 and Skn7 in regulating the expression of genes induced by hydrogen peroxide, (Toone and Jones, 1998; Rodrigues-Pousada *et al.*, 2004).

The transcription factor Yap1, under normal conditions is regulated at the level of subcellular localization. Under normal conditions Yap1 is restricted to the cytoplasm but it is transported to the nucleus in response to oxidative stress. In the nucleus Yap1 binds to the Yap1 response element (YRE: TGACTAA), this element is present in the promoter of a number of genes encoding antioxidant defence enzymes like GL11 and GSH1 (Lee *et al.*, 1999). For the induction of other genes like TRX1, TRX2, TRR1, SOD1, SOD2 and CTT1 both Yap1 and Skn7 are required (Estruch, 2000). Currently it is unknown how oxidative stress signals are transmitted to Skn7.

The Msn2/4 transcriptional regulator also seems to have a significant, yet distinct role from that of YAP1 in tolerance to hydrogen peroxide (Hasan *et al.*, 2002). In response to several stresses (temperature, salt, osmotic stress, ethanol stress) the transcription factor is translocated from the cytosol into the nucleus where it binds and activates genes containing stress response element, (STRE: CCCCT). Only CTT1, several proteases, chaperones, heat shock proteins and metabolic enzymes are activated by Msn2/4 in response to oxidative stress.

Msn2p is required for activation of expression of the cytosolic catalase gene CTT1 in response to oxidative stress. Current models for the regulation of Msn2p/Msn4p argue that these factors are likely to respond to a wide range of stresses and serve in inhibition of growth in order to allow cells to adjust to the imposition of stress (Estruch, 2000). Msn2/4 may be more important during the

period of recovery from oxidative stress where as Yap1 is important to prevent the stress (Martinez-Pastor *et al.*, 1996).

1.12.2 Temperature Stress

Temperature stress is induced by a rapid change in the external temperature, either an increase or a decrease. Since lager fermentations are performed at 7 - 13°C, high temperature stress is not a major issue for lager yeast. However, lager yeast do have a normal heat shock response. Hsps are not induced until temperatures of 37 - 41°C, (Brosnan *et al.*, 2002).

1.12.2.1 Cold Stress

Brewing yeast can also undergo temperature stress during cropping. Exposure of the yeast cells to a rapid decrease in temperature has implications on the cell membrane, a decrease in membrane fluidity occurs. This results in a slower diffusion of membrane proteins, decreased activity of membrane-associated enzymes and a major reduction in membrane transport (Vigh *et al.*, 1998).

The yeasts response and adaptation to low temperatures has not been extensively studied. Cold shock proteins; Tip1, Tir1, Tir2 and Nsr1 have been identified in yeast and are induced in response to cold shock. TIP1 and its homologues TIR1 and TIR2 encode serine- and alanine-rich cell wall proteins and are possibly involved in maintaining cell wall integrity during stress. NSR1 has been shown to be involved in ribosome biogenesis pre-rRNA processing and ribosome biogenesis. OLE1, a fatty acid desaturase gene is also induced during cold shock, which results in an increase in the degree of unsaturation of fatty acids, (Nakagawa *et al.*, 2002). Trehalose is accumulated and leads to the induction of heat shock proteins; Hsp104, Hsp43, Hsp12 during a response to cold stress, (Kandror *et al.*, 2004). Scahde *et al.*, 2004, has classified genes that are induced by cold shock into two distinct groups, early and late cold response genes.

1.12.3 Ethanol Stress

During fermentation, yeast cells convert sugars into ethanol and carbon dioxide. Ethanol can reach a concentration that causes stress to the cells. This is the case when fermentations are performed under high gravity conditions. Ethanol effects cell growth, cell viability and fermentation (Pratt *et al.*, 2004), but to varying extents. Yeast growth is inhibited by ethanol above a concentration of 10-12% (v/v) whereas fermentation capacity is inhibited at approximately 20% ethanol (v/v). The degree of inhibition is strain dependent and is also related to environmental factors like high temperatures. Ethanol tolerance decreases with increasing temperatures.

There have been several proposed mechanisms by which the toxic effect of ethanol may be exerted. These include non-specific osmotic effects and a number of specific cellulase targeted sites (D'Amore *et al.*, 1990). The damage caused to the cell membrane results in alteration of cell membrane organisation and permeability. Ethanol has also been reported to cause the inactivation of enzymes such as hexokinase. Ethanol stress can also induce petite mutations in yeast. These strains have lost their respiratory function. Fermentations with respiratory – deficient yeast can results in a poor utilisation of fermentable sugars, reduced ethanol production and a decrease in the rate of fermentation and viability of the yeast (Powell *et al.*, 2000).

Yeast cells will alter their membrane composition in response to ethanol stress. A noted increase in the content of mono – saturated fatty acids together with a decrease in saturated fatty acids is observed when cells are challenged by ethanol stress (Mishra & Prasad, 1989). The predominant unsaturated fatty acids on *S. cerevisiae* are palmitoleic acid and oleic acid. Oleic acid is the most effective unsaturated fatty acid in overcoming the toxic effects of ethanol in growing yeast cells (You *et al.*, 2003). Yeast cells are not able to synthesise unsaturated fatty acids under anaerobic conditions.

Heat shock proteins are also induced in response to ethanol stress. The threshold concentration from ethanol to cause heat shock protein induction in yeast cultures

growing at 25°C is between 4 and 6% (vv). Above this level, several heat shock proteins are induced such as Hsp104, Hsp30 (Piper, 1995). Trehalose is also synthesised in response to ethanol stress in order to stabilise cell membranes and proteins. Metal ions mostly Mg^{2+} play a significant role in protecting yeast from ethanol stress and conferring ethanol tolerance. The addition of Mg^{2+} in the beginning of a fermentation or a high gravity fermentation results in an increased initial fermentation rate, ethanol production and vitality at the end of the fermentation (Rees & Stewart, 1997). The effect is more pronounced when oxygenated wort is supplemented with Mg^{2+} than non – oxygenated wort.

A large number of genes are up - regulated during alcohol stress to maintain vital cellular functions. This included genes involved in ionic homeostasis, heat production, trehalose production, antioxidant defence, lipid and energy metabolism (James *et al.*, 2003). However little is known about the mechanisms by which alcohol stress is sensed and signalled to its effectors.

The protein Asr1, has recently been shown to accumulate in the nucleus upon exposure to ethanol stress (Betz *et al.*, 2004). The cellular localisation of this protein is not altered by osmotic, oxidative or heat stress or during nitrogen or glucose starvation. Further analysis is required to identify its nuclear targets.

1.12.4 Nutrient Limitation

The use of high gravity wort leads to nitrogen and lipid deficiencies. Nitrogen limitations are due to syrups that are added to the kettle to increase the specific gravity of the wort. This results in decreased carbon to nitrogen ratio. Oxygen is found in decreased levels in high gravity wort because of its solubility decreasing as the content of the wort solids increases. Deficiencies will prematurely terminate yeast growth which leads to significantly prolonged and in some cases stuck fermentations. Supplementing wort with a nitrogen source (yeast extract, peptone, aspartate) or a lipid source (ergosterol and oleic acid) results in an increase of cell mass production, decreased fermentation times and increased ethanol levels (Casey *et al.*, 1984, Cruz *et al.*, 2002, Dragone *et al.*, 2004). Cells

that are limited for nutrients start to accumulate trehalose. Hsp are also induced as a response to severe nutrient limitation.

1.12.5 Pressure Induced Stress – hydrostatic pressure or gaseous pressure

1.12.5.1 Hydrostatic Pressure

Hydrostatic pressure is generated in the tall cylindroconical vessels. As the fermentation proceeds agitation is efficient and cells will circulate continuously through out the vessel. This results in the yeast being subjected to a constantly changing pressure environment. High hydrostatic pressure has impact on the volume of the cell, membrane fluidity and proteins.

Genomic expression patterns in response to high hydrostatic pressure revealed a number of differentially expressed genes. Most of the up regulated genes were defined as genes of unknown function, although genes involved in stress defence and metabolism were also up – regulated. Genes related to protein synthesis and fate (folding, modification and destination) together with genes involved in cell cycle progression were strongly repressed under these conditions.

1.12.5.2 Gaseous Pressure

High gravity wort is more viscous than normal gravity wort, carbon dioxide will escape more slowly during fermentation, resulting in a stressful situation. The effects of high carbon dioxide levels on yeast cells include the loss of biomass yield and fermentation capacity and an inhibition of growth. Little is known about the molecular mechanisms involved in carbon dioxide sensitivity in yeast. Genome wide transcript profiles revealed only a small number of genes that were differentially expressed under high carbon dioxide pressure.

1.13 Summary of Stresses during Industrial Fermentation.

As yeast progresses through the brewing cycle of storage, pitching, fermentation, cropping and storage, it is subjected to a number of stresses, as have been discussed in the above sections.

Optimal growth conditions are avoided during fermentation to achieve the maximum ethanol concentration (theoretically) possible from the wort sugars available. Yeast should adapt to these stresses in order to withstand them.

During an industrial fermentation, yeast encounters multiple stresses simultaneously. In addition, carbon sources are more complex and growth conditions can vary greatly from those under laboratory conditions. To date little research has been done on the response of yeast to the combined stresses occurring in an industrial fermentation process.

Genome-wide expression analysis identified a strong response of genes involved in the biosynthesis of ergosterol and oxidative stress protection during the initial stages of industrial lager fermentation (Higgins *et al.*, 2003, James *et al.*, 2003). All heat shock genes are repressed in the fermenting cells as the industrial fermentation continues (Brosnan *et al.*, 2000, James *et al.*, 2003). These genes can be induced through the binding of the transcription factors HSF1 and Msn2/4 in response to heat and ethanol, oxidative and osmo-stress respectively. Lower expression of the heat shock proteins may be explained by the fact that the transcription level of Hsf1 was reduced in the fermenting cells (James *et al.*, 2003). The reason for the lower transcription level of Hsf1 is unknown.

Industrial and lab strains behave differently under stress conditions. When comparing the stress response of a lab and an industrial strain during high gravity fermentation, it was shown that osmotic and ethanol stress responses occurred in both strains during fermentation. However, the lab strain was most affected by the stresses (Devantier *et al.*, 2005., James, 2002). Industrial strains showed a higher tolerance to heat shock and oxidative environments than the lab strain (Garay-Arroyo *et al.*, 2005). Stress responses are highly dependent on the genetic and environmental background of the strain used.

1.14 Aim and Objectives

The initial aim of this study was to characterise the genomic rearrangements in the lager yeast strain CMBS-33 through an in depth analysis of chromosomes III, VIII and XVI, specifically, to determine the exact location of the recombination events and to ask if novel gene products arise from these rearrangements. Secondly, the question of whether gene dosage compensation occurs in the lager polyploid yeast genomes was explored. The lager yeast genome is a hybrid genome. This work sets out to determine if both genomes present in these strains are expressed and the level of expression of the homologous genes.

The third aim of this study was to characterise at a genome level a series of stress tolerant lager yeast mutants that had previously generated using EMS treatment. As part of this study a second generation of mutants were generated without the use of EMS. The second generation of mutants were characterised using CGH analysis. The cross tolerance of first and second generation lager strains to a variety of stresses were compared. The final objective of this work was to determine if the lager yeast genome is stable and to determine if exposure to environmental stress can influence genome stability.

In summary this work focused on the genomic organisation of an industrial lager strain CMBS-33 specifically looking at mosaic chromosomes and secondly the development of brewer's yeast strains that have a higher resistance to stress conditions present in high gravity fermentation.

Chapter 2
Materials & Methods.

2.1 Yeast Strains.

The yeast strains used during this, study, are listed in Table 2.1.

2.2 Cell culture and growth media

Yeast was cultured in the laboratory in YEPD, (1% yeast extract, 2% Bacto-peptone, 2% glucose) and YEPM (1% yeast extract, 2% Bacto-peptone, 2% maltose) when the cells were subsequently used in fermentations. All strains were grown on YEPD and YEPM agar plates at 30°C, except the lager strains which were grown at room temperature. Broth cultures of the lager strains were grown at 13-15°C and S150 broth cultures were grown at 30°C.

2.2.1 Fermentations.

Wort was prepared from dried spray malt, (Spraymalt, Brewferm, Amber 18EBC, Brouland, Belgium). The stock wort was made from the powder resuspended in distilled water and stirred overnight at a final concentration of 40% (w/v). It was steamed for 4 hours in a steamer and then cooled to room temperature. The insoluble materials were removed by centrifugation and the brix value was measured using a portable refractometer. Prior to starting the fermentation, the wort was diluted to the required concentration with distilled water and zinc sulfate added to 1 mM. Fermentations were carried out at room temperature in 200ml volumes in a 250ml sterile glass cylinder.

The wort was aerated once with compressed air, bubbling from the bottom of the vessel. Anaerobic fermentations were overlaid with mineral oil. Fermentations were pitched at cell densities of $1.5x10^7$ lager yeast cells/ml. For all fermentations the yeast were propagated in 500ml aliquots of YEPM in 2 litre flasks with mild aeration at room temperature. The yeast was centrifuged and all the media removed to avoid dilution of the wort. Flasks were incubated at a 45° angle. The sterility of the fermentations was determined by incubating wort without yeast and checking for contamination. Prior to the pitching of cells at the start of fermentation, one end of a long sterile rubber tubing was inserted into the bottom of the fermentation vessel. The other end was attached to a 50ml syringe outside

Table 2.1 Yeast Strains used in this Study

Strain	Mating Type	Genotype	Source/Notes
S150	MAT**a**	*ura* 3-52, *leu* 2-112, *his* 3-52, *trp* 1-1	Dr. J. Beggs, Edinburgh University
L6437 (n)	MAT**a**	*ura* 3-52, *leu*2::*his*G, *his*3::*his*G	Dr. G.R. Fink, Whitehead Institute for Biomedical Research, M.I.T.
L6438 (2n)	MAT**aa**	*ura* 3-52, *leu*2::*his*G, *his*3::*his*G	Dr. G.R. Fink, Whitehead Institute for Biomedical Research, M.I.T.
L6439 (3n)	MAT**aaa**	*ura* 3-52, *leu*2::*his*G, *his*3::*his*G	Dr. G.R. Fink, Whitehead Institute for Biomedical Research, M.I.T.
L6440 (4n)	MAT**aaaa**	*ura* 3-52, *leu*2::*his*G, *his*3::*his*G	Dr. G.R. Fink, Whitehead Institute for Biomedical Research, M.I.T.
CMBS	Allotetraploid	NA	Belgium lager strain collection
CMBS-45	Allotetraploid	NA	Generated during this study
CMBS-51	Allotetraploid	NA	Generated during this study
C5B	Allotetraploid	NA	Generated previously in lab of Dr. Bond, Trinity College Dublin
C5B-45	Allotetraploid	NA	Generated during this study
C5B-51	Allotetraploid	NA	Generated during this study
C6B	Allotetraploid	NA	Generated previously in lab of Dr. Bond, Trinity College Dublin
C6B-45	Allotetraploid	NA	Generated during this study
C6B-51	Allotetraploid	NA	Generated during this study
C10B	Allotetraploid	NA	Generated previously in lab of Dr. Bond, Trinity College Dublin
C10B-45	Allotetraploid	NA	Generated during this study
C10B-51	Allotetraploid	NA	Generated during this study
6701	Allotetraploid	NA	Guinness Strain collection
S. bayanus	MAT**a**		Collectio de Levuner d'Interet Biotechnologique, Paris, France

the vessel through an airtight stopper. This was used to remove 10ml samples throughout the fermentation.

The samples were transferred to pre-chilled 15ml tubes and the yeast cells were collected by centrifugation at 8000r.p.m for 5mins at 4°C. Attention was given so as to not over-expose the cells to air. The yeast pellet was resuspended in 2ml cold distilled water and centrifuged again. The cells were aliquoted into 2ml eppendorfs and quick frozen and stored at -70°C. The wort brix values were measured at room temperature at each time point.

2.3 Isolation of High Molecular Weight Yeast DNA.

Cells were grown to an optical density of 0.4 – 0.6 at 600nm and pelleted at 16000 x g for 5mins. Cells were resuspended in 500µl of sterile water and transferred to an eppendorf tube. Cells were pelleted at 16000 x g. for 5mins and the supernatant decanted and resuspended in the residual growth media by briefly vortexing. 200µl of a solution containing: 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8.0. 1 mM EDTA was added to the cells. 200µl phenol pH 8.0/ chloroform, 0.3g of 425-600 µM acid washed glass beads added and vortexed for 3 mins. 200µl of 1X TE was added and centrifuged at max speed for 7 mins. The aqueous layer is removed and phenol/chloroform extraction is performed on it again, (500µl). After the second extraction the aqueous layer is removed and placed into a fresh eppendorf tube and 1ml of 100% alcohol was added. The sample was mixed by inversion and centrifuged at maximum speed for 15 mins.

The resulting pellet is resuspended in 400µl 1xTE. 30µg RNAse A was added. The samples were incubated at 37°C for 15 mins. The phenol/chloroform extraction is repeated. To the final aqueous layer, 10µl of 4 M Ammonium acetate, 1ml 100% alcohol and 3µl glycogen were added and the sample mixed by inversion and placed at -70°C for 1 hour. The samples were centrifuged for 20 mins at 16000 x g. The resulting DNA pellet is washed in 70% alcohol. The pellet was air dried and resuspended in 50µl 1xTE and the optical density measured.

2.4 Determination of nucleic acid concentrations

The concentration of RNA and DNA samples were determined by measuring the absorbance at 260nm in an Eppendorf Bio Photometer. The samples diluted 1:500 in dH_2O and the optical density at 260nm measured using a Quartz cuvette and the concentration of the nucleic acid was determined using the conversion factors below:

OD_{260}nm value of 1 equals: 50µg/ml dsDNA

40µg/ml ssRNA

In respect to the concentration and purity of the RNA absorbance readings were take at both OD_{260}nm and OD_{280}nm. In general, the ratio of OD260 to OD280 was approximately 1.8.

2.5 Preparing Large Amounts Of Yeast Samples For Karyotyping.

The plug moulds (*Bio-rad)* were pre-cooled to 4°C and kept on ice prior to use. Low melting point agar (LMP) (200 mg LMP in 0.125 M EDTA), was melted and kept molten at 38°C in a water bath. 2ml of liquid culture were pelleted resulting in a cell pellet approximately 4 mm in size. The supernatant was removed. The pellet was resuspended in 500µl buffer D (0.05 M EDTA pH 8.0) and centrifuged for 3 mins at 3000 r.p.m. The supernatant was removed and this washing procedure was repeated twice. On the last wash the supernatant was removed by aspiration. The pellet was resuspended in 100µl buffer E, (0.5 M EDTA pH 8.0) and place in a 38°C water bath. 240µl of Zymolyase T20™, (20mg/ml in 1xTE, *Seikagaku Corporation Tokojo Japan*) was added to the LMP agar at 38°C. The yeast suspension was mixed with 125µl LMP agarose/ zymolyase solution by pipetting the mixture, and approximately 70µl of the mixture was quickly added to the pre-cooled moulds. The mould was left to set for 30 mins. 1ml of fresh LET buffer (15mg DTT per 10ml buffer E) was added to eppendorfs. After the agarose blocks had set, they were removed from the mould and the solidified blocks were placed in the eppendorf tubes containing LET buffer. The eppendorf tubes were incubated at 37°C for 1 hour, (up to a maximum of 4 hours). The LET buffer was removed by aspiration and replaced by fresh NDS buffer, (0.5mg Proteinase K,

30% N-lauroylsarcosine, buffer E). and incubated at 50°C for 16 hours. The eppendorf tubes containing the agarose blocks in NDS buffer were placed at 4°C for 1 hour, the NDS buffer was removed by aspiration and replaced with buffer F (1xTE), and washed twice in buffer F. The agarose blocks were put into fresh buffer F and left overnight at 4°C to allow the removal of residual salts. The next day the buffer was removed and replaced by fresh buffer F. The agarose blocks were stable for up to 6 months if stored at 4°C.

2.5.2 Running CHEF Gels: Separation of all Chromosomes.

The DR-3 System (*Bio-Rad*), pump and chiller units were switched on. The electrophoresis tank was filled with 2l 0.5X TAE and the temperature set to 14°C on the cooling unit. The running parameters were set, listed in Table 2.2. 1.5 g of PFGE agarose was added to 100ml 0.5X TAE and heated in the microwave till molten. Once cooled slightly it was poured into the gel casting rig and comb inserted, ensuring that there were no air bubbles. Once set the wells were filled with 0.5X TAE to prevent air bubbles and the DNA plugs inserted. The wells were sealed with molten agar and the gel was transferred from the casting stand to the tank and anchored ensuring no air was trapped under the gel and the run begun. After the electrophoresis, the gel was stained in ethidium bromide (10mg/ml) for approximately 15 mins and then destained in the used 0.5X TAE for 30 mins and viewed using a UV box (*Bio-rad*). Southern blots were preformed as described in section 2.6.

2.6 Southern Hybridisation Analysis

Genomic DNA was separated on 1.2% TAE (0.04 M Tris-Acetate, 1 mM EDTA pH 8.0) gel containing 0.5µg/ml ethidium bromide. The DNA fragments were identified by a brief exposure to UV intensity light.

Prior to transfer of the DNA to membrane the agarose gel was soaked in 0.25M HCL for 15 mins to depurinate the DNA, then rinsed twice in sterile water. The gels were then denatured (0.5 N NaOH, 1.5 M NaCl) for 15 mins and neutralised (0.5 M Tris-Cl pH 7.5, 1.5 M NaCl) for 15 mins and finally rinsed in sterile water

Table 2.2 CHEF gel settings

44hour gel runs parameters (For separation of all chromosomes).

Parameters	Block 1	Block 2
Initial Switch	60secs	90 secs
Final Switch	60 secs	90 secs
Runtime	28 hours	16 hours
Pulse Angle	120	120
Voltage	5.8V/cm	5.8V/cm
Flow/ Speed pump	70	70
Set temperature	14	14
Buffer volume	2l	2l

24hour gel runs parameters (For small chromosome separation only).

Parameters	Block 1
Initial Switch	35s
Final Switch	35s
Runtime	24hours
Pulse Angle	120
Voltage	5.8V/cm
Flow/ Speed pump	70
Set temperature	14
Buffer volume	2l

18 hour gel runs parameters (For small and mid weight chromosomes).

Parameters	Block 1
Initial Switch	50s
Final Switch	50s
Runtime	18hours
Pulse Angle	120
Voltage	6.0V/cm
Flow/ Speed pump	70
Set temperature	14
Buffer volume	2l

before transfer. The DNA was transferred to a nylon membrane (*Biodyne B 0.45μ, Pall Corporation)* with a minimum of 12 hours transfer time by capillary transfer using 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). Nucleic acids were cross-linked to the membrane using a UV light (program C3, setting 150mJoule, Genelinker *Bio-Rad*). Membranes were pre-hybridised with in a solution containing 7% SDS, 5X SSC, 2% blocking buffer (10% Casein (Sigma) in 1xMAB [Maleic Acid, pH 7.0 *Sigma]*), 0.1% N-lauroylsarcosine and 50 mM sodium-phosphate (pH 7.0) for 1 hour at 68°C. A fresh aliquot of hybridisation buffer containing 5 ng of denatured digoxigenin-UTP DNA probe (Section 2.14.1) was added and the membranes were incubated over night at 68°C in the hybridisation oven. Following hybridisation, the membranes were washed twice for 15 mins in 2X washing solution (2X SSC, 0.1% SDS) at room temperature, followed by two more washes in 0.5X washing solution (5X SSC, 0.1% SDS) for 15 mins at 50°C. Membranes were washed in wash buffer (1X MAB, pH 7.5, 0.3% (v/v) Tween 20) for 5 mins at room temperature. The membrane was then blocked for 1 hour in blocking buffer (1X MAB with 2% (w/v) Casein), followed by incubation with the antibody solution, (Anti-DIG-alkaline phosphate, *Roche*), diluted 1:10,000 in blocking buffer for 30 mins. Two more stringent washes in Wash Buffer were carried out for 15 mins at room temperature. The membrane was immersed in detection buffer (100 mM NaCl, 100 mM Tris-HCL pH 9.5) for 5 mins at room temperature. This was followed by chemiluminescent detection with CDP-Star (0.25 mM, *Sigma*). The membranes were then exposed to x-ray film, (Hyperfilm, *Amersham Biosciences*).

2.7 DNA extraction for Microarrays –Silica Paramagnetic Particles.

Cells at an optical density of 0.6 at 600nm were pelleted and washed twice in sterile distilled water. Cells were lysed using 10% SDS and the supernatant collected as described in section 2.3. 300μl of buffer A (1% Sorbitol, 100 mM Tris-HCL pH 8.0, 7 M Guanidine thiocyanate) was added and the samples were vortexed for 3 mins. Immediately after vortexing 40μl of silica magnetic particles *(Merck)* were added and the solution mixed by inverting the tube. The solution was incubated at 50°C for 10 mins with the tube flicked from time to time to keep

Table 2. 3 Primers used in Southern Hybridisation Analysis and Colony Hybridisation.

Oligonucleotides	Sequence 5' - 3'	Nucleotide Position
YPR159W FOR S. cerevisiae	TTCCCCGGATGGTATTTCTT	858967 - 858986
YPR159W REV S. cerevisiae	AACCAGCATCTTCCCAAGTAG	859656 - 859676
YPR159W FOR S. bayanus	CACGGATCAGATGTCGAAGA	838 – 857
YPR159W REV S. bayanus	ATTCGATGGCGTACTTTTGG	1725 – 1744
YPR160W FOR S. cerevisiae	TTACTATTTGTCTTTGGAGTTTTT	861667 - 861690
YPR160W REV S. cerevisiae	AACCTGCGTCCGGTTCTTGGTCCAAG	861826 - 861851
YPR160W FOR S. bayanus	CAACCACACCGTTATGCAAG	1287 – 1306
YPR160W REV S. bayanus	CTCCAAATCACTTGCGGGAAT	2191 – 2211
YPR161C FOR S. cerevisiae	TTTGATTGACCACAACGGTG	865797 - 865816
YPR161C REV S. cerevisiae	TGCCGCTGAAGCGATTATTT	864921 - 864940
YPR161C FOR S. bayanus	GAGGTGCCCTCGTATCTTGA	350 – 369
YPR161C REV S. bayanus	CGGTTGTCCTCCCAATCTAA	1290 – 1309
YPR162C FOR S. cerevisiae	GTGGGGCCCAGACAAAGTTA	867981 - 868000
YPR162C REV S. cerevisiae	CGATTTTATGGCAGTGCAGA	867221 - 867240
YPR162C FOR S. bayanus	CGTCGGGATCCTAGAATTGA	307 – 326
YPR162C REV S. bayanus	GAGAAAATCAGCAGCGGTTC	1064 – 1083
YPR175W FOR S. cerevisiae	TTGCATGGTTCTTGTGGAAG	889938 - 889957
YPR175W REV S. cerevisiae	TTGAGGATGGAACGTACTCCA	890987 - 891007
YPR175W FOR S. bayanus	AAACCTGGACTGGAGGGACT	477 – 496
YPR175W REV S. bayanus	AGAATAAATCAGCGCCGAGA	1194 – 1213
YPR184W FOR S. cerevisiae	TTCAGAGAGGCTAATGCAGGT	905652 - 905672
YPR184W REV S. cerevisiae	ATCATCTTCGTAGGCATCCCA	906624 - 906644
YPR184W FOR S. bayanus	GCCGCCAAAAACTACAACAT	542 – 561
YPR184W REV S. bayanus	TAATGGGTTCCCGTTCCATA	1476 – 1495
YPR186C FOR S. bayanus	ACCGTCTTCATCGCTCAGAT	358 – 377
YPR186C REV S. bayanus	GGAATAAGTCCCCATGCAAA	1240 – 1259

YPR189W FOR S. cerevisiae	CGGTTGATAGGCATTTTGGA	916140 - 916159
YPR189W REV S. cerevisiae	ACATTCGTTTAGCGCCTTCA	916933 - 916952
YPR189W FOR S. bayanus	GGGATTGCTCATCTGGAAAA	2147 – 2166
YPR189W REV S. bayanus	TGGATCGGACTGGGTATAGC	3016 – 3080
YPR190C FOR S. cerevisiae	GCAATGCGTTTAAGCCGTT	917510 - 917528
YPR190C REV S. cerevisiae	TGCCATTTGGAAAACTTCCC	917076 - 917095
YPR190C FOR S. bayanus	TTGGAAAACTTCCCAAAGTGA	10 – 30
YPR190C REV S. bayanus	TCCACACTCAGCTTCGTTGA	619 - 638

Nucleotide position for *S. cerevisiae* primers were derived from SGD website.
Nucleotide position for *S. bayanus* primers were derived from the Yeast Comparative Genomics site at the Broad Institute, MIT.
http://www.broad.mit.edu/cgi-bin/annotation/fungi/comp_yeasts/blast_page.cgi

the particles in suspension. Using a magnetic rack (*Merck*) the particles were pulled to the side of the eppendorf and any liquid was removed.

The tube was removed from the magnetic rack and the particles resuspended in 500µl buffer A, magnetized and the liquid discarded. This step was again repeated but using buffer B, (70% ethanol, 50 mM Tris-HCL pH 7.2, 1 mM EDTA).

The particles were allowed to air dry for 10 mins. 50µl of 1xTE (10 mM Tris-HCL pH 8.0, 1 mM EDTA) was added to the particles and the samples were incubated for 5 mins at 50°C. The beads were removed using the magnetic rack and the DNA containing supernatant was transferred to a fresh eppendorf and the optical density measured as described in section 2.4.

2.8 Preparation of Fluorescent labelled Yeast genomic DNA by Random Priming for use on DNA Microarrays

(Courtesy of C. Sather, Genomics Resource, Fred Hutchinson Cancer Research Centre, Seattle).

2.8.1 Random Priming of Sample DNA

For each sample, 300ng of high molecular weight genomic DNA was dissolved in sterile water to give a final concentration of 30ng/µl. 20µl of 2.5X Random Primer Mix (*Bioprime Kit Invitrogen*) was added and the DNA/Primer mix was denatured at 100°C 10 mins and immediately quenched on ice.

2.8.2 Labelling Reaction

The following reagents were added to the: 30µl DNA/Random Primer mix: 5µl 10 x dNTP Mix, 11µl sterile water, 3µl Cy3-dUTP or Cy5-dUTP, 1µl Klenow (40-50U/µl). The mix was incubated at 37°C overnight.

2.8.3 Removal of unincorporated label and the addition of blocking agents.

The Cy3- and Cy5- labelled samples were combined and 450µl of 1X TE added. The mix was transferred to a Microcon YM-30 concentrator and centrifuged at 10,000x g for 8 mins or until the volume was reduced to approximately 25µl. The following was added directly to the Microcon YM-30 concentrator containing the concentrated DNA sample: 10µl Yeast tRNA (10µg/µl) and 400µl 1xTE buffer. The column was centrifuged at 10,000 x g for 8 mins or until the volume was approximately 15µl. The column was transferred to a fresh eppendorf and the sample eluted by centrifuging at 10,000 x g for 2 mins.

2.8.4 Preparation of Samples for Hybridisation

The volume of the sample was adjusted to 22µl with sterile water. 4µl of 20x SSC was added and the sample filtered through a 0.45µm filter to remove particles. The sample was stored at -20°C in the dark until just prior to hybridisation.

2.9 Microarray Hybridisation and Wash Protocol.

(Courtesy of C. Sather, Genomics Resource, Fred Hutchinson Cancer Research Centre, Seattle).

2.9.1 Array (Slide) Examination

The Array Slide was pre-scanned with an Axon scanner at low resolution. The arrays were pre-hybridised in 3% milk solution for 1 hour.

2.9.2 Sample Preparation

The labelled samples were allowed to thaw. 0.6µl of 10% SDS was added and samples were heated at 99.9°C for 2 mins on a heating block. Then centrifuged at 14,000 r.p.m. for 3 mins and cooled to room temperature.

2.9.3 Hybridisation Set-up

The array slide was placed in the hybridisation chamber. 10µl of 3X SSC was added to the slide, away from the spotted array. The probe sample was added onto

the array area ensuring the pipette did not touch the surface. A cover slip was promptly added over the array and the hybridisation chamber was sealed. The arrays were incubated at 63°C for approximately 16 hours by submerging in a water bath.

2.9.4 Post-Hybridisation Washes

The array slide was removed from the hybridisation chamber and placed in a slide rack and submerged in wash 1, (1X SSC, 0.03% SDS) for 2 mins. The slide rack was then dipped 15 times in wash 2 (1X SSC) to ensure any excess SDS has been removed. The slide was then soaked and shaken at 60 r.p.m. (protected from light) for 20 mins in 0.2X SSC. The slide was again soaked and shaken at 60 rpm (protected from light) for 10 mins. During this process the slides were immediately transferred through the wash steps to avoid drying effects. The slides were then dried via centrifugation at 1000 r.p.m. for 5 mins. The arrays were scanned using a GenePix 4000B dual – laser scanner (*Molecular Devices*) and the data was extracted using GenePix 6.0 software.

2.10 Normalisation of Microarray Data.

Prior to the normalisation of the data, it was sorted using Microsoft Excel to remove any flagged spots i.e. spots that a value of 50 (absent spots) –75 (empty spots) and –100 (bad spots that are filtered out). The remaining conserved spots had a flag value of equal to or greater than 0. The data obtained from the DNA microarrays was normalised using the software program VARAN (www.bionet.espci.fr/varan/). Once the flagged spots were removed that data was formatted into a VARAN input file. VARAN accepts only one annotation column therefore the gene name was used. The data was saved as a test tabulated file and imported into VARAN. On the VARAN index page, VARAN generator was launched and normalisation performed following the on-screen links.

2.11 RNA extraction using the hot phenol method.

10ml aliquots of cells at an optical density of 0.6 at 600nm were pelleted and washed with 10ml of ice-cold sterile distilled water and centrifuged at 1470 x g

for 5 mins at 4°C. The pellets were resuspended in 500µl of ice-cold AE buffer (50 mM sodium acetate, pH 5.3, 10 mM EDTA) and 50µl of 10% (w/v) SDS and vortexed for 30 seconds. 550µl of AE buffered phenol pH 5.3 pre-warmed to 65°C, was added to the samples and the tubes vortexed for 30 seconds. The samples were incubated at 65°C for 30 mins with 10-second vortexing every 5 mins. The samples were centrifuged at 1470 x g for 5 minutes. The aqueous layer was removed extracted once more with an equal volume of AE buffered phenol (pH 5.3) and once more with an equal amount of chloroform. The aqueous layer was transferred to a fresh tube and the nucleic acid was precipitated at –70°C by adding 0.1vol 4 M sodium acetate pH 5.3 and 2.5 volumes ethanol.

The RNA was resuspended in 50µl DEPC-treated sterile distilled water and the nucleic acid concentration determined, see section 2.4.

2.12 Formaldehyde – containing agarose gel electrophoresis

RNA was separated on 1.2% (w/v) formaldehyde agarose gels. RNA samples of the desired concentration usually 30µg, were precipitated at -70°C with 0.1 vol Sodium Acetate, pH 5.3 and 2.5 vol 100% ethanol. The pellet was resuspended in the following denaturing loading buffer; 1µl DEPC- treated SDW, 3.5µl formaldehyde, 10µl formamide, 2µl 10X MOPS buffer (200 mM MOPS, 300 mM Sodium citrate pH 7.0). 3.5µl formaldhyde gel loading buffer (0.1% bromophenol blue, 0.1% Xylene cyanol, 50% (w/v) glycerol and 10 mM EDTA pH 8.0). The samples were mixed well and incubated at 65°C for 15 mins to denature the RNA, then quick chilled on ice prior to loading the gel. Gels were run overnight at 20V in 1X MOPS buffer (20 mM MOPS, 30 mM sodium citrate, pH 7.0) to ensure good separation of the RNA. The gels were subsequently blotted as described in section 2.13.

2.13 Northern Hybridisation Analysis

RNA was transferred from the agarose gel to a nylon membrane (*Biodyne B 0.45µ, Pall Corporation)* with a minimum of 6 hours transfer time by capillary transfer using 20X SSC (3 M NaCl, 0.3 M sodium citrate pH 7.0). Nucleic acids

were cross-linked to the membrane using a UV light (program C3, setting 150mJoule, Genelinker *Bio-Rad*). Membranes were pre-hybridised with hybridisation solution [7% SDS, 5X SSC, 2% blocking buffer, 0.1% N-lauroylsarcosine and 50 mM sodium-phosphate (pH 7.0)] for 1 hour at 68°C. a fresh aliquot of hybridisation buffer containing 5ng of denatured digoxigenin-UTP DNA probe, section 2.14.1, was added and the membranes were incubated over night at 68°C in the hybridisation oven. Following hybridisation, the membranes were washed twice for 15 mins in 2X washing solution (2X SSC, 0.1% SDS) at room temperature, followed by two more washes in 0.5X washing solution (5X SSC, 0.1% SDS) for 15 mins at 50°C. Membranes were washed in wash buffer (1X MAB, pH 7.5, 0.3% (v/v) Tween 20) for 5 mins at room temperature. The membrane was then blocked for non-specific binding for 1 hour in blocking buffer (1X MAB with 2% (w/v) Casein), followed by incubation with the antibody solution, (Anti-DIG-alkaline phosphate, *Roche*), and diluted 1:10,000 in blocking buffer for 30 mins. Two more stringent washes were carried out using wash buffer for 15 mins at room temperature. The membrane was immersed in detection buffer (100 mM NaCl, 100 mM Tris-HCL pH 9.5) for 5 mins at room temperature. This was followed by chemiluminescent detection with CDP-Star (0.25 mM, *Sigma*). The membranes are then exposed to x-ray film, (Hyperfilm, *Amersham Biosciences*).

2.13.2 Northern Blot Stripping

Membranes were washed twice in DEPC-treated water for 10 mins at room temperature and then incubated in stripping solution, [DEPC-treated water, 0.1% SDS (w/v)] for 1 hour at 68°C in a hybridisation oven. The solution was changed after 30 mins. Following stripping, the membrane was rinsed in DEPC-treated water and then in 2X SSC. After the washes the membranes went through a repeat hybridisation procedure.

2.14 DNA amplification by polymerase chain reaction.

When necessary the amplification of DNA products was carried out by polymerase chain reaction. The recombinant form of the enzyme *Taq* DNA

Table 2.4 Primers used in Northern Hybridisation Analysis.

Oligonucleotides	Sequence 5' - 3'	Nucleotide Position
YCR005C FOR	AAGGCAAAAGGAAGCTCACA	122010 - 122029
YCR005C REV	CTTTGACTCGCTTTCCAAGG	121808 - 121827
YCR031C FOR	CTTAACGTGAACGGCAGTGA	177664 - 177683
YCR031C REV	CCCAAGTTTTTGGTGTTGCT	177857 - 177878
YCR046C FOR	TCAAGCGGTCCTTGTTCTTT	210209 - 210228
YCR046C REV	AGCAGGAACGTCAGATTGCT	210394 - 210413
YCR057C FOR	CCGCTCGCAGATAATAGAGC	220805 - 220824
YCR057C REV	GCGCTACGAGAAAAGCAGTT	221021 - 221040
YCR077C FOR	AACGCATGATGTGGACGTTA	250549 - 250568
YCR077C REV	CGTTTCGTTCTTGACCACCT	250747 - 250766
TDH3 FOR	CTGTCAAGTTGAACAAGGAAACCAC	883061 – 883085
TDH3 REV	CAACGTGTTCAACCAAGTCGACAA	882828 – 882851
5.8s RNA FOR	CGGATCTCTTGGTTCTCGC	464678 – 464696
5.8s RNA REV	TGACGCTCAAACAGGCATG	464555 – 464573

Nucleotide position for *S. cerevisiae* primers were derived from SGD website.

polymerase from the thermophilic eubacterium *Thermus aquaticus* BM *(Promega)* was routinely used for the generation of labelled DNA probes. This enzyme is free of non-specific endo – or exonucleases and is highly processive 5' – 3' DNA polymerase. When DNA amplification was necessary for cloning or PCR products to be sequenced *Pfu* polymerase was used, which harbours a 3' – 5' proof reading exonuclease activity resulting in reduced error rate compared to *Taq* DNA polymerase.

25µl reactions containing 1X *Taq* DNA polymerase buffer (50 mM KCl, 10 mM Tris-HCL pH 9.0, 0.1% Triton x-100, *Promega*), 2.5 mM $MgCl_2$, 2 mM of each dNTP, 0.5pmol of each oligonucleotide primers, 1U *Taq* DNA polymerase (*Promega*) and 25 ng of the desired DNA template were carried out in 0.2ml PCR tubes using a Perkin Elmer Thermo Cycler. Sterile water was added to obtain a final volume of 25µl. Reaction were subjected to the following conditions:

Initial denaturation at 94°C for 5 mins
Denaturation at 94°C for 30 sec
Annealing (at the appropriate temperature) 1 min
Extension at 72°C for 1 min
Final extension at 72°C for 10 min

2.14.1 Amplification of Digoxygenin-UTP labelled DNA probes.

Labelled DNA probes were generated by the incorporation of the Digoxigenin-UTP during a standard PCR amplification. PCR labelling was carried out using *Taq* DNA polymerase as described in section 2.4 with the exception of the dNTP mix which was replaced by a DIG probe synthesis mix (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.3 mM dTTP and 0.7 mM alkali-labile DIG-11-UTP, pH 7.0).

2.15 cDNA synthesis and Polymerase Chain Reaction amplification of cDNA

2.15.1 Purification of RNA

RNA samples were treated with DNase I to remove any contaminating DNA before proceeding with RT-PCR. RNA (30µg) was incubated with 0.02U RNasin

ribonuclease inhibitor, 1X DNase I buffer (40 mM Tris HCl pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$, *Promega*) and 10U DNase I (*Promega*) in a final volume of 50µl at 30°C for 1 hour. This was followed with a further purification of the RNA using the RNeasy mini kit (*Qiagen*), described briefly herein. The lysate buffer (350µl) (*Qiagen*) was added to the RNA sample followed by 250µl of 100% ethanol. The mixture was added to an RNeasy column and centrifuged for 15 secs at 13,200 r.p.m. The flow through was discarded and 500µl of RPE (wash) buffer (*Qiagen*) was added to the column and centrifuged. The flow through discarded and this washing step was repeated. The empty column was centrifuged again to remove any residual wash solution. The column was transferred to a fresh eppendorf and 30µl DEPC-water was added to the column and after 1min incubation at room temperature, was centrifuged for 1 min at 16000 x g. The concentration of the RNA was then determined as described in section 2.4.

2.15.2 Reverse Transcriptase of RNA

1.5µg of RNA samples were used in each reverse transcription reaction. The RNA was reverse transcribed in a mixture containing 1X ImProm-II buffer (*Promega*), 6 mM $MgCl_2$, 0.5 mM dNTP mix, 1 mM reverse primer, 0.02U RNasin ribonuclease inhibitor (*Promega*) and 0.5µl ImProm-II reverse transcriptase (*Promega*) and nuclease free water to a final volume of 20µl. Samples were incubated at the following; 70°C for 5 mins, chilled 4°C for 5 mins, 25°C for 5 mins, 42°C for 1 hour followed by inactivation at 70°C for 15 mins. The cDNA product was amplified as described below.

2.15.3 Amplification of DNA by Polymerase Chain Reaction (PCR)

PCR reactions were carried out in a final volume of 25µl containing 0.2mM dNTPs, 1X Mg free buffer, (20 mM Tris HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.004U Taq polymerase (*NEB*), 400 nM forward primer, 400 nM reverse primer and 10ng of the desired DNA templates or 2µl of the cDNA prepared as outlined above, using a GeneAmp PCR System 2700 machine. The primers used are listed in Table 2.4.

Table 2.5 Primers used in RT-PCR.

Oligonucleotides	Sequence 5' - 3'	Nucleotide Position
Universal FOR†	CAAGGAGACTTACGGGGTTTC	3803 - 3823
Universal REV†	TCACGAATACTCATCGAAGCA	4005 - 4025
Experimental FOR†	CAAGGAGACTTACGGGGTTTC	3803 - 3823
Experimental REV†	TTGGTTCTCCCTTTGAGGC	4188 - 4206
YPR161C FOR‡	GAGGTGCCCTGGTATCTTGA	350 - 369
YPR161C REV‡	GAAAACCGTCGCACCACCATC	542 - 562
YPR175W FOR‡	AAACCTGGACTGGTGGGACT	477 - 469
YPR175W REV‡	CGCTGTTTTGGAAGTTCTCG	687 - 706
YPR186C FOR‡	ACCGTCTTCATCGCTCAGAT	358 - 377
YPR186C REV‡	AGCCTTGTGTAGGGGAAAATG	579 - 599
Ampicillin FOR*	AATGCTTAATCAGTGAGGCA	7949 - 7968
Ampicillin REV*	TCACAGAAAAGCATCTTACG	8469 - 8488

†Nucleotide position was derived from sequence data, with nucleotide 1 representing the start site of YPR159W ORF.

‡Nucleotide position for *S. bayanus* primers was derived from sequence available at Yeast Comparative Genomics, Broad Institute, MIT, with nucleotide 1 representing the start codon in each ORF.

*Nucleotide position was derived from the available pYAC55 vector sequence.

The reaction was performed using the following amplification parameters which consisted of an initial denaturing of 95°C for 8.5 mins, 30 cycles of denaturing 95°C for 30 secs, annealing at 60°C (depending on Tm of primer set) for 1 min, extension at 72°C for 45 secs and a final extension of 72°C for 7 mins. PCR products were subsequently electrophoresised on a 1.2% TBE agarose gel with 1X TBE running buffer.

The *Wallace rule* was used to calculate the melting temperature for short oligonucleotides (<20 nucleotides). A, T, G, and C refer to the base composition of the oligonucleotides.

Tm = 2*(A+T) + 4*(G+C)
The annealing temperature was then set to 5°C below that of the Tm.

2.16 Real Time PCR

Total genomic DNA was prepared as per section 2.5 and serial dilutions were prepared to give a range of DNA concentrations from 100 – 2ng/µl. 7.5µl of 2x SYBR® Green Jumpstart™ *Taq* ReadyMix™ (*Sigma,* S4438), (20 mM Tris-HCL pH 8.3, 100 mM KCl, 7 mM $MgCl_2$ 0.4 mM each dNTP, 0.05U/µl *Taq* DNA polymerase), was added to 4µl DNA template and 2µl of primers (10 µM of each forward and reverse primer). Tubes were briefly centrifuged to eliminate any bubbles. The real time PCR was performed on a Robocycler machine (*Corbett*) under the following conditions: 94°C for 2 mins followed by 40 cycles of 94°C for 15 secs, 55°C for 60 secs and 72°C for 60s ecs.

Prior to performing the quantitative analysis of the data, the efficiency of the reaction (Eff) was determined by plotting the data on a graph with a fitted line to determine the slope. The formula Eff = $(10^{-1/slope})$-1 was used. Data sets that had an efficiency of less than 0.98 were discarded.

The fluorescence data was analysed by quantitative analysis to calculate the threshold value, (Ct). This involves comparing the C_t values of the samples of interest with a control sample. The C_t values of both the control and the samples

Table 2.6 Real-time PCR primer sets.

Oligonucleotides	Sequence 5' - 3'	Nucleotide Position
YDL171C FOR (NADH - Housekeeping gene)	GTTTAGGGGCCAAATCCAAT	152744 – 152763
YDL171C REV (NADH - Housekeeping gene)	GCTTAGCACCCTGAGCAATC	152581 – 152600
YAL051C FOR	CCCCGTCGATATAATTGGTG	127749 – 127769
YAL051C REV	TTGTGTGGAACCCAACTGAA	127485 – 127504
YBR134W FOR	AAGCCTGGTTTGACACCAAC	504244 – 504263
YBR134W REV	ATCACAAATGGAACCCGAAA	504520 – 504263
YCR105W FOR	GCAAAAGTGACAACGAGCAA	309383 – 309402
YCR105W REV	ATACCACCGATGCCAACAAT	309622 – 309641
YFL053W FOR	CCACAAACCTGAAGCCATTT	24034 - 24053
YFL053W REV	CCTCTGCAGCATAACCCAAT	24262 - 24281
YGR097W FOR	GAACGCTCCCGTTGTACCTA	681674 - 681693
YGR097W REV	CGCTATTATTGCCCCGTTTA	681890 - 681909
YHL047C FOR	AGGTATGCTGCTGGAGCTGT	9715 - 9734
YHL047C REV	AAAATGAACGCCCACATAGC	9511 - 9530
YJR154W FOR	CACGATTCCTGTGGGAACTT	726435 - 726454
YJR154W REV	GCAAGGCTTCCAATGGTAAA	726647 - 726666
YPL056C FOR	ATGAAGAACGCAGCATTGTG	453713 - 453732
YPL056C REV	AGAGAAGAGACGCAGCAAGC	453532 - 453551

Nucleotide position for *S. cerevisiae* primers were derived from SGD website.

of interest are normalized to an appropriate endogenous housekeeping gene, (NADH). The comparative C_t method is also known as the $2^{-[delta][delta]Ct}$ method, where $[delta][delta]C_t = [delta]C_{t,sample} - [delta]C_{t,control}$ Here, $[delta]C_{T,sample}$ is the C_t value for any sample normalized to the endogenous housekeeping gene and $[delta]C_{t,\ control}$ is the C_t value for the control also normalized to the endogenous housekeeping gene. For the $[delta][delta]C_t$ calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal.

2.17 Generation of total Genomic DNA Library.

High molecular weight DNA from the lager strain CMBS-33 was isolated, as described in Section 2.3.

2.17.1 Partial Cleavage of Genomic DNA.

10µg of total genomic DNA was mixed with 10µl of 10X *Sau*3A buffer (10 mM Bis-Tris-Propane-HCL, pH 7.0, 10 mM $MgCl_2$, 1 mM Dithiothretiol) and the volume adjusted to 100µl with sterile water. 2µg was dispensed into a sterile eppendorf tube (tube 1) and 1µg into additional tubes (tubes 2-9) and placed on ice. The enzyme was diluted to give a final concentration of 1unit per 1µl in buffer (10 mM Bis-Tris-Prpane-HCL, pH 7.0, 10 mM $MgCl_2$, 1 mM Dithiothretiol). 1µl of enzyme was added to tube 1 and mixed (0.5U per µg), 10µl was removed and mixed with the contents of tube 2. The serial dilution was continued until tube 8. Tube 9 was kept as an undigested DNA control. The eppendorfs were incubated at 37°C for 1 hour. The reaction was stopped with the addition of 20 mM EDTA. The samples were separated on a 1.5% TAE gel at 2V per cm to give improved resolution of high molecular weight DNA.

2.17.2 Ligation of Genomic DNA into the CopyControl pCC1Bac Cloning-Ready Vector.

Size fractionated *Sau*3A digested genomic DNA was ligated into the CopyControl pCC1BAC cloning Ready Vector (*Epicentre*), (Fig. 2.1), the protocol is described herein. The following were mixed in an eppendorf, 1µl CopyControl pCC1BAC

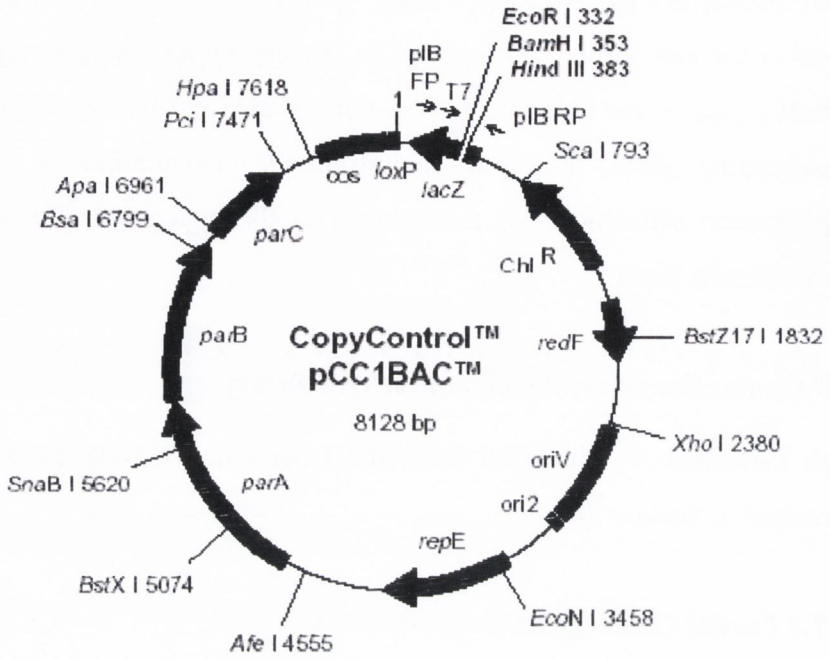


Figure 2.1: CopyControl pCC1BAC Vector. The vector contains the following features, chloramphenicol resistance as an antibiotic selectable marker, and both a single-copy and high-copy oriV origin of replication. Not I sites surrounding the BamH I cloning site

Cloning-Ready Vector (25ng/µl, pre-cut with *BamH*I), 100ng partially digested genomic DNA fragments made up to a final volume of 87µl with sterile water and incubated at 55°C for 10 mins. The solution was then allowed to cool to room temperature for 15 mins. To the cooled solution, the following were added; 1X Fast-Link Ligation buffer, 10 mM ATP, 2µl Fast-Link DNA Ligase. The ligation mixture was incubated at 16°C for 4 hours. The reaction was then heated to 65°C for 15 mins to inactivate the Fast-Link DNA Ligase. The ligation reaction was desalted using an agarose cone on ice for 1 hour.

2.17.3 Transformation and Selection of CopyControl BAC Clones

1ml of SOC medium (Tryptone 2% (w/v), Yeast extract 0.5% (w/v), 8.6 mM NaCl, 2.5 mM KCl, $MgSO_4$, 20 mM Gluose) not containing an antibiotic was prepared for each electroporation to be performed. Electroporation cuvettes and 1.5ml eppendorf tubes were pre-chilled on ice. The TransforMax EPI300 Electrocompetent *E. coli* cells (*Epicentre*) were allowed to thaw on ice. 2µl of the desalted ligation reaction and 50µl of cells were added to a pre-chilled eppendorf, the rest of the ligation reaction was stored at 4°C. The cells and DNA were mixed via pipetting and transferred to an electroporation cuvette, ensuring no air bubbles were present. An electrical pulse was applied at the following parameters 2.5V,125µFd (resistance) and 200 Ohms. Immediately after electroporation, 950µl of SOC medium was added to the cuvette and mixed. The cells were transferred to a 15ml tube and incubated at 37°C with shaking for 1 hour. This allowed for the cells to recover and for expression of the antibiotic resistance marker. 100µl of the transformation reaction was plated on LB containing chloramphenicol (12.5µg/ml), X-GAL (40µg/ml) and IPTG (0.4mM) plates and incubated overnight at 37°C.

2.17.4 Sizing the CopyControl BAC Clones

25µl of EpiLyse solution (*Epicentre*), was added to eppendorfs for each CopyControl BAC clone that was to be sized. Individual clones were picked from chloramphenicol plates since clones contain single copy number plasmids an entire colony was required from the overnight plates to obtain enough BAC DNA

to be seen on the agarose gel. The solution was mixed via pipetting to completely resuspend the cells. 10µl of EpiBlue Solution (*Epicentre*) was added to each tube and mixed then centrifuged briefly. 20µl of each sample was loaded onto a 1% TAE agarose gel along with 10µl of BAC-Tracker Super-coiled DNA ladder (*Epicentre*). The gel was run at 4.5V/cm for 3 hours.

Once the approximate size of the DNA had been determined the remaining 900µl of the transformed cells were plated onto selective media. Recombinant clones (white) were individually picked and inoculated into wells on a 96 well micro-titre plate containing L-Broth and chloramphenicol (12.5µg/ml) and glycerol (20% v/v) to allow for freezing and further investigation. To obtain a representative genomic DNA library, approximately 4800 clones were required; this is based on the haploid genome size of *S. cerevisiae* of $1.2x10^7$ bp and the brewery strain having 4-6 times more DNA than that of the haploid strain. Therefore with an insert size of 100kb (10^5 bp) then 480 clones will represent 1 genomic equivalent and in general 10 genome equivalents are required to ensure a 99.9% chance of cloning a given sequence.

2.18 Characterisation of BAC Library

2.18.1 Colony Lifts

Nylon membrane was laid on top of chloramphenicol/ X-GAL/ IPTG plates and clones from the 96 well plates were replica-plated directly onto the membrane. The plates were grown over night at 37°C. The membranes and plate were marked for orientation. The piece of nylon membrane, cell side up was placed over 4 sheets of blotting paper soaked successively in the following solutions: 10% SDS for 3 mins, 0.5 N NaOH, 1.5 M NaCl for 5 mins, 0.5 M Tris-Cl, 1.5 M NaCl pH 7.4 for 5 mins and finally 2xSSC for 5 mins. The membrane DNA side-up was then left to air dry for 30 mins and UV cross-linked.

2.18.2 Colony Hybridisation

Hybridisation analysis was achieved using standard methods as described for Southern blotting techniques, Section 2.4, with the exception of the pre-

hybridisation step, which was increased to 2 hours at 68°C. Following hybridisation, with a selection of DIG-dUTP labelled DNA probes, listed in Table 2.3, positive clones were identified, isolated and grown in 2X YT media (Tryptone 16g, Yeast extract 10g, NaCl 5g in 1l) supplemented with chloramphenicol (12.5µg/ml) at 37°C overnight.

2.19 Isolation of BAC DNA

For BAC minipreparations, 3ml of LB media (Luria - Bertain) containing chloramphenicol (12.5µg/ml) was inoculated with a single colony and incubated with shaking at 37°C overnight. The overnight culture was transferred to a 1.5ml eppendorf tube and centrifuged at 16000 x g, at 4°C for 3 mins. The supernatants were removed. The cell pellets were resuspended by vortexing in 150µl of solution I (50 mM Glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0). Then 200µl solution II (0.2 N NaOH, 1% SDS) was added and mixed by inversion. The solution was left to stand at room temperature for 5 mins until translucent. Then 150µl of ice cold solution III (0.5M Potassium acetate, 7 mM glacial Acetic Acid) was added and the tubes placed on ice. After a 15 min incubation on ice, the tubes were centrifuged at 16000 x g at 4°C for 10 mins. The supernatant was removed to a fresh 1.5ml tube and 400µl of phenol/chloroform (1:1) was added and mixed by vortexing. The tubes were centrifuged at 13,000 r.p.m. for 5 mins. The supernatant was carefully removed and transferred to a new eppendorf. 750µl of prechilled 100% ethanol was added into the supernatant and mixed by vortexing.

The samples were centrifuged at 13,000 r.p.m. at 4°C for 10 mins. The supernatant was discarded and the pellet washed in 70% ethanol. The pellets were dried and 20µl of sterile distilled water added to the dried pellets. The samples were incubated at 37°C to dissolve the DNA.

2.20 Sequencing

Sequencing of PCR products and direct sequencing from BAC clones was preformed at GATC, Constance, Germany. Specific primers were designed and

Table 2.7 Primers used in YPR159 – Intergenic – YPR160 Sequencing Reactions.

Oligonucleotides	Sequence 5' - 3'	Nucleotide Posit
YPR159_P3 FOR	ATACTGGACAGCCCCTGATG	1041 - 1060
YPR159_P3 REV	TTTGCAAGGATTGAGAGGCTA	1538 - 1558
YPR159_P4 FOR	TCATCCGAATCAAGGTGTTG	1455 - 1474
YPR159_P4 REV	CGCATGTCACGGATATAGCA	1989 - 2008
YPR159_P5 FOR	CTTGGTATGAATTCGGTGAG	1694 - 1713
YPR159_P5 REV	GGATAACTTGAACTTCGAGC	2138 - 2157
YPR159-INTERGENIC_P1 FOR	CTGAACGATGACGACAATGG	1750 - 1769
YPR159-INTERGENIC_P1 REV	TTATCCAGGAGCGCCTCTAA	2576 - 2595
YPR159-INTERGENIC_P2 FOR	TGTGTTTGCTCGCTCATCTT	2714 - 2733
YPR159-INTERGENIC_P2 REV	GGAAGGAACACCGACGAATA	3439 - 3458
INTERGENIC FOR	TTATCCAGGAGCGCCTCTAA	2576 - 2595
INTERGENIC REV	GTAGCGGCATCGCCTAGTAG	3183 - 3202
INTERGENIC_P2 FOR	CAACGGAAGAGGAAGCTCTG	3229 - 3248
INTERGENIC_P2 REV	CCGTCAGTCTCCTGGTCAAT	3798 - 3817
INTERGENIC-YPR160 FOR	GGAAGGAACACCGACGAATA	3439 - 3458
INTERGENIC-YPR160 REV	TTTTGATGGGTAGGGCTTTG	4109 - 4128
YPR160_P1 FOR	GAGACTGACGCTGTGGAACA	3807 - 3827
YPR160_P1 REV	GACGACCTAGCCCACCATTA	4287 - 4306
YPR160_P2 FOR	TCAAAGGGAGAGCCAAGAGA	4189 - 4208
YPR160_P2 REV	AAACTCGGTGGTTGGTCTTG	4646 - 4665
YPR160_P3 FOR	CACAATGGATTGGTGGTGAA	4547 - 4566
YPR160_P3 REV	CTTGCATAACGGTGTGGTTG	5010 - 5029
YPR160_P4 FOR	ATGCAAGAAGCCTTGGAAAA	5361 - 5380
YPR160_P4 REV	GCTTGACTTGGTTCCATTCC	5945 - 5964
YPR160_P5 FOR	GGAATGGAACCAAGTCAAGC	5918 - 5937
YPR160_P5 REV	CTCAAAGCACTTGCGGGAAT	6412 - 6431

YPR160_P6 FOR	TTGCAAAGAAATTCCCCAAG	5126 - 5145
YPR160_P6 REV	CGCTTCTGTACCAGCAGTTG	5944 - 5964
YPR160_P7 FOR	CGTTACTTGGCAATGAAAAATATG	5674 - 5697
YPR160_P7 REV	AGTCACTGGTTCGACGTTCC	6419 - 6438

Nucleotide position was derived from sequence data obtained through this study, with nucleotide 1 representing the start site of YPR159W ORF through to the stop codon on YPR160W.

used in the reactions, as listed in Tables 2.7 and 2.8. BAC DNA used in sequencing reactions was isolated as described in section 2.19.

2.21 Generation of chromosome XVI pYAC55 library in ploidy yeast strains.

2.21.1 Recovery of Insert DNA from BAC Clone

BAC DNA was isolated as described in section 2.17. To isolate the genomic DNA inserts, 0.6µl *Not*I (10U/ µl) (*New England Biolabs*), 1µl 10x buffer for *Not*I (50 mM Tris – HCl pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol) and 0.5µl sterile distilled water was added to 8µl of BAC clone DNA. The mix was incubated at 37°C for 2 hours for digestion. After the digestion the mixes were loaded onto a 1% CHEF gel and run under the conditions of 6.0V/cm, 5-15s pulse, 14°C with a 16 hour runtime. The following day the gel was removed and the bands visualised with ethidium bromide staining. The bands of insert DNA were excised from the gel and electroeluted from the gel slice into dialysis bag. This DNA was then ethanol precipitated as described in section 2.3 and used in the ligation reactions.

2.21.2 Ligation

Prior to the ligation, the vector, pYAC55 (Figure 2.2), was digested with *BamH*I, which cleaved the DNA to expose DNA telomeres at the end of the DNA sequence. The two telomere sequences flank a *HIS3* gene on the vector, which is removed following digestion. In addition to this feature the cloning site is located in a gene called *sup4-O*. *Sup4-O* is a suppressor tRNAtyr gene that reads the stop codon UAA as a tyrosine. This gene has a natural *Sma*I site, that is used to clone the large DNA fragments. If a DNA fragment is inserted in this site the sup4-o gene is disrupted and cannot suppress the UAA stop codon anymore. When an empty YAC is transformed into a yeast host that carries a modified *ADE2* gene with a UAA stop codon in its sequence, the sup4 gene product suppresses the stop codon and the yeast make a functional *ADE2* gene product. The cells are white. If a DNA insert has disrupted the *SUP4* gene, the cells do not make *ADE2* and the cells are pink. This is a simple colour test to see if the YAC carries an insert in the cloning site.

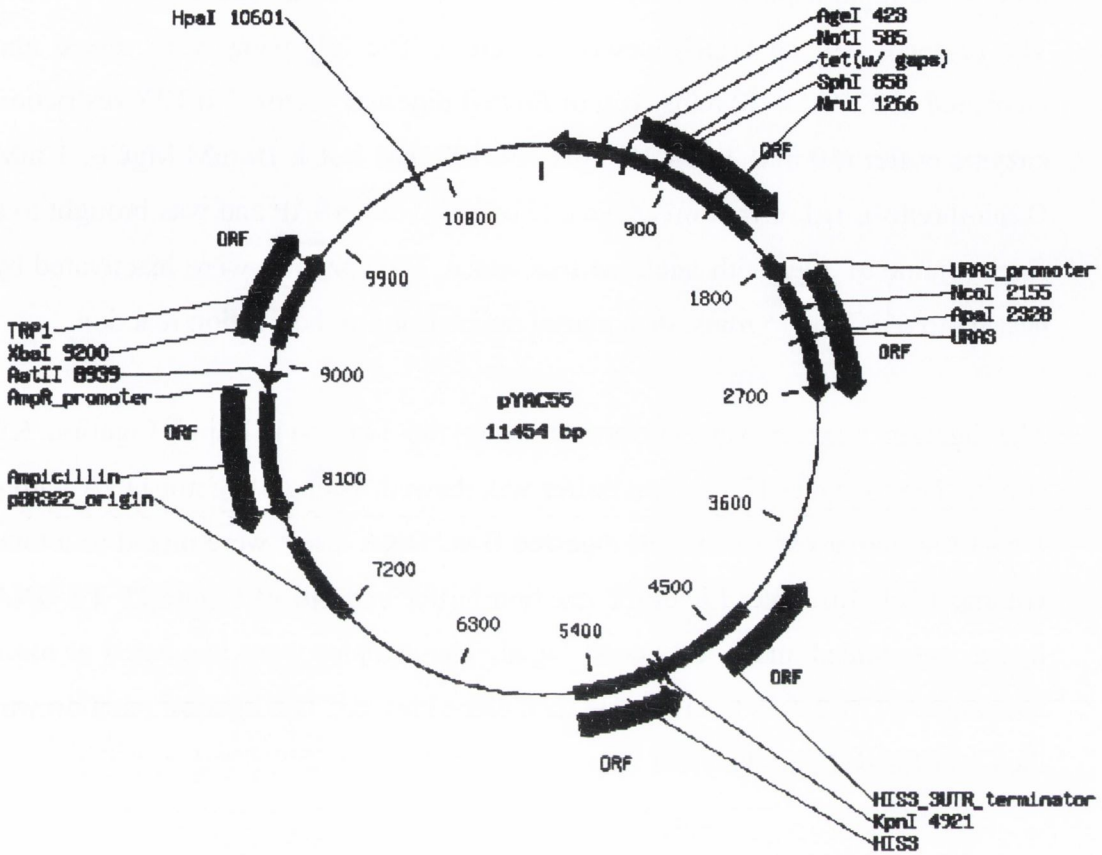


Figure 2.2: pYAC55 vector. YACs contain three essential parts, a centromere, an ARS (autonomously replicating sequence)and two telomeres. The telomeres are not true teleomeres and are generated by cleaving the sequence that leaves a teloemere seeding sequence on the end of the DNA. The HIS3 gene is removed by BamH1 digestion to expose the telomere seed sequences.

Following *BamHI* digestion, the vector was further treated using SuperSAP (*USB*). SuperSAP is an enhanced formulation of Shrimp Alkaline Phosphatase used in the rapid dephosphorylation of DNA prior to being used in cloning work. The protocol will be briefly described herein. The following were mixed and incubated at 37°C for 30 mins, 1µg of *BamHI* digested vector, 3µl 10X restriction enzyme buffer (50 mM Tris – HCl pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol), 1µl restriction enzyme (*NotI*), 1µl SuperSAP and was brought to a final volume of 30µl with nuclease free water. The enzymes were inactivated by heating to 65°C for 15 mins, then placed on ice prior to the ligation reaction.

The ligation reaction was performed using the Ligate-IT Rapid Ligation Kit (*USB*). The 5x ligate-IT reaction buffer was thawed. 100ng of vector DNA with a 1-to-3 fold molar excess of *NotI* digested BAC DNA insert were mixed to a total volume 15µl. 4µl of 5x Ligate-IT reaction buffer and 1µl of Ligate-IT T4 DNA ligase were added and briefly centrifuged. The samples were incubated at room temperature (20-25°C) for 10 mins, then placed on ice. The ligation reaction was then transformed into the yeast strains.

2.21.3 Electroporation

A 50ml YEPD culture was inoculated with a single colony and grown with shaking at 30°C to early stationary phase. The ploidy strains used and their phenotype are listed in Table 2.1. The cells were harvested and pelleted and kept on ice through the protocol. Cells were washed first in 40mls of ice-cold sterile water, with the wash repeated with 20mls of sterile water. The cells were then resuspended in 5ml of 1 M Sorbitol (ice-cold) and pelleted. The cells were resuspended with 150µl 1 M ice cold Sorbitol and placed on ice. 40µl of the yeast suspension was mixed with 5µl of DNA (~5µg) in a prechilled electroporation cuvette, ensuring there were no air bubbles. The cuvette was given one pulse: V= 1.5kV, 25µF, 200Ohms. Immediately 1ml 1 M Sorbitol (ice cold) was added and the solution transferred to an eppendorf. The cells were plated on URA^- selective media. Individual colonies were picked and grown in YEPD broth for DNA and RNA extractions.

2.22 Yeast FACS.

Cells were grown to an optical density of less than 1, so as to be in exponential phase. 500µl of the cell culture was pelleted and the supernantant was discarded. The cell pellet was resuspended in 300µl of sterile water and the cells resuspended with vortexing. Immediately 700µl of 95% alcohol was dropwise added and the cells were briefly vortexed again. The cells were left in incubate at 4°C overnight. The cell pellet was centrifuged for 5 minutes at 15,000rpm and the supernantant was discarded and the cell pellet was resuspended in 1ml of 50mM citrate buffer pH 7.4. The cells were sonicated for 10 secs at 5 amplitude microns on a Soniprep sonicator (MSE). The cells were pelleted and the supernantant removed and resuspended in 1ml of 50mM citrate buffer pH 7.4 with RNase (0.25mg/ml) and incubated for 2hrs at 50°C followed by the addition of 100µl of Proteinase K (10mg/ml) and a futher incubation at 50°C for 2hrs. The cells were pelleted and the supernatant discarded and the cell pellet resuspeneded in 1ml 50mM citrate buffer with propidium iodide (8µg.ml) and incubated at room temperature for 30mins.The cell sorting was carried out on a Beckman Coulter Excis XL Facs analysis machine and analysis was performed using Beckman Coulter Expo 32 ADC Software.

Chapter 3
Characterisation of the Mosaic Chromosomes of the lager yeast CMBS-33.

3.1 Introduction.

The lager yeasts are thought to have arisen from a natural genome fusion of two yeast strains *S. cerevisiae* and possibly *S. bayanus* or another related species. The two genomes either co-exist as separate entities or their individual chromosome homeologs may have recombined and are maintained as hybrids. Indeed classical genetic studies using the technique of single chromosome transfer from lager strains to well defined haploid *S. cerevisiae* concluded that lager yeast genomes consist of *S. cerevisiae* chromosomes, non-*S. cerevisiae* and mosaic chromosomes consisting in part of *S. cerevisiae* and non-*S. cerevisiae* genes as discussed in detail in Chapter 1. Analysis of individual genes in many lager strains of yeast has revealed at least two or more copies of most genes, one closely related to the equivalent gene in *S. cerevisiae* with the other showing a higher degree of divergence, i.e. *S. cerevisiae*-like (*Sc*-like) and non-*S. cerevisiae*-like chromosomes. The overall analysis of the lager yeast genome indicates that the genomes are polyploid.

While some information of the genetic content of lager strains has been obtained little is known about the organisation of the genome or the copy number of *S. cerevisiae* and non-*S. cerevisiae* genes. Initially DNA microarray technology was used to obtain information on the gene copy number of the *S. cerevisiae* like genes in the lager strains. Here competitive genomic hybridisations were carried out with differentially labeled DNA from a lager yeast strain and a haploid *S. cerevisiae* strain. Hybridisation to the *S. cerevisiae* microarrays depends on the degree of complementarity and the number of copies of each gene present in the genome. Using stringent hybridisation conditions which do not allow binding to the non-*S. cerevisiae* genome, the copy number of each individual *S. cerevisiae* gene can be deduced. The data obtained from this work showed that there are dramatic shifts in the gene copy number at specific locations in eight of the sixteen chromosomes, (Fig. 1.3). The areas where 'jump' locations are present may represent sites of intra- and inter - chromosomal translocations between the *S. cerevisiae* like chromosomes or the homeologous chromosomes. Using this

method, Bond *et al*, 2004 showed that the 'jump' regions in some cases align with known Ty elements, ARS sites or recombination sites, as discussed in chapter 1.

The full sequential organisation of genes in the lager strains at present is unknown, sequencing and alignment of closely related genomes of *S. bayanus* and *S. paradoxus* indicated that the genome organisation and sequential order of the ORFs in the sixteen chromosomes are conserved within the species, in general.

The goal of this project is to contribute to the current knowledge of the genomic organisation of lager brewery strains of yeast. Chromosome composition of the lager yeasts has been determined using pulse field gel electrophoresis (PFGE). While chromosomes with similar electrophoretic characteristics to those present in *S. cerevisiae* were evident, the chromosome composition was more complex than that of the haploid *S. cerevisiae*.

The specific aims of this chapter are to characterise the chromosomal rearrangements in the CMBS-33 lager strain, specifically those associated with chromosomes III, VIII and XVI. Secondly in order to characterise the regions where rearrangements have occurred a total genomic library of the CMBS-33 genome was prepared. The region surrounding a specific rearrangement event occurring on chromosome XVI at YPR159W was cloned and sequenced, with a view to determining if novel genes arose at recombination sites.

3.2 Results

3.2.1. The composition of hybrid chromosomes

The method of PFGE coupled with Southern blot analysis experiments can provide a gross picture of the chromosome composition in the lager strains of yeast and is therefore a pre-requisite for a more focused study of the fine details of the chromosomes. Southern blotting of the PFGE gels using probes to genes on either side of the recombination sites was carried out, to determine if the rearrangements identified by CGH analysis represented sites of chromosomal translocations between *S. cerevisiae* like chromosomes or inter-chromosomal recombination between homeologus pairs.

3.2.1.1 Chromosome III.

Previous work with a number of lager strains, (Bond *et al.*, 2004), utilising competitive genomic hybridisation (CGH) revealed that the ratio of hybridisation for genes on chromosome III varied on either side of the MAT locus. For the strain 6701, genes to the left of the MAT locus show an average ratio of hybridisation of 0.5, while genes to the right show a ratio of hybridisation of 1.7, (Fig. 1.3). Likewise, for the strain CMBS-33, genes to the left of the MAT locus had an average ratio of hybridisation of 1.0, while genes to the right had a ratio of hybridisation of 1.7. Subsequent analysis by real-time PCR indicated that the ratio of hybridisation is directly proportional to the *S. cerevisiae* gene copy number as the non-*S. cerevisiae* like genes do not hybridise under the conditions used. Using the relationship between ratio of hybridisation and gene copy number, it was possible to deduce that the 6701 and CMBS-33 strains contained one copy and two copies of *S. cerevisiae* like genes to the left of the MAT locus respectively, while there were four copies of the *S. cerevisiae* like genes to the right of the MAT locus in both strains. Thus suggesting that a recombination event has occurred at the MAT locus in the two strains. A number of possible events could explain these results. Firstly, recombination between homeologus chromosome III could have occurred leading to a number of mosaic chromosome IIIs.

Alternatively sections of chromosome III to the right of the MAT locus may have recombined with another chromosome or linear amplification of this region may have occurred. To distinguish between these possibilities and to confirm the CGH data, PFGE analysis was performed followed by Southern blotting using DNA probes to either side of the MAT locus.

A representative 24hour CHEF gel is shown in Figure 3.1a, this separation was used as it gives a good resolution of the low molecular weight chromosomes. The three smallest chromosomes I, VI and III are clearly separated on this gel. The gel was transferred to a nylon membrane and probed with a DIG-labelled DNA probes to YCR007C which lies to the left of the MAT locus, (Fig. 3.1B). The same membrane was stripped following the hybridisation and was re-probed with a DIG-labelled probe for the region YCR047, a region that lies to the right of the MAT locus, (Fig. 3.1C). The results show an approximate equal intensity of hybridisation with the YCR007 probe with both DNA from the haploid *S. cerevisiae* strain, (Fig. 3.1B lane 1) and the lager strain CMBS, (lane 2). Hybridisation with the YCR047 probe shows a greater degree of hybridisation to CMBS DNA, (Fig. 3.1C, lane 2) compared to the signal obtained from *S. cerevisiae* DNA, (Lane 1). Thus the observed hybridisation on CHEF gels confirms the CGH data and the increase in gene copy number for *S. cerevisiae*-like genes to the right of the MAT locus. Furthermore probes to either side of the MAT locus hybridise to the same chromosome band indicating that genes on either side of the MAT locus are contiguous. From, this it is possible to ascertain the chromosome type and the minimum number of chromosome III in the lager strain CMBS-33, (Fig. 1.4B).

3.2.1.2 Chromosome X

CGH analysis also revealed a change in the ratios of hybridisation on chromosome X at the ORF YJR009C, that was specific to the lager strain 6701. The ratio of hybridisation changed from 0.9 to 1.3 in 6701 strain while CMBS-33 showed a constant ratio of 1.1 for chromosome X. To examine the composition of chromosome X in CMBS, CHEF gels were electrophoresised for 18hours, a

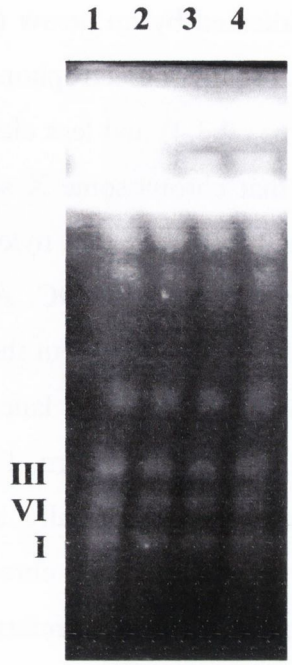


Figure 3.1A

Figure 3.1B

Figure 3.1C

Figure 3.1A: 24 hours CHEF gel (short protocol) that separates only low molecular weight chromosomes. Lane 1: *S. cerevisiae* strain S150 Lane 2: CMBS-33 lager strain, Lane 3: 6701 lager strain, Lane 4: DT Lager strain

Figure 3.1B: Southern blot of 24hour gel probed with DIG-dUTP labelled YCR007. Lane 1: *S. cerevisiae* strain S150, Lane 2: CMBS-33.

Figure 3.1C: Southern Blot of 24hour gel probed with DIG dUTP labelled YCR047 Lane 1: *S. cerevisiae* strain S150, Lane 2: CMBS-33.

protocol that separates chromosomes ranging in size from 225 – 825Kbp. The separated chromosomes are shown in Figure 3.2a. The position of chromosome X on the EtBr stained gel in *S. cerevisiae* is indicated by an arrow (lane 1), one distinct band is apparent. Chromosomes in this region of electrophoresis are more diffuse in the three lager strains examined, (lanes 2,3,4) and less clearly defined. In the lager strains, it was previously shown that chromosome X separates into three bands, (Casey *et al.,* 1988). The gel was transferred to a nylon membrane and probed with DIG-labelled DNA to the region of YJR009C. At least three distinct bands are apparent in both strains 6701 and CMBS-33 but the intensity of hybridisation varies between the two strains, (Fig. 3.2B lanes 2 and 3, respectively). The CMBS-33 strain shows a more diffuse pattern. The difference in intensity of hybridisation between the three bands especially band 2 again suggests the presence of both *S. cerevisiae* and mosaic-type chromosomes. A third lager strain, which was isolated from a lager bottle and is referred to here as strain DT. This strain appears to contain just two hybridising bands.

3.2.1.3 Chromosome XVI.

The microarray data for chromosome XVI, (Bond *et al.,* 2004) is shown in the schematic in Figure1.5a. The data shows an area of over 30 genes where there is a significant lack of hybridisation to the *S. cerevisiae* microarrays. The ratio of hybridisation between the lager strains and the haploid *S. cerevisiae* strain falls to less than 0.2, encompassing the region YPR160W to YPR190C. This region lies directly before the telomere. The lack of any significant hybridisation indicates a high sequence divergence in this region or possibly that the whole region has been deleted from the two lager strain's genomes.

To distinguish between these two possibilities, lager yeast chromosomes were electrophoresised using the 44 hour protocol, which separates all chromosomes in yeast. In total 25 bands can be distinguished in the lager strains ranging in molecular weight from 225 – 2220Kbp with chromosome XVI having a molecular weight of 1020Kbp. From the ethidium bromide staining of the gel it is difficult to see if there is any variance in the chromosome, as the higher molecular

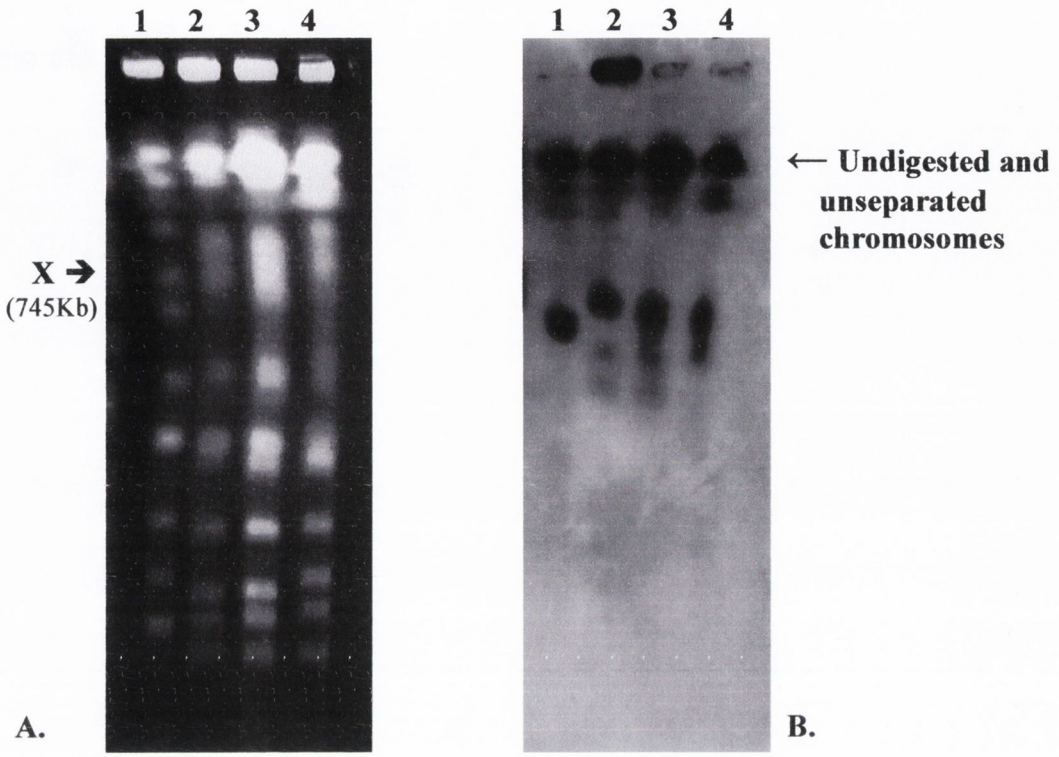


Figure 3.2 A: 18hour CHEF gel.
Lane 1: *S. cerevisiae* strain S150, Lane 2: 6701 lager strain, Lane 3: CMBS-33 lager strain, Lane 4 DT Lager strain. The position of chromosome X is indicated by an arrow.

Figure 3.2 B: Southern blot of 18hr CHEF gel probed with YJR009C.
Lane 1: *S. cerevisiae* strain S150, Lane 2: 6701 lager strain, Lane 3: CMBS-33 lager strain, Lane 4 DT Lager strain. The three hybridising bands are indicated by the numerals 1,2 and 3.

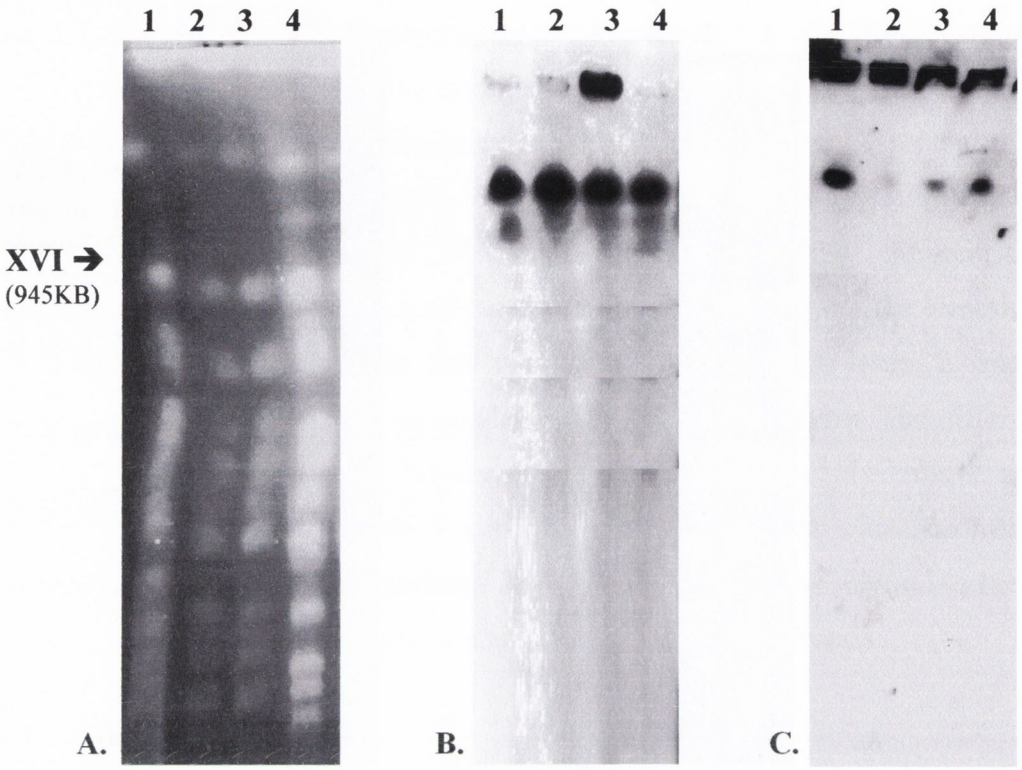


Figure 3.3 A: 44hour CHEF gel which separates all chromosomes.
Lane 1: *S. cerevisiae* strain S150, Lane 2: 6701 lager strain. Lane 3: CMBS-33 lager strain. Lane 4: DT lager strain.

Figure 3.3 B: Southern blot of 44 hour gel using DIG- dUTP labelled YPR159W probe.
Lane 1: *S. cerevisiae* strain S150, Lane 2: 6701 lager strain. Lane 3: CMBS-33 lager strain. Lane 4: DT lager strain.

Figure 3.3 C: Southern blot of 44 hour gel using DIG- dUTP labelled YPR160C probe.
Lane 1: *S. cerevisiae* strain S150, Lane 2: 6701 lager strain. Lane 3: CMBS-33 lager strain. Lane 4: DT lager strain.

weight chromosomes are difficult to visualise. The gel was transferred to a nylon membrane and first probed with DIG-labelled DNA to the YPR159W ORF, (Fig. 3.3B). Hybridisation is observed with all of the lager DNA samples (Fig 3.3B lanes 2 – 4), as well as to the *S. cerevisiae* DNA. The intensities of the hybridising band in 6701 and CMBS-33 is greater than that observed in the *S. cerevisiae* and DT samples despite the under loading of DNA in these samples, (compare lanes 2 and 3 with lanes 1 and 4, Fig. 3.3A). This indicates that a higher copy number of YPR159W is present in the two lager strains. When the DNA was probed with DIG labelled DNA for the *S. cerevisiae* YPR160C ORF the hybridisation signal for the lager strains, is much less intense than that observed for the *S. cerevisiae* laboratory strain. The Southern blot results suggest that there has been significant sequence divergence in this region of chromosome XVI in the lager strains. However, there have been no gross chromosomal rearrangements in the case of chromosome XVI, as both YPR159W and YPR160W hybridise to the same band.

3.2.1.4. Construction of BAC Library.

To characterise the area of sequence divergence observed in the region of YPR160W – YPR190W at a nucleotide resolution it was necessary to generate a genomic library of CMBS DNA. The BAC vector (pCC1BAC, Epicentre) is designed to allow cloning of large segments of DNA up to 100Kbps size inserts, (Fig 2.2). To ensure complete coverage of the genome a library size of approximately 4800 clones was required, based on the genome size of *S. cerevisiae* times four to allow for the tetraploid nature of the lager yeast genome, times ten to ensure a 99% chance of recovering the fragments of interest; in fact 7000 clones were recovered, as described in the methods section. This unamplified library was arrayed in 96-well micro-titre plates. The arrayed BAC genomic library was screened using a pool of probes to ORFS on either side of the recombination site, (Table 2.2). The screening of the library was carried out under firstly high stringency and then low –stringency conditions, through the variation of hybridisation temperature and the washing steps, (Section 2.18). Previous reports have indicated that the *S. cerevisiae* genome component of the lager strains is approximately 98% homologous to the published *S. cerevisiae*

sequence while the non-*S. cerevisiae* genome is approximately 85% homologous. Therefore through using different stringency conditions it was possible to identify clones representing the non-*S. cerevisiae*-type DNA. The set of pooled probes resulted in signals of different intensities, (Fig. 3.4), which implies one of two things. Firstly the clones only contain part of the region of interest or alternatively that *S. cerevisiae* and non-*S. cerevisiae* DNA is present in the clones.

Colonies that gave a positive signal from the colony hybridisation were picked and DNA isolated as described in section 2.19. The DNA was electrophoresised on a 1.2% gel (Fig.3.5A). The DNA was probed with individual DIG-labelled DNA probes, (YPR159W, YPR160W, YPR175, YPR189 and YPR190), to screen out false positive clones and to determine if any of the clones contained the entire region on interest from chromosome XVI, (Fig 3.5 B – F). Clone A11 from plate 27, herein referred to as P27A11, was shown to be true positive clones containing an insert with the region of low hybridisation on chromosome XVI and was used in further analysis of the BAC clone.

3.2.2 Sequencing of Junctions

To examine the region on chromosome XVI where recombination has occurred between the two homologous chromosomes, clones were analysed at a nucleotide level by DNA sequencing.

Initial sequencing attempts of the BAC clone was unsuccessful so a new rationale was designed. This involved amplifying the region of YPR159W to YPR160W including the intergenic region into small PCR fragments with specific primers either derived from the *S. cerevisiae* and/or the *S. bayanus* gene databases, (Table 2.2) and then in a stepwise manner sequencing each PCR fragment. Each section was designed so as to overlap the previous and subsequent section by approximately 50 – 100bps as seen in the schematic in Figure 3.6. After the first round of sequencing was completed, it was noted that certain region had not been sequenced such as the ATG site for YPR160W, therefore a revised strategy was developed. Using these initial sequencing results specific primers were designed

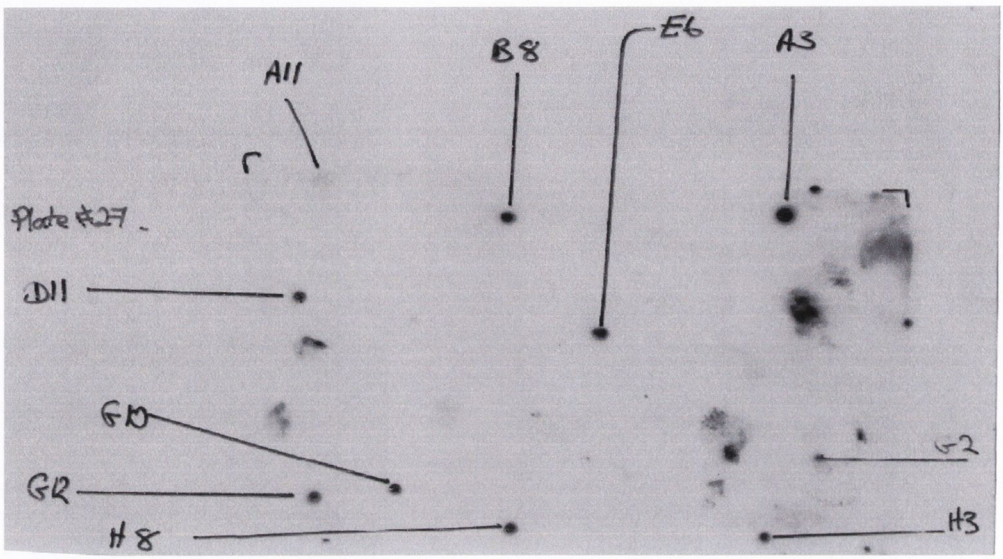


Figure 3.4: Colony lift from plate 27 which under went Southern hybridisation analysis using a mix of probes from Chromosome XVI.

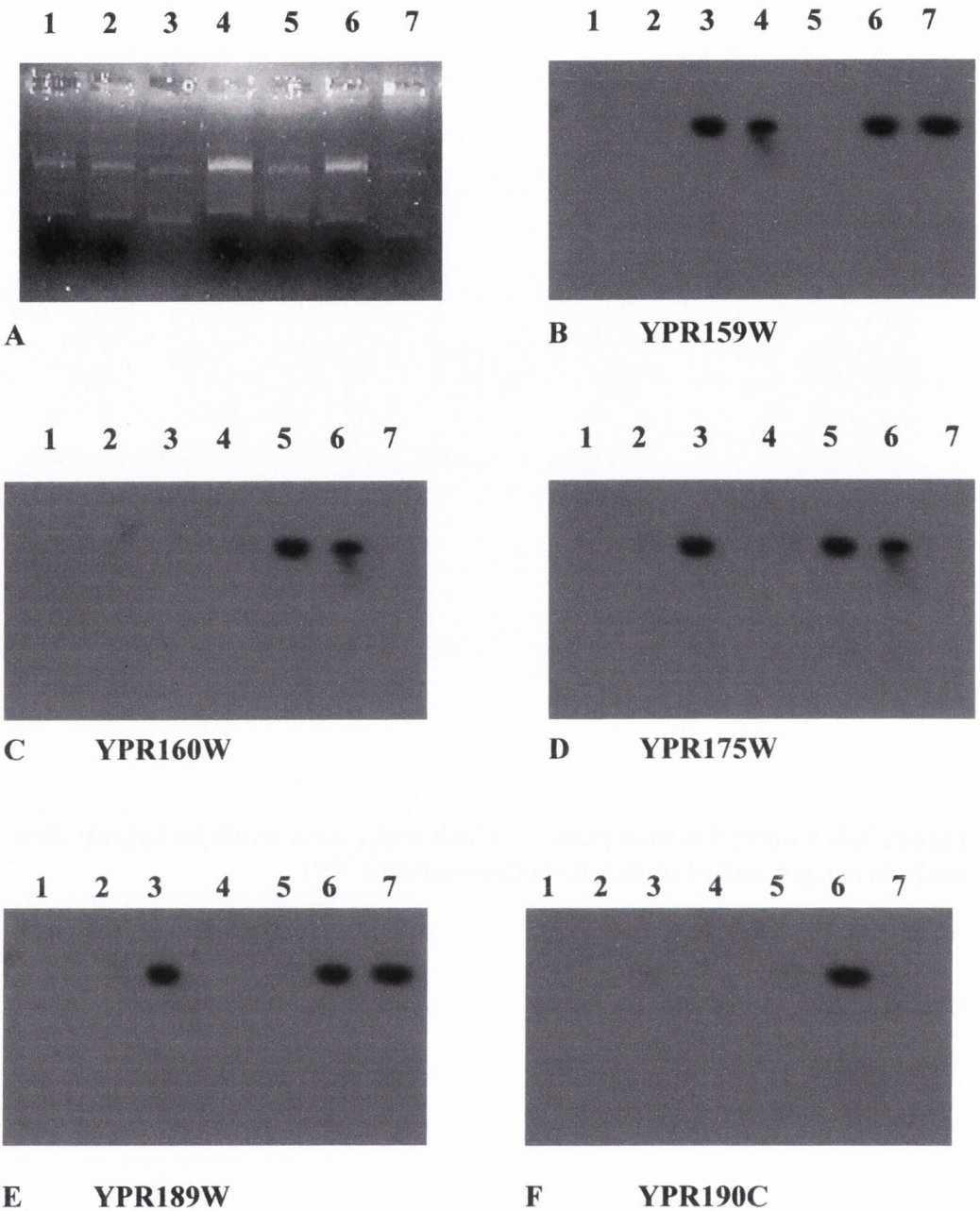


Figure 3.5A: Isolated BAC DNA from Plate 27 on 1.2% EtBr stained agarose gel. Lane 1:E6, Lane 2:G2, Lane 3:H3, Lane 4:D11, Lane 5:H8, Lane 6:A11, Lane 7:B8.

Panel B: Southern blot of gel probed with YPR159W DIG-dUTP labelled probe.
Panel C: Southern blot of gel probed with YPR160W DIG-dUTP labelled probe.
Panel D: Southern blot of gel probed with YPR175W DIG-dUTP labelled probe.
Panel E: Southern blot of gel probed with YPR189W DIG-dUTP labelled probe.
Panel F: Southern blot of gel probed with YPR190C DIG-dUTP labelled probe.

and a second round of sequencing was performed, directly from the BAC clone, therefore further reducing the chances of sequencing errors, introduced from PCR amplification.

The revised sequencing strategy is shown in Figure 3.7, only new regions sequenced are shown in this figure. Sequences obtained for the region of YPR159W were not re-sequenced. Analysis of the sequences obtained for the regions of YPR159W confirmed that the sequence was almost identical to the YPR159W *S. cerevisiae* sequence, (99%), as available of the *Saccharomyces* Genome Database, (www.yeastgenome.org), as can be seen in Table 3.1 with the exception of P3B where the percentage identity drops to 92%. This did not result in a change in the protein coding sequence. From this work the ORF YPR159W in the lager yeast genome is *S. cerevisiae*-like and the sequence has not diverged. The deduced sequence of the CMBS YPR159W gene is shown in Figure 3.8.

Following from the examination of the ORF YPR159W, the intergenic region was sequenced. This is a region 1561bp in length in the *S. cerevisiae* genome. Originally, it was proposed that a recombination event between the *S. cerevisiae* and *S. bayanus* genomes may have occurred in this region. The intergenic region was divided into four sections to which *S. cerevisiae*–like primers were designed for the initial sequencing, (Fig 3.6). The deduced sequence was then aligned against both the *S. cerevisiae* and *S. bayanus* genomes, the percentage identities for these alignments can be seen in Tables 3.2A and 3.2B. Each region was sequenced in duplicate or triplicate. As can be seen from this data, the intergenic region up to and including the first 375bp of the ORF YPR160W show a stronger percentage similarity to the *S. cerevisiae* genome than to the *S. bayanus* genome.

The initial sequencing of the ORF YPR160W was performed on PCR products obtained from DNA isolated from the BAC clone, the percentage identities for these sequences can be seen in Tables 3.3A and 3.3B. After this had been completed it was clear that this method was not satisfactory as there were gaps in the regions sequenced and the start site of the ORF had not been sequenced. However the data does indicate that the ORF shows a higher percentage identity

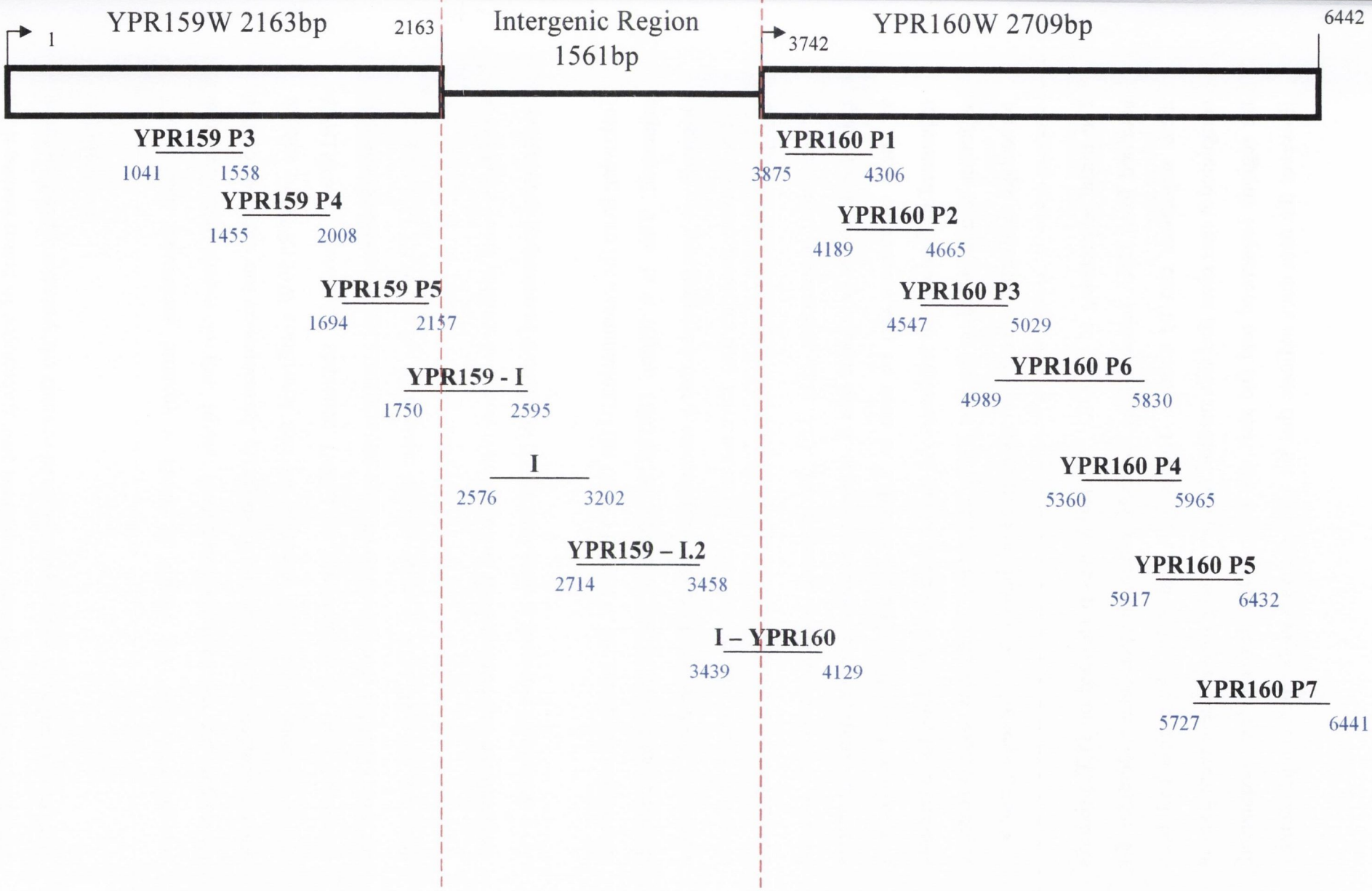


Figure 3.6: Schematic representing initial sequencing outline. The names above each line refers to the primer pair used for amplification. (Table 2.6). The numbers below refer to the nucleotide position from the start of YPR159W which is set at 1.

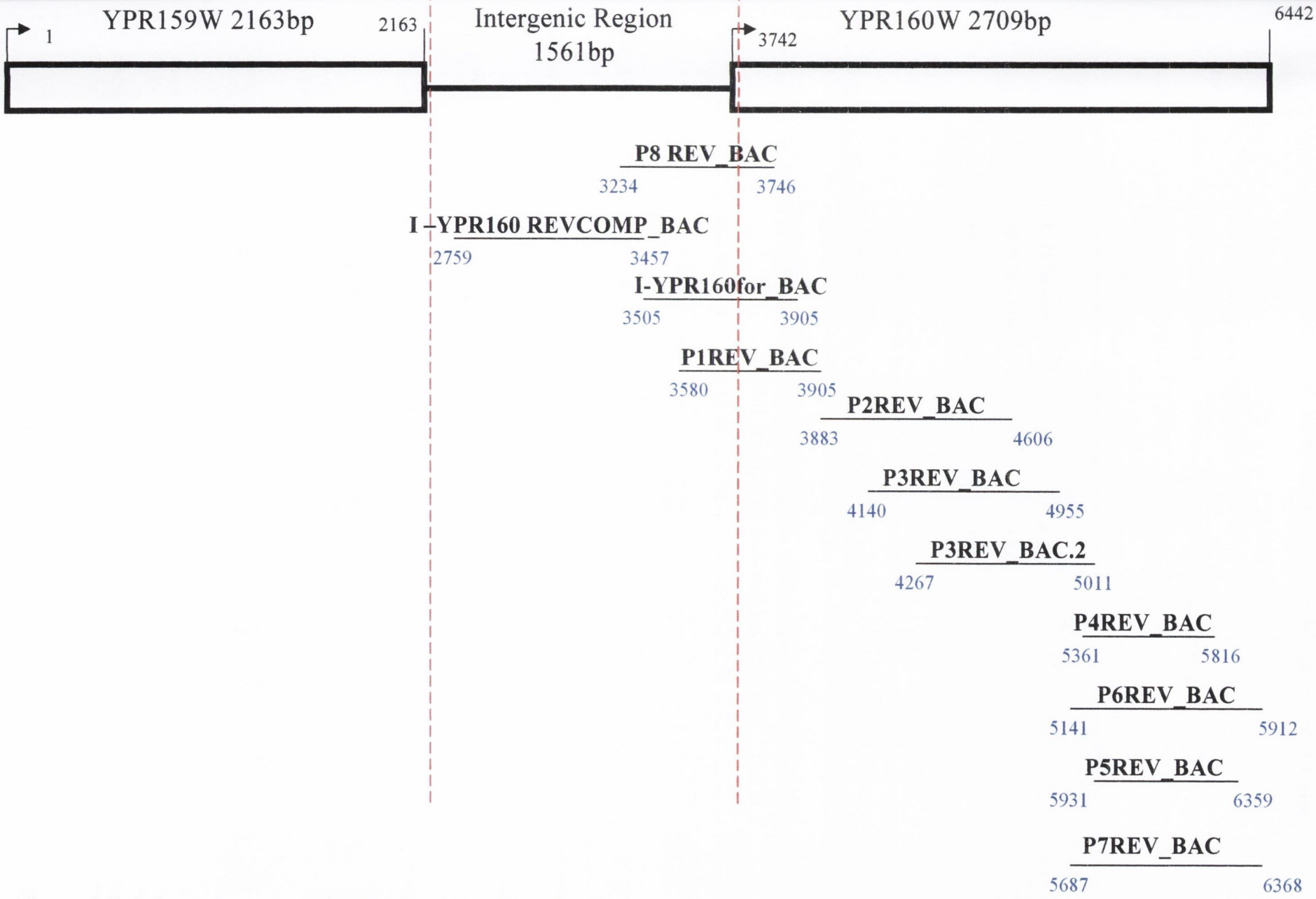


Figure 3.7: Schematic representing BAC sequencing rationale.

Table 3.1 Percentage Identities for YPR159W.

		% Identities	Co- ordinates *
YPR159W	**P3 A**	99	858742 - 859133
	P3 B	92	858713 - 859118
	P3 C	98	858683 - 859133
	P4 A	99	859090 - 859574
	P4 B	99	859102 - 859586
	P4 C	99	859090 - 859598
	P5 A	99	859328 – 859734
	P5 B	99	859326 – 859734
	P5 C	99	859326 – 859734

co-ordinates in relation to SGD website (www.yeastgenome.org).
A, B and C represent independent sequencing runs.

Figure 3.8: Alignment of *S. cerevisiae* YPR159W sequence against the Lager specific YPR159W sequence.

The alignments begins from nucleotide 1164 (858742, SGD co-ordinates) to the stop codon.
The two mismatched nucleotides at positions 1642 and 1668 are highlighted in red.

```
Lager           ----------------------CTTATATTACAGATCAGGTATGTTGCAGAGTTGGAAT
S.c.            ATGGATGCCTTTAAGAACCATGGCTTATATTACAGATCAGGTATGTTGCAGAGTTGGAAT
                                      ************************************

Lager           AAGGTTTGTTTCACGCAGGGTGCTCTAGAAATCTCTGCAAATCTACCAAATTATGGCCGT
S.c.            AAGGTTTGTTTCACGCAGGGTGCTCTAGAAATCTCTGCAAATCTACCAAATTATGGCCGT
                ************************************************************

Lager           GTTTCAGGTTTGTGGCCCGGTCTTTGGACTATGGGTAATTTAGGGAGGCCCGGTTATTTG
S.c.            GTTTCAGGTTTGTGGCCCGGTCTTTGGACTATGGGTAATTTAGGGAGGCCCGGTTATTTG
                ************************************************************

Lager           GCTAGTACCCAAGGTGTCTGGCCTTATTCTTATGAATCGTGTGATGCTGGTATTACACCC
S.c.            GCTAGTACCCAAGGTGTCTGGCCTTATTCTTATGAATCGTGTGATGCTGGTATTACACCC
                ************************************************************

Lager           AACCAAAGTTCCCCGGATGGTATTTCTTACTTGCCGGGACAAAAGTTAAGTATTTGTACT
S.c.            AACCAAAGTTCCCCGGATGGTATTTCTTACTTGCCGGGACAAAAGTTAAGTATTTGTACT
                ************************************************************

Lager           TGTGATGGTGAAGATCATCCGAATCAAGGTGTTGGTAGAGGAGCTCCAGAAATTGATGTT
S.c.            TGTGATGGTGAAGATCATCCGAATCAAGGTGTTGGTAGAGGAGCTCCAGAAATTGATGTT
                ************************************************************

Lager           TTGGAAGGTGAAACTGATACTAAGATTGGTGTAGGTATAGCCTCTCAATCCTTGCAAATT
S.c.            TTGGAAGGTGAAACTGATACTAAGATTGGTGTAGGTATAGCCTCTCAATCCTTGCAAATT
                ************************************************************

Lager           GCACCTTTTGATATCTGGTACATGCCTGACTATGATTTTATCGAGGTTTACAATTTCACA
S.c.            GCACCTTTTGATATCTGGTACATGCCTGACTATGATTTTATCGAGGTTTACAATTTCACA
                ************************************************************

Lager           ACAACGACCATGAACACTTATACTGGCGGTCCATTTCAACAAGCCGTGTCTGCTGTCTCA
S.c.            ACAACGACCATGAACACTTATGCTGGCGGTCCATTTCAACAAGCCGTATCTGCTGTCTCA
                ********************* ************************* ************

Lager           ACTTTGAACGTAACTTGGTATGAATTCGGTGAGTACGGTGGCTACTTTCAAAAATATGCC
S.c.            ACTTTGAACGTAACTTGGTATGAATTCGGTGAGTACGGTGGCTACTTTCAAAAATATGCC
                ************************************************************

Lager           ATTGAGTATCTGAACGATGACGACAATGGTTATATCCGTTGGTTTGTTGGTGATACTCCA
S.c.            ATTGAGTATCTGAACGATGACGACAATGGTTATATCCGTTGGTTTGTTGGTGATACTCCA
                ************************************************************

Lager           ACTTATACTATTCATGCTAAAGCCTTACATCCTGATGGTAATATTGGTTGGAGAAGAATC
S.c.            ACTTATACTATTCATGCTAAAGCCTTACATCCTGATGGTAATATTGGTTGGAGAAGAATC
                ************************************************************

Lager           AGTAAAGAACCAATGTCGATTATTCTAAATCTGGGTATTTCCAACAATTGGGCTTATATT
S.c.            AGTAAAGAACCAATGTCGATTATTCTAAATCTGGGTATTTCCAACAATTGGGCTTATATT
                ************************************************************

Lager           GATTGGCAGTATATTTTCTTCCCAGTGGTTATGTCGATTGACTATGTTAGAATATACCAA
S.c.            GATTGGCAGTATATTTTCTTCCCAGTGGTTATGTCGATTGACTATGTTAGAATATACCAA
                ************************************************************
Lager           CCAAGTAATGCTATATCCGTGACATGCGATCCAAGTGACTATCCAACATATGATTATATT
S.c.            CCAAGTAATGCTATATCCGTGACATGCGATCCAAGTGACTATCCAACATATGATTATATT
                ************************************************************
```

Figure 3.8 cont'd.

```
Lager           CAATCGCACTTAAATGCATTCCAGAATGCAAACTTAACTACTTGGGAAGATGCTGGTTAC
S.c.            CAATCGCACTTAAATGCATTCCAGAATGCAAACTTAACTACTTGGGAAGATGCTGGTTAC
                ************************************************************

Lager           ACGTTCCCTAAGAATATCCTAACTGGTAAATGTACTAGCTCGAAGTTCAAGTTATCCTCT
S.c.            ACGTTCCCTAAGAATATCCTAACTGGTAAATGTACTAGCTCGAAGTTCAAGTTATCCTCT
                ************************************************************

Lager           TAA
S.c.            TAA
                ***
```

Table 3.2A Percentage Identities for the Intergenic Region of YPR159W – YPR160W to *S. cerevisiae* genome.

		% Identities	Co- ordinates *
S. cerevisiae	**YPR159W - I P1 A**	97	859393 - 860013
	B	96	859400 - 860054
	YPR159W - I P2 A	98	860342 – 861035
	B	99	860337 – 861023
	INTERGENIC A	98	860208 – 860780
	B	98	860207 – 860768
	INTERGENIC - YPR160W A	97	861076 – 861633
	B	99	861065 – 861694

* co-ordinates in relation to SGD website (www.yeastgenome.org).
A and B represent independent sequencing runs.

Table 3.2B Percentage Identities for the Intergenic Region of YPR159W – YPR160W to *S. bayanus* genome.

		% Identities	Co- ordinates *
S. bayanus	**YPR159W - I P1 A**	86	1820 – 2435
	B	86	1822 – 2435
	YPRW - I P2 A	61	2764 – 3454
	B	62	2767 – 3445
	INTERGENIC A	78	2630 – 3202
	B	78	2629 – 3190
	INTERGENIC - YPR160W A	81	3498 – 4046
	B	84	3487 – 4116

*co-ordinates in relation to nucleotide 1 corresponding to the first nucleotide of YPR159W through to the last nucleotide of YPR160W.
A and B represent independent sequencing runs.

Table 3.3A: Percentage Identities for the ORF YPR160W from the lager yeast to *S. cerevisiae.*

		% Identities	Co- ordinates *
S. cerevisiae	**YPR160W P1 A**	85	861494 - 861764
	B	86	861506 - 861764
	C	85	861494 - 861764
	YPR160W P2 A	86	861888 - 862232
	B	86	861828 - 862216
	C	86	861856 - 862233
	YPR160W P3 A	87	862364 - 862598
	B	85	862163 - 862589
	C	86	862349 - 862599
	YPR160W P4 A	84	862983 - 863533
	B	84	862983 - 863533
	C	84	862977 - 863521
	YPR160W P5 A	83	863537 - 863854
	B	83	863548 – 863988
	C	84	863555 – 863990
	YPR160W P6 A	85	862756 – 863520
	B	85	862756 – 863482
	C	84	862791 – 863482
	YPR160W P7 A	84	863299 – 863668
	B	84	863325 – 863668
	C	84	863325 – 863668

A, B and C represent independent sequencing runs.

Table 3.3B: Percentage Identities for the ORF YPR160W form the lager yeast to *S. bayanus.*

		% Identities	Co- ordinates *
S. bayanus	**YPR160W P1 A**	92	3919 - 4277
	B	91	3927 - 4306
	C	92	3919 - 4277
	YPR160W P2 A	95	4328 - 4663
	B	94	4316 - 4647
	C	95	4256 - -4647
	YPR160W P3 A	94	4795 - 5029
	B	94	4601 - 5020
	C	94	4781 - 5030
	YPR160W P4 A	93	5414 - 5961
	B	93	5414 - 5961
	C	93	5414 - 5952
	YPR160W P5 A	92	5979 - 6285
	B	91	5979 - 6419
	C	92	5986 - 6421
	YPR160W P6 A	93	5187 - 5951
	B	93	5187 - 5913
	C	93	5224 - 5913
	YPR160W P7 A	93	5732 - 6086
	B	93	5756 - 6086
	C	93	5756 - 6086

A, B and C represent independent sequencing runs.

to the *S. bayanus* sequence. Along with these caveats there was also the problem of mismatched nucleotides due to the error rate of using Pfu PCR products. Therefore using this initial data as a starting point, the region was re- sequenced directly from the BAC clone, to reduce the error rate and the get a better coverage of the region.

Using the previously obtained sequences, primers were designed to allow sequencing was performed directly from the BAC clone, as represented in the schematic Figure 3.7. The newly obtained sequence included the start site of the ORF YPR160W and part of the intergenic region. Looking at the intergenic region first, this second run of sequencing confirmed the previous results that the intergenic region shows a high percentage homology to *S. cerevisiae* sequence, therefore it can be deduced that the recombination event between the two genomes did not occur in this region. More interestingly analysis of the sequence for YPR160W showed that the first 330bp of YPR160W from the lager genome showed a 98% sequence identity to the *S. cerevisiae* YPR160W and 91% sequence identity to the *S. bayanus* YPR160W ORFs. Within this 330bp region there are 59 nucleotide differences between the lager DNA sequence and published *S. cerevisiae* and *S. bayanus* sequence of which 52 are identical to *S. cerevisiae*, 2 are identical to *S. bayanus* and 5 nucleotide changes are unique to the lager specific YPR160W ORF, (Fig 3.9). After this 330bp, there is an increase in the number of nucleotide differences, 261 of which are identical to *S. bayanus*, 53 of which are identical to *S. cerevisiae*. There are 87 nucleotide differences that are unique to the lager specific YPR160W ORF, (highlighted in pink in Fig. 3.9). Of these unique differences 4 result in a single nucleotide insertion at positions 390, 1195, 1223 and 2242 and 2 result in the deletion of single nucleotides at positions 2703 and 2704. These lager specific nucleotide changes were noted in 2 out of the 3 sequencing runs performed directly from the BAC clone. The insertion and deletion of nucleotides results in the sequence appears to knock the proteins coding sequence out of frame , resulting in a non-functional gene in the lager yeast strain.

Table 3.4A: Percentage Identities for ORF YPR160W isolated from lager yeast, sequenced directly from BAC clone aligned against *S. cerevisiae*.

		% Identities	Co- ordinates *
S. cerevisiae	**I - YPR160W A**	98	861076 - 861487
	A.2	97	861512 - 861629
	A.3	95	861669 - 861773
	B	98	861076 - 861487
	B.2	97	861512 – 861629
	B.3	95	861669 – 861773
	YPR160W P1 A	99	861158 – 861487
	A.2	97	861512 – 861629
	A.4	95	861669 – 861759
	B	99	861158 – 861487
	B.2	97	861512 – 861629
	B.3	95	861669 – 861759
	YPR160W P2 A	74	861555 – 862182
	B	75	861555 – 862183
	C	75	861555 – 862183
	YPR160W P3 A	86	861771 – 862539
	B	84	861734 – 862521
	C	86	861771 – 862539
	YPR160W P4 A	84	862930 – 863385
	B	84	862942 – 863385
	YPR160W P5 A	85	863500 – 863935
	B	85	863500 – 863935
	YPR160W P6 A	85	862710 – 863481
	B	84	862787 – 863481
	YP160W P7 A	85	863256 – 863945
	B	85	863256 – 863944
	YPR160W P8 A	99	860812 – 861324
	B	99	860812 – 861320

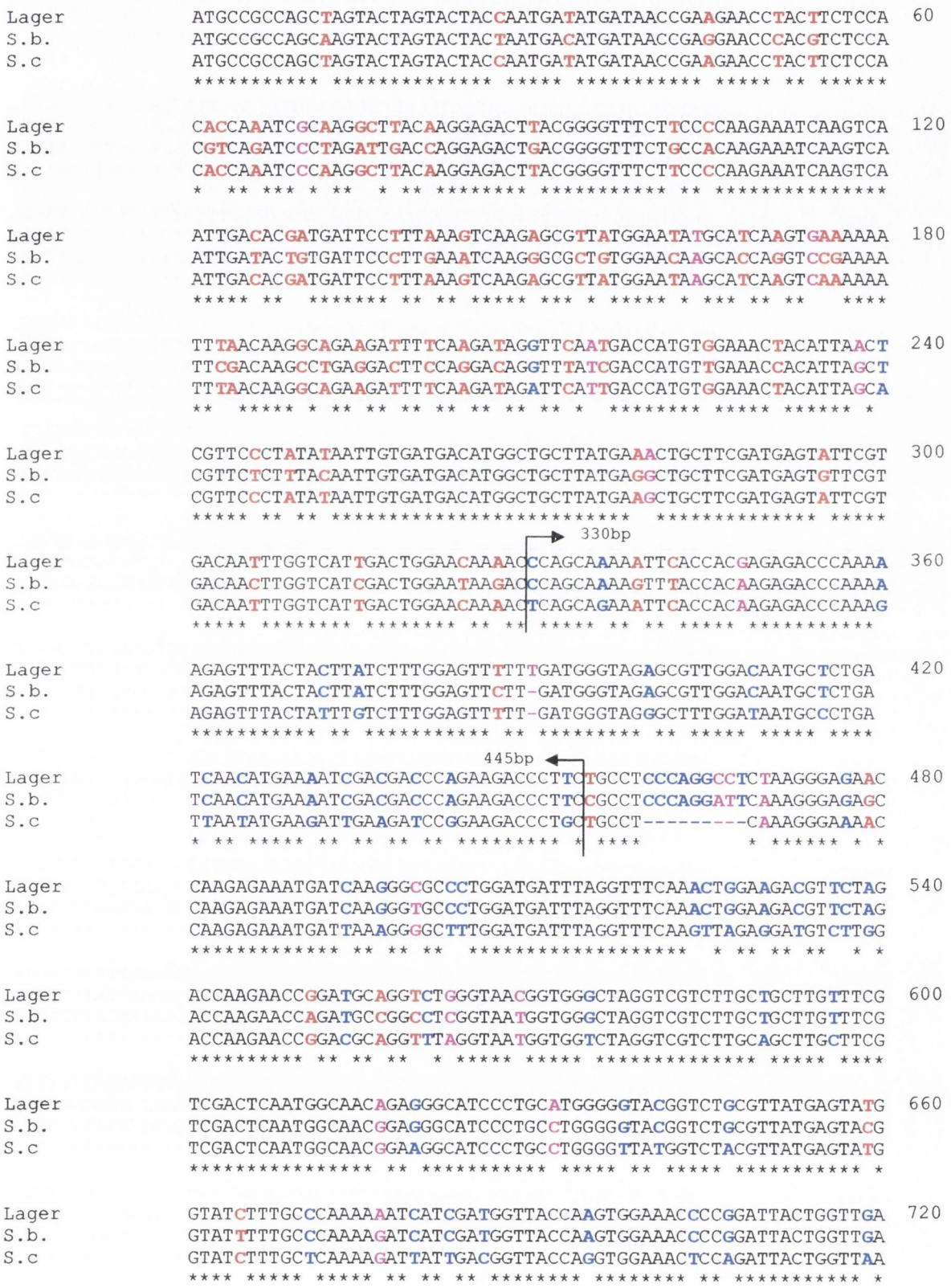
A and B represent independent sequencing runs.

Table 3.4B: Percentage Identities for ORF YPR160W isolated from lager yeast, sequenced directly from BAC clone aligned against *S. bayanus*.

		% Identities	Co- ordinates *
S. bayanus	**I - YPR160W A**	91	3505 - 3905
	A.2	87	3940 - 4046
	B	91	3505 - 3905
	B.2	87	3940 - 4046
	YPR160W P1 A	89	3580 - 3905
	A.2	87	3940 - 4046
	B	89	3580 - 3905
	B.2	87	3940 - 4046
	YPR160W P2 A	95	3883 - 4606
	B	95	3942 - 4606
	C	94	4267 - 5011
	YPR160W P3 A	94	4202 - 4970
	B	94	4140 - 4952
	C	94	4202 - 4970
	YPR160W P4 A	93	5361 - 5861
	B	93	5373 - 5816
	YPR160W P5 A	93	5931 - 6359
	B	93	5931 - 6359
	YPR160W P6 A	93	5141 - 5912
	B	93	5218 - 5912
	YP160W P7 A	93	5687 - 6368
	B	93	5687 - 6386
	YPR160W P8 A	99	3234 - 3746
	B	99	3234 - 3745

A and B represent independent sequencing runs.

Figure 3.9: Alignment of *S. cerevisiae* YPR160W, *S. bayanus* YPR160W sequences against the Lager specific YPR160W sequence.
The *S. cerevisiae* changes are highlighted in red. The *S. bayanus* changes are highlighted in blue. Lager specific changes are highlighted in pink.



```
Lager          ATTCTGGTAATCCATGGGAAATTGAACGTAACGAAGTTCAAATTCCAGTCACATTTTATG
S.b.           ATTCTGGTAACCCATGGGAAATCGAACGTAACGAAGTTCAAATTCCAGTCACATTTTACG
S.c            ATTCTGGTAATCCATGGGAAATTGAACGTAACGAAGTGCAAATTCCTGTCACCTTTTATG
               ********** *********** ************** ******** ***** ***** *

Lager          GTTACGTCGATAGGCCAGAAGGTGGTAAGACGACATTGAGTGCATCACAATGGATTGGTG
S.b.           GTTACGTCGACAGGCCAGAAGGCGGTAAGACGACACTAAGTGCATCACAATGGATTGGTG
S.c            GTTATGTTGATAGACCAGAAGGCGGTAAAAACTACACTGAGTGCGTCACAATGGATCGGTG
               **** ** ** ** ******** ***** ** *** * ***** *********** ****

Lager          GTGAACGTGTTCTTGCCGTTGCCTACGATTTCCCCGTTCCAGGTTTCAAAACTTCCAATG
S.b.           GTGAACGTGTTCTTGCCGTTGCCTACGATTTCCCCGTTCCAGGTTTCAAGACTTCCAATG
S.c            GGGAAAGAGTTCTTGCTGTCGCGTATGATTTCCCAGTTCCGGGTTTCAAGACTTCCAATG
               * *** * ******** ** ** ** ******** ***** ******** **********

Lager          TTAATAATTTGAGATTGTGGCAAGCAAGACCAACCACCGAGTTCGATTTTGCTAAATTCA
S.b.           TTAATAATTTGAGATTGTGGCAGGCAAGACCAACCACCGAGTTTGATTTTGCCAAGTTCA
S.c            TAAATAACTTAAGACTATGGCAAGCAAGGCCAACAACAGAATTTGATTTTGCAAAATTCA
               * ***** ** *** * ***** ***** ***** ** ** ** ******** ** ****

Lager          ATAATGGTGACTATAAGAACTCTGTGGCTCAACAGCAAAGTGCCGAGTCCATTACCGCTG
S.b.           ATAATGGTGACTACAAGAACTCCGTGGCTCAACAGCAAAGTGCCGAATCCATAACCGCTG
S.c            ATAATGGTGACTATAAAAACTCTGTGGCTCAGCAACAACGCGCAGAGTCTATAACCGCTG
               ************* ** ***** ******** ** *** * ** ** ** ** *******

Lager          TGCTGTATCCAAACGACAACTTTGCCCAAGGTAAAGAACTGAGATTGTAACAGCAATACT
S.b.           TGCTGTATCCAAACGACAACTTTGCCCAAGGTAAAGAACTGAGATTGAAACAGCAATACT
S.c            TGTTGTATCCAAACGATAACTTTGCTCAAGGTAAGGAGTTGAGGTTGAAACAGCAGTACT
               ** ************* ******** ******** **  **** *** ******* ****

Lager          TCTGGTGTGCTGCATCCCTGCACGATATCCTAAGAAGATTCAAAAAATCCAAGAGGTCAT
S.b.           TCTGGTGTGCTGCATCCTTGCACGATATCCTAAGAAGATTCAAAAAGTCTAAGAGATCAT
S.c            TCTGGTGTGCTGCATCCTTACACGACATCTTAAGAAGATTCAAAAAATCCAAGAGGCCAT
               ***************** * ***** *** ***************** ** *****  ***

Lager          GGACCGAGTTCCCTGAACAAGTGGCTATTCAATTGAACGATACTCATCCAACCTTTAGCT
S.b.           GGACCGAGTTCCCTGAGCAAGTGGCTATTCAATTGAATGATACCCATCCAACGTT-AGCT
S.c            GGACTGAATTTCCTGACCAAGTGGCTATTCAGTTGAATGATACTCATCCAACTTT-AGCC
               **** ** ** ***** ************** ***** ***** ******** ** ***

Lager          ATCCTTGAATTGCAAAGAGTCTTTGGTTGATTTGGAAAAATTGGACTGGCACGAAGCTTG
S.b.           ATCGTTGAATTGCAAAGAGTCTT-GGTCGATTTGGAGAAATTAGATTGGCACGAAGCTTG
S.c            ATCGTTGAATTACAGAGAGTTTT-GGTCGATCTAGAAAAACTAGATTGGCACGAGGCTTG
               *** ******* ** ***** ** *** *** * ** *** * ** ******** *****

Lager          GGACATTGTCACCAAGACCTTTGCTTATACCAACCACACCGTTATGCAAGAAGCCTTGGA
S.b.           GGACATTGTCACCAAGACCTTTGCTTACACCAACCACACCGTTATGCAAGAAGCCTTGGA
S.c            GGACATCGTGACCAAGACTTTTGCTTATACTAACCACACTGTTATGCAAGAGGCCCTGGA
               ****** ** ******** ******** ** ******** *********** *** ****

Lager          AAAATGGCCCGTTGGCTTATTCGGCCATTTGTTGCCCAGACATCTGGAAATTATCTACGA
S.b.           AAAATGGCCCGTTGGCTTATTCGGCCATTTGTTGCCCAGACATCTGGAAATTATCTACGA
S.c            AAAATGGCCCGTCGGCCTCTTTGGCCATTTGCTACCCAGACATTTGGAAATTATATATGA
               ************ *** * ** ********* * ********* ********** ** **

Lager          TATTAATTGGTTCTTCTTACAGGATGTTGCAAAGAAATTCCCCAAGGATGTTGATCTTTT
S.b.           TATTAATTGGTTCTTCTTACAGGATGTTGCAAAGAAATTCCCCAAGGATGTTGATCTTTT
S.c            TATCAACTGGTTCTTCTTGCAAGATGTGGCCAAAAAATTCCCCAAGGATGTTGATCTTTT
               *** ** *********** ** ***** ** ** **************************

Lager          GTCTCGTATATCCATCATCGAGGAAAACTCTCCAGAGAGACAGATCAGAATGGCCTTTTT
S.b.           GTCTCGTATATCCATCATCGAGGAAAACTCTCCAGAAAGACAGATCAGAATGGCCTTTTT
S.c            GTCTCGTATATCCATCATCGAAGAAAACTCTCCAGAAAGACAGATCAGAATGGCCTTTTT
               ********************* ************** ************************
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```
Lager           GGCTATTGTTGGTTCTCATAAAGTCAACGGTGTTGCGGAATTGCACTCTGAATTAATTA
S.b.            GGCCATTGTTGGTTCTCATAAGGTCAACGGTGTTGCTGAATTGCACTCTGAATTAATTA
S.c             GGCTATTGTTGGTTCACACAAGGTTAATGGTGTTGCTGAATTGCACTCTGAATTAATCA
                *** *********** ** ** ** ** ******** ****** ************** *

Lager           AGACCACCATCTTCAAAGATTTCGTCAAATTCTACGGTGCATCAAAGTTTGTCAACGTTA
S.b.            AGACCACCATTTTCAAAGATTTCGTCAAATTCTACGGTGCATCAAAGTTCGTAAATGTTA
S.c             AAACGACCATATTTAAAGATTTTGTCAAGTTCTATGGTCCATCAAAGTTTGTCAATGTCA
                * ** ***** ** ******** ***** ***** *** ********** ** ** ** *

Lager           CTAACGGTATCACACCAAGAAGATGGTTGAAGCAAGCTAACCCTAACTTGGCTAGATTGA
S.b.            CTAACGGTATCACACCAAGAAGATGGTTAAAGCAAGCCAACCCTGCCTTGGCTAGGTTGA
S.c             CTAACGGTATCACACCAAGGAGATGGTTGAAGCAAGCTAACCCTTCATTGGCTAAACTGA
                ******************** ******** ******** ******   *******   ***

Lager           TTAGCAAAACTCTTAACGATCCTACAGAGGACTATCTACTAGACATGACAAAGTTAACTC
S.b.            TTAGTGAAACTCTTAACGATCCTACAGAGGAGTATCTACTAGATATGACAAAGTTAACTC
S.c             TCAGTGAAACCCTTAACGATCCAACAGAGGAGTATTTGTTGGACATGGCCAAACTGACCC
                * **  **** *********** ******** *** *  * ** *** * **  * ** *

Lager           AACTGGCAAAGCACCTTGAGGATAAGAAGTTTTTGAAAGAGTGGAATCAAGTCAAACTCA
S.b.            AATTGGCAAAACACATTGAGGATAAGAAGTTTTTGAAGGAATGGAACCAAGTCAAGCTCA
S.c             AGTTGGGAAAATATGTTGAAGATAAGGAGTTTTTGAAAAAATGGAACCAAGTCAAGCTTA
                *  *** ***  *  **** ****** **********  * ***** ******** ** *

Lager           ATAATAAGATCAGATTGGTGGACCTAATCAAAAAAGAAAATGGTGGTGAAGACATCATTA
S.b.            ACAATAAGATCAGATTAGTGGATCTAATCAAAAAAGAAAATGACGGTGAAGACATCATTA
S.c             ATAATAAGATCAGATTAGTAGATTTAATCAAAAAGGAAAATGATGGAGTAGACATCATTA
                * ************** ** **  ********** *******  ** * ***********

Lager           ACAGAGAGTATCTAGACGATACTTTGTTTGATATGCAAGTTAAACGTATTCACGAGTATA
S.b.            ACAGAAAGTATCTAGATGACACTTTGTTTGATATGCAAGTTAAACGTATTCATGAGTATA
S.c             ACAGAGAGTATTTGGACGACACCTTGTTTGATATGCAAGTTAAACGTATTCATGAATATA
                ***** ***** * ** ** ** ****************************** ** ****

Lager           AACGTCAACAACTAAACGTCTTTGGTATTATTTACCGTTACTTAGCAATGAAAAATATGC
S.b.            AGCGTCAACAACTAAACGTCTTTGGTATTATTTACCGTTACTTGGCAATGAAAAATATGC
S.c             AGCGTCAACAGCTAAACGTCTTTGGTATTATATACCGTTACCTGGCAATGAAGAATATGC
                * ******** ******************** ********* * ******** *******

Lager           TAGAGAACGGTGCTTCCATCGAGGAAGTGGCCAAGAAATATCCACGTAAGGTTTCTATCT
S.b.            TAGAGAACGGTGCTTCTATCGAAGAAGTGGCCAAGAAATATCCACGTAAGGTTTCTATCT
S.c             TGAAGAACGGTGCTTCGATCGAAGAAGTTGCCAAGAAATATCCACGCAAGGTTTCAATCT
                *  ************* ***** ***** ***************** ******** ****

Lager           TCGGTGGTAAGAGTGCACCCGGTTACTACATGGCTAAGTTGATCATCAAACTGGTCAACT
S.b.            TCGGTGGTAAGAGTGCACCCGGTTACTACATGGCTAAGTTGATCATCAAATTGGTCAACT
S.c             TTGGTGGTAAGAGTGCTCCTGGTTACTACATGGCTAAGCTGATCATAAAATTGATCAACT
                * *************** ** ****************** ******* *** ** *******

Lager           CTGTCGCTGAAATTGTTAACAACGACGAATCAATCGACGACTTATTGAAAGTTGTCTTCA
S.b.            CTGTCGCAGAAATTGTTAACAACGACGAATCAATTGATGATTTATTAAAAGTCGTATTCA
S.c             GTGTTGCTGACATTGTTAATAACGACGAGTCAATTGAGCATTTGTTGAAGGTTGTCTTTG
                 *** ** ** ******** ******** ***** **  * ** ** ** ** ** ** **

Lager           TTGCTGATTATAATGTATCCAAGGCCGAAATTATTATTCCAGCAAGTGATTTAAGTGAAC
S.b.            TTGCTGATTATAATGTGTCCAAGGCTGAGATTATTATTCCCGCAAGTGATTTGAGTGAAC
S.c             TTGCTGATTATAATGTTTCTAAGGCTGAAATCATTATTCCAGCAAGTGACTTGAGTGAGC
                **************** ** ***** ** ** ******** ******** ** ***** *

Lager           ATATTTCAACTGCTGGTACAGAAAGCGTCAGGTACTTCTAATATGAAGTTTGTCATGAAT
S.b.            ATATTTCAACTGCTGGTACAGAA-GCGTCAGGTACTTCTAATATGAAGTTTGTCATGAAT
S.c             ATATTTCTACTGCTGGTACTGAA-GCGTCTGGTACTTCTAATATGAAGTTTGTTATGAAC
                ******* *********** *** ***** ********************** *****
```

```
Lager           GGTGGTTTGATTATTGGAACCGTCGACGGTGCCAATGTGGAAATTACCAGAGAAATCGGT
S.b.            GGTGGTTTGATTATTGGTACCGTCGACGGTGCCAATGTGGAAATTACCAGAGAAATCGGT
S.c             GGTGGTTTGATTATTGGTACTGTTGATGGTGCCAATGTGGAAATCACAAGGGAAATTGGT
                ***************** ** ** ** ***************** ** ** ***** ***

Lager           GAATATAACGTCTTCCTATTTGGTAATTTAAGTGAAAATGTCGAAGATTTGAGATATAAC
S.b.            GAAGATAACGTCTTCTTATTTGGTAATTTGAGTGAAAACGTCGAAGATTTGAGATATAAC
S.c             GAAGATAATGTCTTCTTGTTTGGTAACCTAAGTGAAAATGTCGAAGAATTGAGATACAAC
                *** **** ****** * ********  * ******** ******** ******** ***

Lager           CTTCAATACCACCCACAGGATTTGCCATCGAGCTTGTAATCCGTCCTATCTTACATTGAC
S.b.            CATCAATACCATCCACAGGAGTTGCCATCAAGTTTGGAATCCGTGCTAACTTACATTGAA
S.c             CATCAATACCATCCACAAGATTTACCATCTAGTTTGGATTCTGTTTTATCCTACATTGAA
                * ********* ***** ** ** ***** ** *** * ** **  ** * ********

Lager           ACTGGTCAATTCTCCCCAGAGAATCCAAACGAATTCAAACCATTGGTTGACAGTATCAAA
S.b.            AGTGGCCAGTTTTCTCCAGAGAATCCAAACGAATTCAAACCGTTAGTTGACAGTATCAAA
S.c             AGTGGACAATTTTCTCCAGAAAATCCAAATGAATTCAAACCTTTAGTCGACAGTATTAAG
                * *** ** ** ** ***** ******** ********** ** ** ******** **

Lager           TATCATGGGGATTACTATCTAGTTAGTGACGACTTTGAATCCTACTTGGCCACCCAAGAG
S.b.            TATCACGGTGATTACTATTTAGTTAGTGATGACTTTGAATCCTACTTGGCCACCCAAGAT
S.c             TACCACGGCGATTATTACCTGGTCAGTGATGACTTTGAATCCTATCTGGCCACCCATGAA
                ** ** ** ***** **  * ** ***** **************  ********** **

Lager           TTAGTCGACCAGGAGTTCCACAACCAAAGACCGGAATGGTTGAAGAAGAGTGTCCTGAGT
S.b.            TTAGTAGACCAAGAGTTCCACAACCAAAGATCGGAATGGCTAAAGAAGAGTATCTTGAGT
S.c             TTAGTGGACCAGGAGTTCCACAATCAAAGGTCAGAATGGTTAAAAAAGAGTGTCCTGAGC
                ***** ***** ********** *****  * ****** * ** ****** ** ****

Lager           GTTGCAAACGTTGGTTTCTTCAGTAGTGACCGTTGCATCGAGGAATATTCTGATACAATC
S.b.            GTTGCAAACGTTGGTTTCTTCAGTAGTGACCGTTGTATCGAAGAATACTCTGATACAATC
S.c             GTTGCAAACGTCGGCTTCTTTAGCAGTGATCGTTGTATCGAGGAATACTCCGATACCATT
                ********** ** ***** ** ***** ***** ***** ***** ** ***** **

Lager           TGGA--GTCGAACCAGTGACTTAA
S.b.            TGGAACGTCGAACCAGTGACTTAA
S.c             TGGAACGTTGAACCAGTGACTTAG
                ****  ** **************
```

Another region of interest identified through sequencing was an 80bp region that encompass the region 4351 – 4431bp in the lager specific YPR160W [shaded region], Fig. 3.9, (nucleotides are numbered in relation to YPR159W where number 1 refers to the ATG of this ORF). The *S. cerevisiae* genome map as annotated at the Saccharomyces Genome Database shows the presence of a 'dubious ORF' YPR160W-A within YPR160W, (861930 – 862010, SGD co-ordinates), as shown in Figure 3.10A. A full schemata illustrating the sequencing results and a proposed structure of the lager specific YPR160W ORF can be seen in Figure 3.10B. In the model, it is proposed that a recombination event occurs within the ORF, resulting in a hybrid gene. The full consensus sequence for the lager specific YPR160W is shown is Figure 3.9.

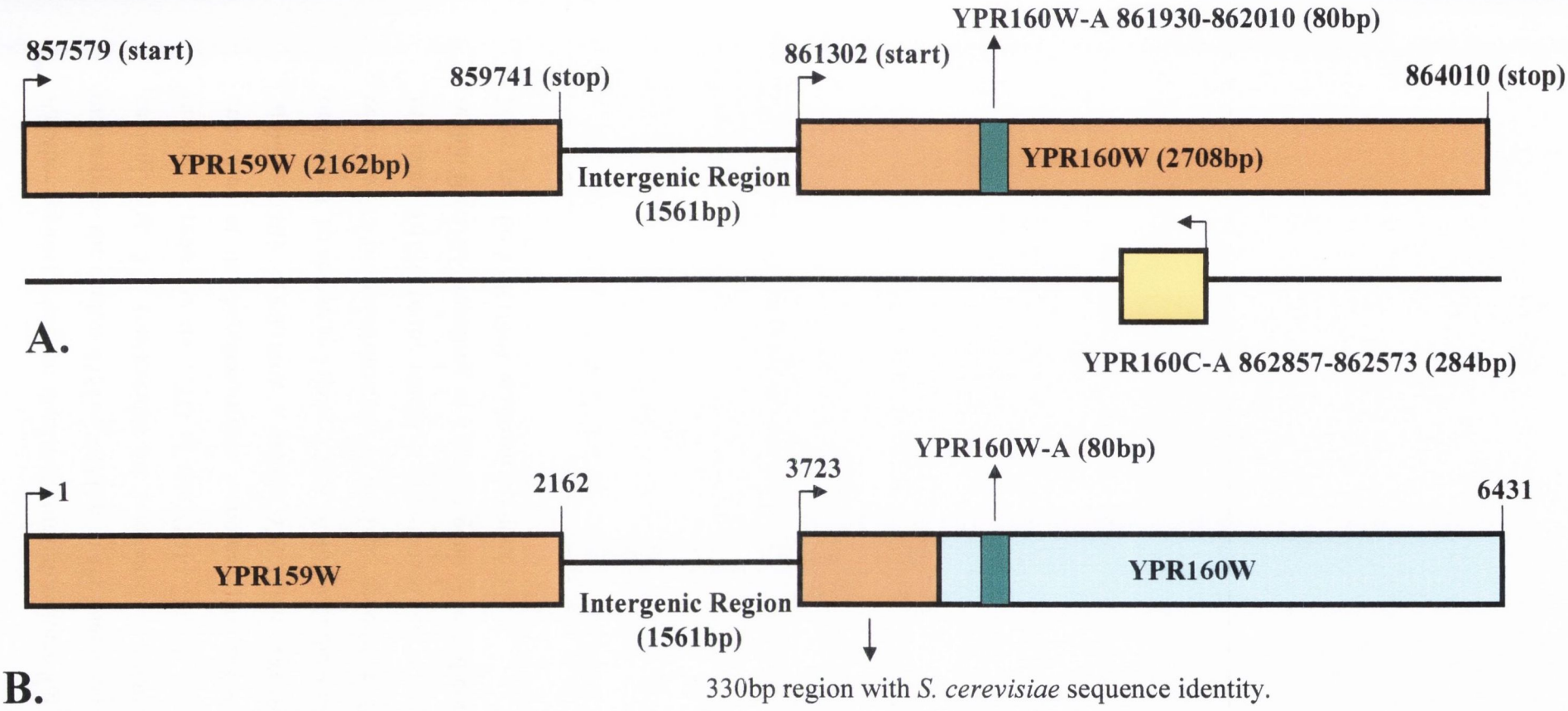


Figure 3.10: A) Schematic of *S. cerevisiae* YPR159w and YPR160W.
B) Proposed model for lager specific region of ORFs YPR159W and YPR160W. The green box represents YPR160W-A.

3.3 Discussion

3.3.1. Chromosome III.

Southern hybridisation analysis of chromosome III, confirms previous microarray data. As can be seen in the Figures 3.1b and 3.1c the probe YCR007 hybridises to the same chromosome as the YCR047 probe. This shows that despite the difference in copy number, the *S. cerevisiae* gene organisation for chromosome III is the same in the lager strain. Furthermore it suggests that genes to the left of the MAT locus are present in a single copy similar to the *S. cerevisiae* copy number and genes to the right of the MAT locus (e.g. YCR047) show a higher degree of hybridisation equating to a higher copy number of these genes. These results show that recombination has occurred between homeologous chromosomes pairs. The recombination site on chromosomes III is located at a site of high genetic recombination, the MAT locus. The results presented here show that two types of chromosome III exist in lager yeast strains. The first consists of *S. cerevisiae*-like genes on both sides of the MAT locus (*S. cerevisiae* type), while the second consists of non- *S. cerevisiae* genes to the left of the MAT locus and *S. cerevisiae* genes to the right of the MAT locus (mosaic type). The relative hybridisation to *S. cerevisiae* DNA both by CGH and CHEF gel analysis suggests a ratio of 1:3 S. cerevisiae type: mosaic type chromosome IIIs.

3.3.2 Chromosome X

On chromosome X in the lager yeast strains, there is a rearrangement after YJR009C in both strains examined, 6701 and CMBS-33. These changes in ratios of hybridisation at specific locations represent regions where homeologous chromosomes have undergone inter- or intra- chromosomal translocations. As seen in Figure 3.2b, at least three different chromosomes hybridise to the YJR009 probe. These results confirm the work of Casey (Casey, 1986) in another industrial lager strain. The different intensities of bands suggests that the *S. cerevisiae*, non-*S. cerevisiae* components of the chromosome result in a mosaic chromosome X in both lager yeast strains. One may propose that the relative

intensities of the bands reflect the chromosome composition, with the big more intense band *S. cerevisiae*-like and the lower intensity bands *S. bayanus*-like. However, nothing further can be deduced about the composition of the chromosome types from this method, as the gel cannot be quantitified due to the uneven loading of the DNA samples. This can occur in PFGE gels as the plug size can vary and there may also have been uneven digestion of the cell membranes during the zymolyse and proteinase K steps, as described in Chapter 2. Therefore it would be essential to obtain cloned copies of these regions to deduce the exact make up of the chromosomes.

3.3.3 Chromosome XVI.

For chromosome XVI the region from YPR160W to YPR190C has a unique ratio of hybridisation of 0.2 in the CGH data. This region encompasses 30 genes, many of which are essential in the haploid *S. cerevisiae* species. The low level of hybridisation to *S.* cerevisiae DNA was confirmed using CHEF gel analysis as demonstrated in Figure 3.3b where one can see a much less intense signal was observed in the lager yeast strain DNA when hybridised to a *S. cerevisiae* YPR160W DNA probe. The low hybridisation signals in the region YPR160W-YPR190C suggest that the *S. cerevisiae* genes in this region have significantly diverged from the *S. cerevisiae* sequence but that non-*S. cerevisiae* homeologus genes are present. Following from this data that confirms the CGH analysis, it is possible for one to deduce that in the lager yeast strains, the complement of chromosome XVI is three *S. cerevisiae* like chromosomes containing the non-*S. cerevisiae* region YPR160W- YPR190C and one non-*S. cerevisiae* chromosome.

3.3.4. Sequencing the Junctions.

Following construction of a CMBS genomic library, a screening protocol was developed which allowed the selection of specific clones of interest using a pool of specific primers for the 'jump' loci of chromosome XVI. Screening of 32 of the 73 total library plates, resulted in 106 chromosome XVI positive clones being obtained, Clones were picked due to the intensity of signal observed. 10 clones

were worked with at a time for ease. During individual hybridisation the number of positive clones was again reduced as false positives were screened out. Clones A11 from plate 27 were chosen for further work, as this clone gave a hybridisation signal when probed with a selection of *S. cerevisiae* and *S. bayanus* DNA probes.

After initial unsuccessful sequencing of the entire BAC clone, DNA isolated from the BAC clones was used in PCR reactions. As the clones gave a signal when individually probed it was possible to PCR amplify the region from YRP159W and YPR160C. The difference in intensities may be due to the fact that the primers were designed using *S. cerevisiae* genome sequence as a template; therefore the primers may not show complete homology to the BAC DNA as these genes have significantly diverged from the *S. cerevisiae* genome.

The analysis of the sequences obtained for the regions YPR159W, has shown that in the lager yeast strain studied, that the ORF YPR159W is *S. cerevisiae* – like and has not undergone any rearrangements during the fusion of the *S. cerevisiae* and *S. bayanus* genomes. There were two mismatches observed in the sequence, which did not change the protein coding sequence. The intergenic region of 1.5Kb showed an average percentage identity of 97% to the *S. cerevisiae* intergenic sequence, in the lager yeast strain CMBS-33 the intergenic region is highly conserved which is very unusual. The obtained sequence for YPR159W and the intergenic region implies that a recombination event has not occurred in this region of sequence.

For the sequencing of the ORF YPR160W, initially PCR products were sequenced using DNA from the BAC clone with a 100kb insert containing the region of interest from chromosome XVI. This first round of sequencing was successful, however it was decided to perform a second round of sequencing directly from the BAC clone. This was because there is an error rate associated with the sequencing of PCR products. The first round of sequencing showed that not all of the primer sets overlapped correctly resulting in regions of DNA not being sequenced, most notably the ATG site of YPR160W. Sequencing directly

from the BAC clone also allowed for sequencing to be performed in the forward and reverse directions. The sequence obtained firstly confirmed the data for the intergenic region that is was *S. cerevisiae* – like DNA.

The sequencing data results for the lager specific YPR160W ORF showed that the first 330bp of the lager specific YPR160W, were *S. cerevisiae* like, having 98% sequence identity to *S. cerevisiae* and 89% sequence identity to *S. bayanus.* A 9-basepair insertion observed at position 448 – 457 in the published *S. bayanus* sequence and the lager sequence is characteristic of the *S. bayanus* nature of this region and may affect the secondary structure of the protein if the gene is functional in the lager yeast.

From the sequencing data obtained, in the lager yeast it appears that the recombination event has occurred in the region of 330 to 445bp, resulting in a genuine hybrid gene in the lager yeast strain. From this data the recombination event has lead to a non-functional YPR160W gene as the sequence revealed a number of lager specific deletions and insertions in the sequence, which were deemed to be correct upon careful examination of the DNA traces, that result in the sequence being knocked out of frame for translation to a protein. Further sequencing will be required to confirm this along with the production of an antibody for use in western blots for validation.

In *S. cerevisiae* YPR160W is a non-essential glycogen phosphorylase (GPH1) required for the mobilization of glycogen and is regulated by cyclic AMP-mediated phosphorylation. Gene expression is regulated by stress response elements and the Hog1 pathway. In the lager yeast it appears that YPR160W is non-functional gene, however the genes YNL307C, YDL079C, YMR139W and YOL128C encode for glycogen syntheases.

Chapter 4
Dosage compensation of transcription in lager yeasts.

4.1 Introduction

The transcription profile of the lager yeast is important in understanding the performance of the lager yeast strains in the stressful environment of an industrial fermentation. Previous transcriptome studies, (James *et al.,* 2003., Bond *et al.,* 2002, Higgins *et al.,* 2003 Oelson *et al.,* 2002), looked at the transcription profiles of the lager yeast through the examination of the temporal expression of genes during a standard fermentation. However, a cavaet of all of these studies is that there is insufficient information about the *S. bayanus* contribution to the overall transcription profile. Using very stringent hybridisation conditions allows the analysis of the *S. cerevisiae* genome but the specific hybridisation conditions required to allow the *S. bayanus* genome to be detected are currently not resolved. Decreasing the stringency of hybridisation can result in non-specific hybridisation on the array slide. Recent studies have seen the developemnt of DNA microarrays containing both *S. cerevisiae* and *S. bayanus* ORF DNA, (Kodama, 2005., Dunn, B., Stanford University, personal communications and unpublished results), however to date no transcriptional data has been published using these microarrays.

Since the lager yeast contain multiple alleles of both *S. cerevisiae* and non-*S. cerevisiae* genes, one question to be addressed is 'Are all alleles expressed in the lager yeasts?' To address this question, firstly the expression of a number of *S. cerevisiae* genes having different copy number of alleles were examined during a standard fermentation. The expression profile and relative level of expression was compared to the expression in a haploid *S. cerevisiae* strain. Cells were collected at specific timepoints on Day 1 when the specific gravity of the wort is still high and the alcohol concentration low, Day 3 during the mid to late fermentation stage and Day 8 at the end of fermentation. This is usually the time point when the commercial brewers stop the brew and harvest the yeast for repitching.

Another important question to be addressed concerns the effects of ploidy on the expression of both the *S. cerevisiae* and the non-*S. cerevisiae* alleles of a given gene. In Chapter 3, a genomic library of the lager yeast CMBS-33 DNA was

prepared using bacterial artifical chromosomes. This enabled the isolation for the first time of a large region of the mosaic chromosome XVI. Sequencing across the region encompassing the known rearrangement site revealed that the recombination event unexpectantly occurs within the YPR160W gene and not the preceeding intergenic region. The resultant rearrangement generated a unique lager specific gene. It appears from the sequence information that the ORF has been disrupted and that this gene does not code for a functional protein. YPR160W encodes for glycogen phosphorylation and is a non-essential gene as four other genes (YNL307C, YDL079C, YMR139W and YOL128C) encode for glycogen syntheases.

With the cloning for the first time of a region of the mosaic chromosome XVI, it was now possible to ask questions regarding the expression of both the *S. cerevisiae* and the non-*S. cerevisiae* alleles within the lager yeast genome, specifically in relation to dosage compensation. With the known caveats of microarray use for transcription profiling, an alternative strategy was adopted. A yeast artificial chromosome containing over 100 Mb of *S. bayanus* chromosome XVI from a lager yeast was introduced into isogenic 1n, 2n, 3n, and 4n polyploid strains of *S. cerevisiae.* Analysis of the expression of the newly introduced *S. bayanus* genes revealed that increasing the copy number of *S. cerevisiae* genes, caused a decrease in the expression of the *S. bayanus* homeologus genes, indicating the gene dosage effects may prevent overexpression of genes in polyploid strains of yeasts.

4.2 Results

4.2.1 Expression levels of Homologeous genes.

In this Chapter, the expression of both the *S. cerevisiae* and non-*S. cerevisiae* alleles in the lager yeast strain CMBS-33 was examined. Firstly, expression levels for genes on chromosome III were examined. As previously discussed, there is a significant difference in the ratio of hybridisation at a DNA level between the left and right of the MAT locus observed in the lager yeast strains (Fig. 1.4), indicating that the *S. cerevisiae* content differs either side of the MAT locus.

The *S.* cerevisiae S150 strain and the lager yeast strain CMBS-33 were pitched into a standard fermentation at 16°P under aerobic and anaerobic conditions. Cells were collected at days 1, 3 and 8 during the fermentation. RNA was extracted using the protocol as described in Chapter 2, section 2.11. Northern blots were performed using a selection of DIG – dUTP labelled probes from either side of the MAT locus. Two control probes were also used, the first being 5.8s ribosomal RNA and the second being TDH3 gene (YGR192C). TDH3 was chosen as a control as it is involved in glycolysis and plays a role in glucose fermentation. The steady state levels of 5.8s and TDH3 mRNAs in the haploid *S. cerevisiae* strain S150 and the lager yeast strain CMBS-33 is as shown in Figures 4.1 and 4.2. The levels of TDH3 are low at day 1 and day 3 of the fermentation in the *S. cerevisiae* strain and then rise significantly on day 8, under aerobic and anaerobic conditions. The 5.8s RNA levels were similar throughout the fermentation under both conditions. In the lager strain CMBS-33 levels of TDH3 increased significantly on day 3 of a fermentation and remained high for the remainder of the fermentation (Fig 4.2C), under anaerobic conditions. The 5.8s RNA levels remained constant throughout the fermentation (Fig 4.2B). Note that only the CMBS-33 samples fermented under aerobic conditions were probed with the 5.8s RNA probe and those fermented under anaerobic conditions were probed with the TDH3 probe.

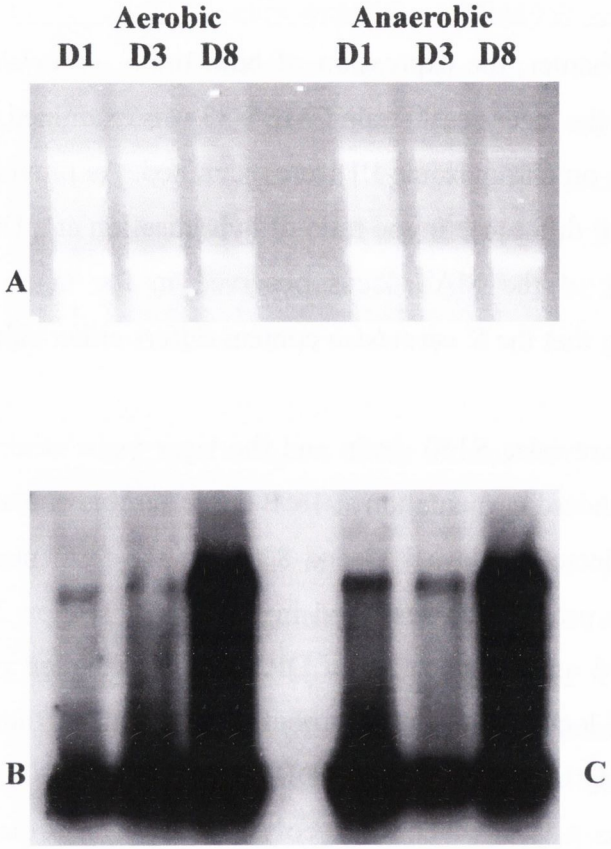


Figure 4.1: Expression of the TDH3 and 5.8s RNA genes by the laboratory strain S150. **(A)**Total RNA was extracted from cells at days 1, 3 and 8 of aerobic and anaerobic fermentations and 30μg were electrophoresised in a denaturing agarose gel. Following transfer to a nylon membrane, the blots were probed with DIG-dUTP labelled TDH3 and 5.8sRNA probes, **(B)** The expression of TDH3 and 5.8sRNA in aerobically fermented cultures,**(C)** The expression of TDH3 and 5.8s RNA in anaerobically fermented cultures.

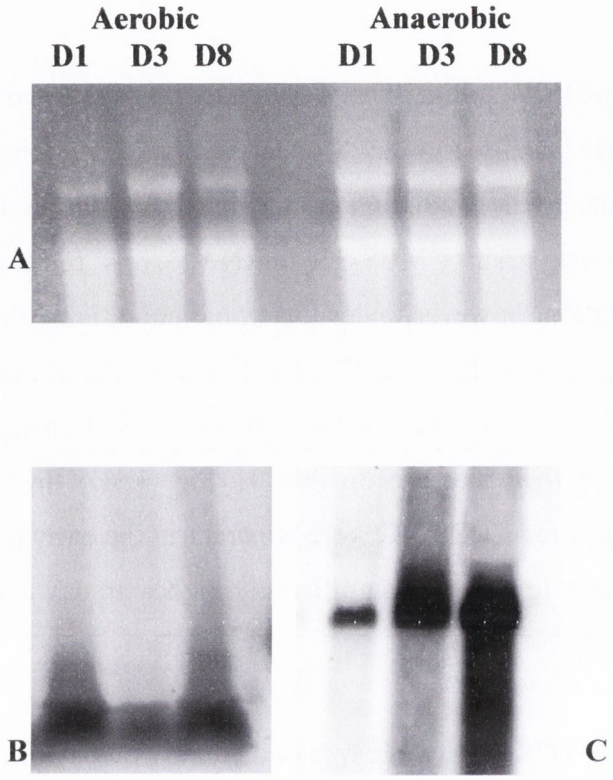


Figure 4.2: Expression of the TDH3 and 5.8s RNA genes by the lager yeast strain CMBS-33. **(A)**Total RNA was extracted from cells at days 1, 3 and 8 of aerobic and anaerobic fermentations and 30μg were electrophoresised in a denaturing agarose gel. Following transfer to a nylon membrane, the blots were probed with DIG-dUTP labelled TDH3 and 5.8sRNA probes, **(B)** The expression of 5.8sRNA gene in aerobically fermented cultures,**(C)** The expression of TDH3 gene in anaerobically fermented cultures.

The two probes chosen from the left hand side of the MAT locus were YCR012W (PGK1) and YCR031C (RPS14A) and from the right hand side were YCR057C (PWP2) and YCR077C (PAT1). The northern hybridisation analysis was also performed on the *S. cerevisiae* S150 strain.

The YCR012W mRNA levels were low on days 1 and 3 in the *S. cerevisiae* strain S150 and increased at day 8 under aerobic conditions. The pattern of expression under anaerobic conditions is indecipherable due to a smudge on the gel. In the lager yeast strain CMBS-33; mRNA levels remain low until day 8 of the fermentation, however under anaerobic conditions, the levels increase by day 3, (Fig. 4.3C). For the gene YCR077C, the levels of expression were low in day 1 and day 3 of both the aerobic and anaerobic fermentations in the *S. cerevisiae* strain and then increase on day 8, (Fig. 4.3D). In CMBS-33, higher levels of expression for YCR077C were apparent in the early days of the fermentation and particularly levels were significantly higher on day 3 under anaerobic conditions, (Fig. 4.3E).

The gene YCR031C was expressed equally on days 1, 3 and 8 in the *S. cerevisiae* strain under both aerobic and anaerobic conditions, (Fig. 4.4B), while there is a different temporal expression of this gene in CMBS fermented under identical conditions, (Fig. 4.4C). Finally for the gene YCR057C, is mostly expressed in the *S. cerevisiae* strain on day 8, with little expression apparent on days 1 and 3. However, the gene is highly expressed on day 3 of the fermentation in the lager strain particularly under anaerobic conditions. The level of hybridisation for all genes is significantly higher in CMBS-33 suggesting that the two *S. cerevisiae* alleles are being expressed. Taken together, the data indicates that the expression pattern of the four genes is differential in the *S. cerevisiae* S150 strain and the lager yeast strain CMBS-33 strain despite the fact that the fermentations were carried out under identical conditions. The hybridisation signal between the different genes cannot be compared as the hybridisation levels are dependent on the probe homology, the gene copy number and the influence of the environmental conditions to which the cells were exposed.

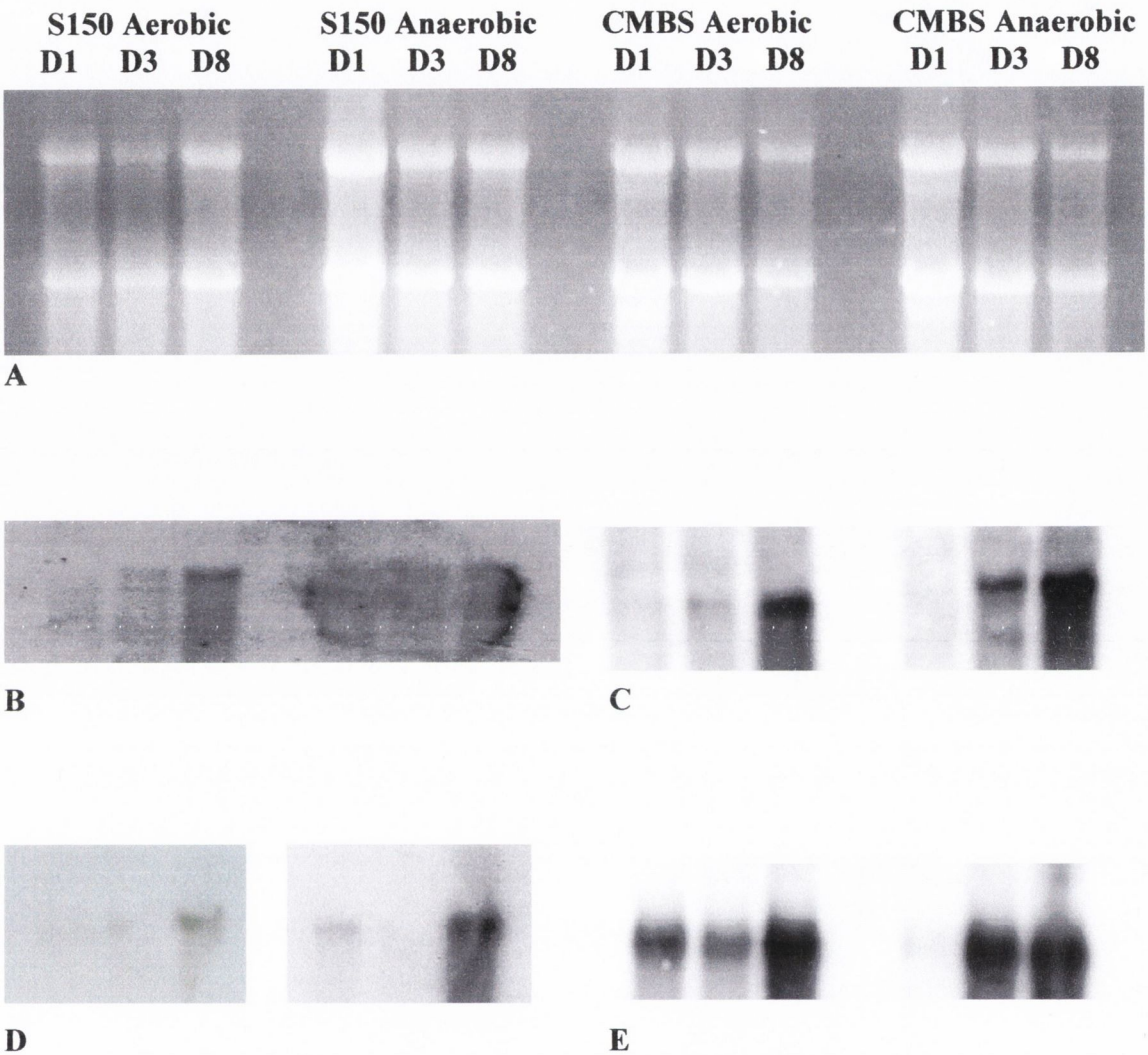


Figure 4.3: Expression of the YCR012 and YCR077 genes by the laboratory strain S150 and the lager yeast strain CMBS-33. **(A)**Total RNA was extracted from cells at days 1, 3 and 8 of aerobic and anaerobic fermentations and 30μg were electrophoresised on a denaturing agarose gel. Following transfer to a nylon membrane, the blots were probed with DIG-dUTP labelled probes. **(B)** The expression of YCR012 in aerobic and anaerobically fermented cultures of S150. **(C)** The expression of YCR012 in aerobically and anaerobically fermented cultures of the lager strain CMBS-33. **(D)** The expression of YCR077 in aerobic and anaerobically fermented cultures of S150. **(E)** The expression of YCR077 in aerobic and anaerobically fermented cultures of the lager strain CMBS-33.

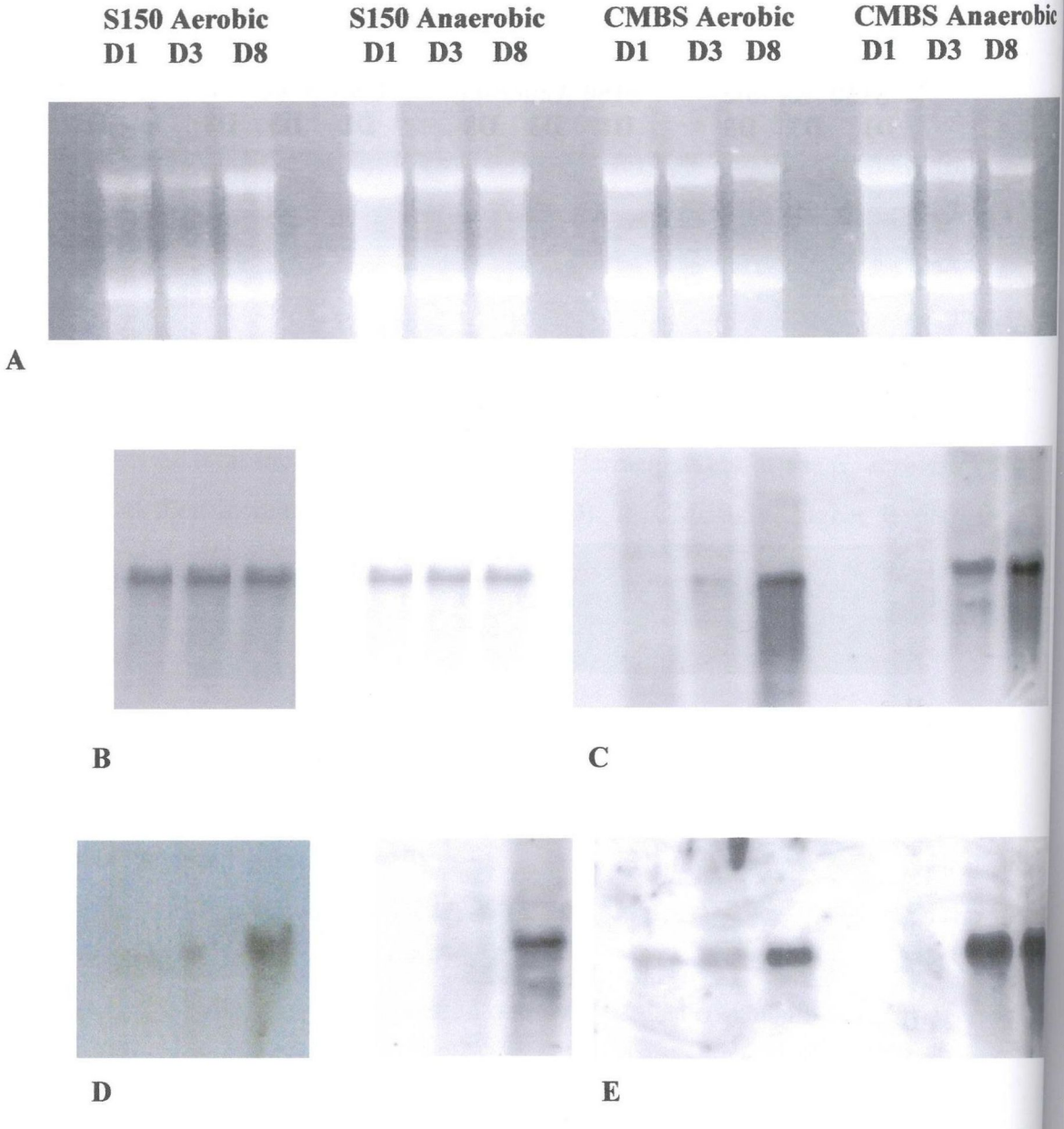


Figure 4.4 : Expression of the YCR031 and YCR057 genes by the laboratory strain S150 and the lager yeast strain CMBS-33. **(A)**Total RNA was extracted from cells at days 1, 3 and 8 of aerobic and anaerobic fermentations and 30µg were electrophoresised on a denaturing agarose gel. Following transfer to a nylon membrane, the blots were probed with DIG-dUTP labelled probes. **(B)** The expression of YCR031 in aerobic and anaerobically fermented cultures of S150. **(C)** The expression of YCR031 in aerobically and anaerobically fermented cultures of the lager strain CMBS-33. **(D)** The expression of YCR057 in aerobic and anaerobically fermented cultures of S150. **(E)** The expression of YCR057 in aerobic and anaerobically fermented cultures of the lager strain CMBS-33.

4.2.2. The differential expression of genes in polyploid strains of yeast

From this initial analysis of gene expression in the lager yeast strains the transcription profiles appear reflect the gene copy number of the homeologous alleles. To fully understand the nature of gene expression in the hybrid genome of the lager yeast strains, transcriptome microarrays have been performed, (James *et al.*, 2003, James *et al.*, 2002). Transcriptome studies have provided a picture of the gene expression contributed by the *S. cerevisiae*-like complement of genes in the genome, only as the *S. bayanus* complement does not hybridise under the conditions used.

The results obtained in Chapter 3, indicate that chromosome XVI in the lager yeasts lacks any *S.* cerevisiae genes in the region YPR160W – YPR190C and these strains contain at least four mosaic chromosome XVIs, each containing *S. bayanus* genes in this region. To determine if the copy number of *S. cerevisiae* genes can influence the expression profile of the *S. bayanus*-like genes, a strategy was employed in which a large section of one of the mosaic copies of chromosome XVI, on a YAC vector, was cloned into a series of isogenic polyploid *S. cerevisiae* strains, (Table 2.1). This allows the analysis of *S. bayanus* gene expression in a model system where the copy number of the *S. cerevisiae* allele can be varied while the *S. bayanus* allele remains constant.

Prior to conducting experiments with these strains, the DNA content of each strain was analysed using flow cytometric techniques. The cells were stained with propidium iodide (PI), a fluorescent biomolecule that binds to nucleic acids, as described in Chapter 2 section 2.21. There is a distinct difference in the DNA content in each of the isogenic strains, (Fig. 4.5).

Using the DNA sequence obtained from the sequencing of the junction at YPR159W – YPR160W as discussed in Chapter 3, section 3.2.3, specific lager yeast primers were designed which are used in this section of work, (Table 2.5). An initial set of universal primers were designed from the available sequences of *S. cerevisiae*, *S. bayanus* and the sequence obtained from the BAC clones as

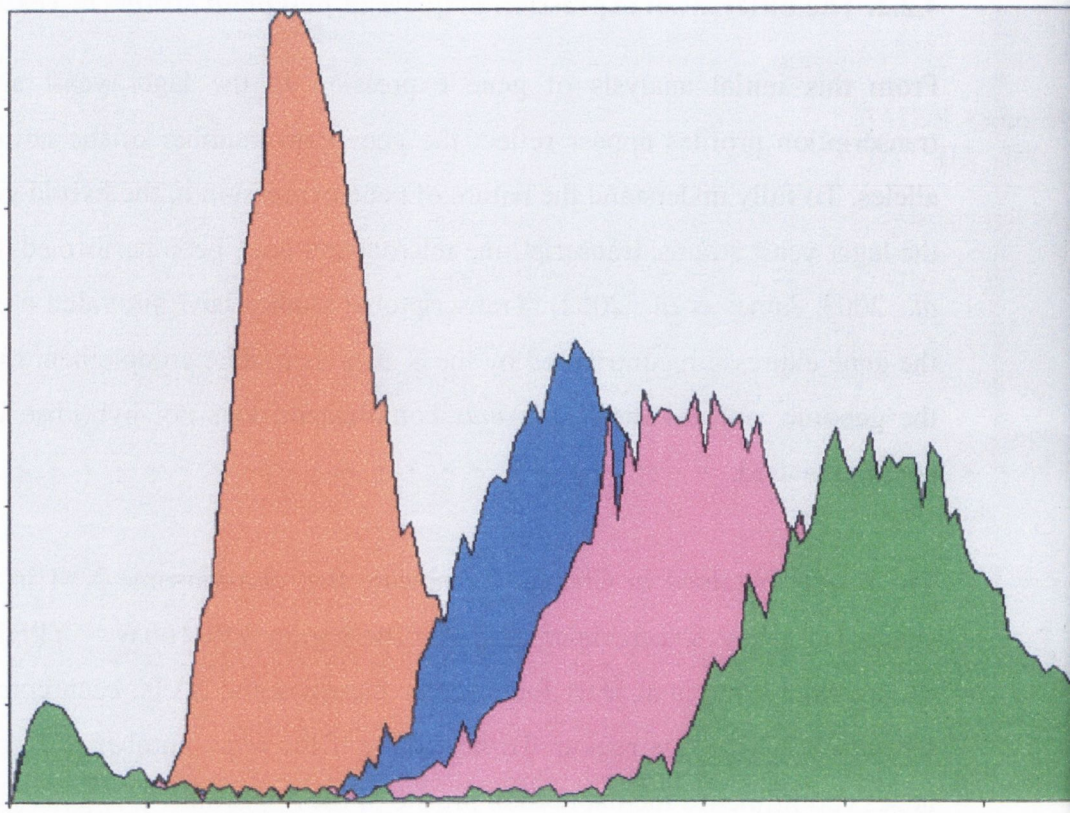


Figure 4.5 Graphical representation of the DNA contents of the isogenic ploidy cells.

The orange peak represents the haploid cells (n).
The blue peak represnts the diploid cells (2n).
The pink peak represents the triploid cells (3n).
The green peak represnts the tetraploid cells (4n).

described in Chapter 3. This universal set of primers allowed amplification of the YPR160W gene DNA from all three DNA backgrounds, *S. cerevisiae, S. bayanus* and Lager genomic DNA, (Fig. 4.6, lanes 1, 4, 7, 10 and 11). Likewise, this primer allows amplification of cDNA copies of RNA isolated from all three strains, (Fig. 4.6 lanes 2, 5 and 8). An experimental set of primers were also designed using the sequences obtained in Chapter 3 for the lager type specific ORF YPR160W. These primers should amplify the *S. bayanus*-like gene present in the lager yeast and not the *S. cerevisiae* alleles. PCR amplification of total genomic DNA and cDNA prepared from RNA isolated from the lager strain, CMBS-33 and the two parental strains were performed, (Fig. 4.7). It was observed that the ORF YPR160W is expressed at an RNA level in *S. bayanus* and in the lager yeast strain CMBS, (Fig. 4.7 lanes 5 and 8). However, reverse transcriptase PCR with the specific lager YPR160W primer set does not give a product in the *S. cerevisiae* strain, (Fig 4.7 lane 2), indicating that the primers can discriminate between the *S. cerevisiae* and *S. bayanus* like genes. It can be deduced that the DNA sequence for YPR160W ORF in the lager yeast is *S. bayanus*-like and that the gene is expressed in both *S. bayanus* and in CMBS-33.

Next the specific region of chromosome XVI containing the *S. bayanus* genes in the region of YPR160W – YPR190C from the CMBS-33 lager strain was recovered from the BAC clone; clone A11, as described in Chapter 2. The DNA was then ligated with a yeast artificial chromosome, (YAC) pYAC55. The YAC was introduced via electroporation into the various isogenic polyploidy *S. cerevisiae* strains. The pYac55 vector contains an ampicillin marker to which a specific set of primers were designed, (Table 2.5). After the transformed colonies were recovered, DNA was extracted and PCR performed to ensure that the transformation was successful, as can be seen in Figure 4.8. The original polyploid strains give no PCR product (data not shown), however, the transformed strains give a PCR product of 514bp, showing that the transformation was successful.

Using the universal set of primers to YPR160W, it was observed that a PCR product and a reverse transcriptase PCR product was obtained in the polyploid

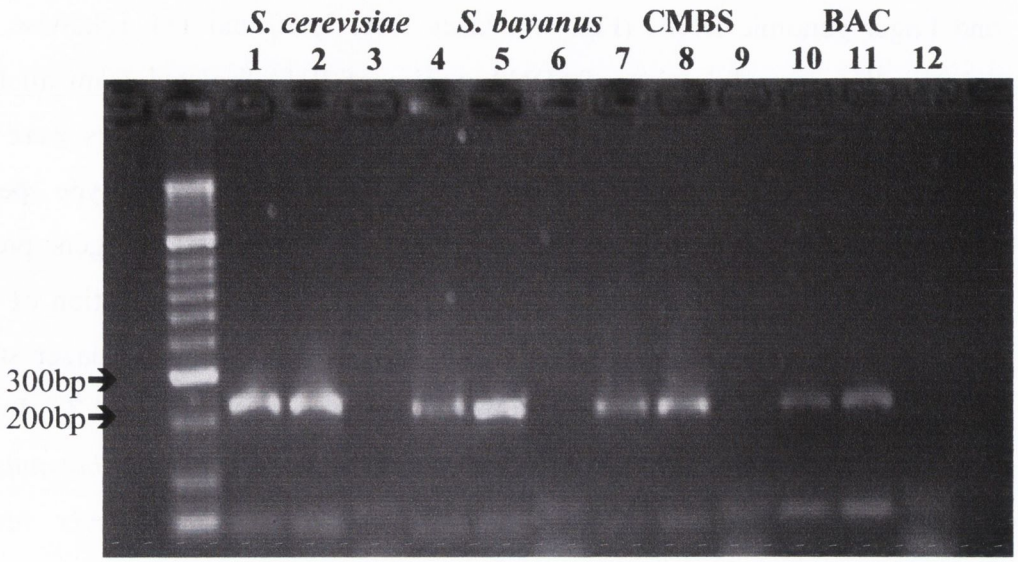


Figure 4.6: PCR products and RT-PCR products using the universal set of primers to the ORF YPR160W.
Lanes 1, 4, 7, 10, 11: PCR products (DNA). Lanes 2, 5, 8: RT-PCR (RNA). Lanes 3, 6, 9: Negative control (RT-PCR minus reverse transcriptase). Lane 12: PCR negative control for BAC clone (no DNA template).

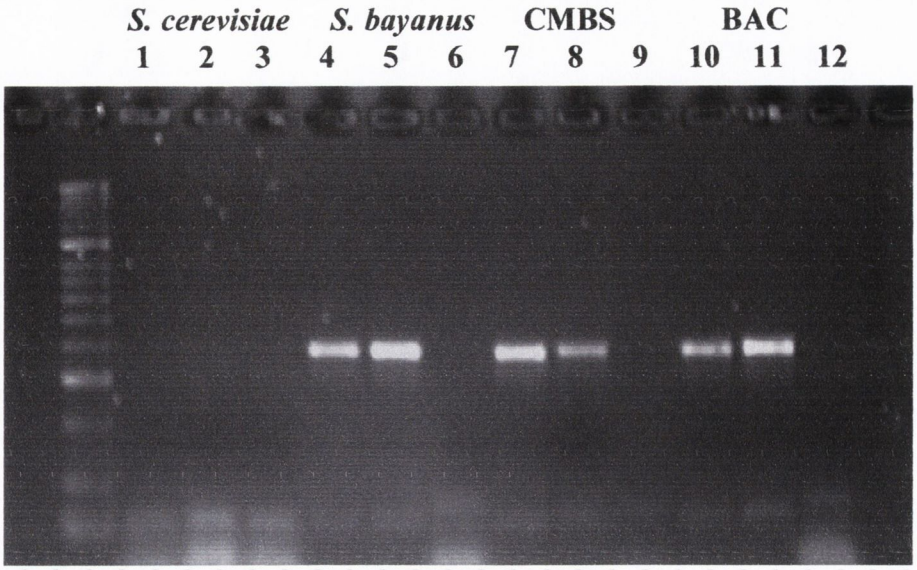


Figure 4.7: PCR products and RT-PCR products using the experimental set of primers obtained from sequencing of BAC clones for the ORF YPR160W. Lanes 1, 4, 7, 10, 11: PCR products (DNA). Lanes 2, 5, 8: RT-PCR (RNA). Lanes 3, 6, 9: Negative control (RT-PCR minus reverse transcriptase). Lane 12: PCR negative control for BAC clone (no DNA template).

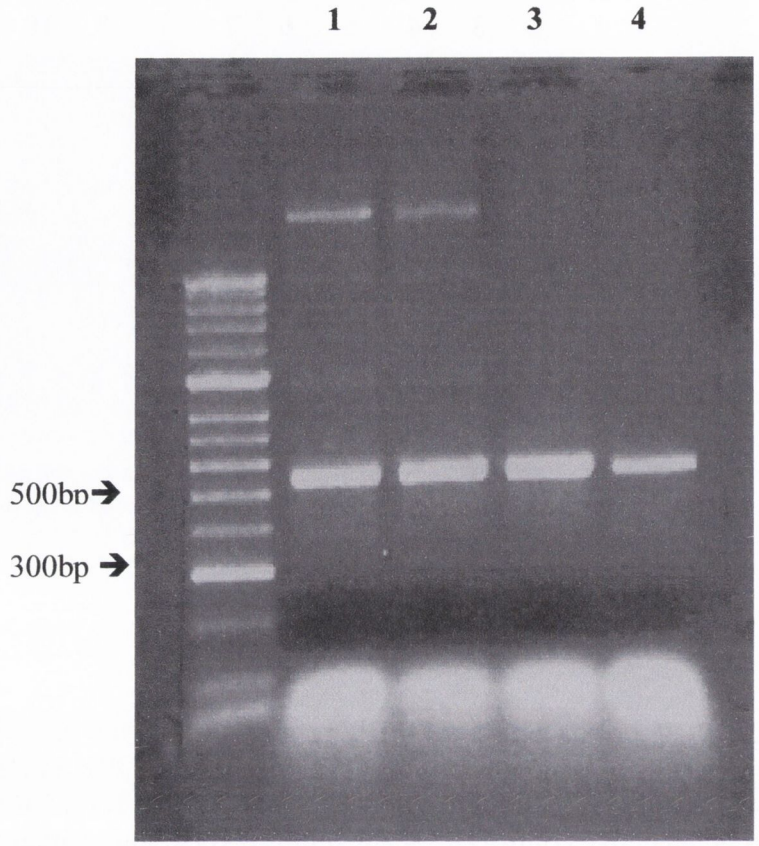


Figure 4.8: PCR amplification of the ampicillin gene using primers in Table 2.4 in isogenic polyploid *S. cerevisiae* strains transformed with the pYAC55 clones containing a region of chromosome XVI.
Lane 1: haploid strain, n. Lane 2: Diploid strain, 2n. Lane 3: Triploid strain, 3n. Lane 4: Tetraploid strain 4n.

strains either containing or lacking the added region of chromosome XVI from the lager yeast, (Fig. 4.9). It is also notable to see that the intensity of the bands for the reverse transcriptase PCR product is stronger than the band obtained in the PCR reactions, which was performed using total DNA extracts. This same experiment was performed using the lager specific set of primers and as expected no products were obtained for the polyploid strains, n, 2n, 3n and 4n, (Fig. 4.10). However in the case of the polyploid strains containing the region of chromosome XVI, it can be observed that there was a PCR product obtained for each strain, (Fig. 4.10). An interesting observation from this experiment was that the expression of the newly introduced *S .bayanus* – like genes decreases as the copy number of the *S. cerevisiae* genes increased. Following from this result, a set of *S. bayanus* – like primers were designed for the ORFs YPR161C, YPR175W and YPR186C, (Table 2.5) that represent the *S. bayanus* genes in the lager yeasts as discussed in Chapter 3.

PCR reactions performed using primer sets for YPR161W, YPR175W and YPR186C showed a similar pattern to that observed with YPR160W. In the case of the polyploid strains no products were apparent for any of the ORFs, (Figs. 4.11, 4.12 and 4.13 respectively). In the polyploid strain containing the introduced *S. bayanus* – like genes from the lager yeast genome, a PCR product was obtained for each of the ORFs, (Figs. 4.11, 4.12 and 4.13). In the case of the reverse transcriptase PCR reactions, a similar pattern was observed in the expression of the *S. bayanus* – like genes in each of the alter polyploid strains. There is a significant decrease in the expression of the *S. bayanus* – like genes as the *S. cerevisiae* genes copy number increases, (Figs. 4.11, 4.12 and 4.13). For the ORF YPR161C, which is involved in cyclin dependent protein kinase activity there is a low level of expression of the gene in the haploid strain, (n + XVI) which steadily decrease until no expression is observed in the tetraploid altered strain, (4n + XVI). In the ORF YPR175W, an essential gene that is required for normal chromosomal replication, the level of expression steady decreases as the *S. cerevisiae* gene copy number decreases. This is again the case observed in ORF YPR186C, another essential gene involved in transcription initiation from RNA

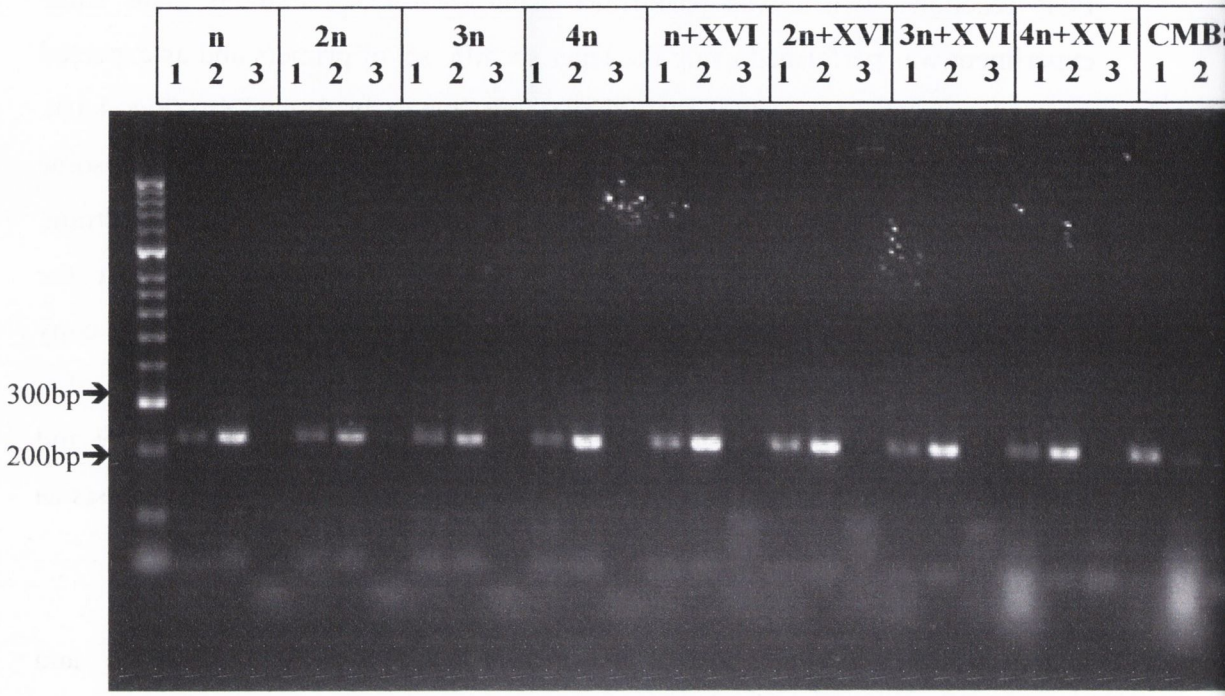


Figure 4.9 Universal Primer Set.
n represents haploid *S. cerevisiae* strain increasing to tetraploid, 4n. n+XVI represents the haploid *S. cerevisiae* strain with an additional chromosome XVI, containing *S. bayanus*-like genes from the lager yeast genome, CMBS-33.

1: PCR reaction, (DNA only). 2: RT-PCR reaction, (RNA only). 3: Negative control (RT-PCR reaction minus reverse transciptase).

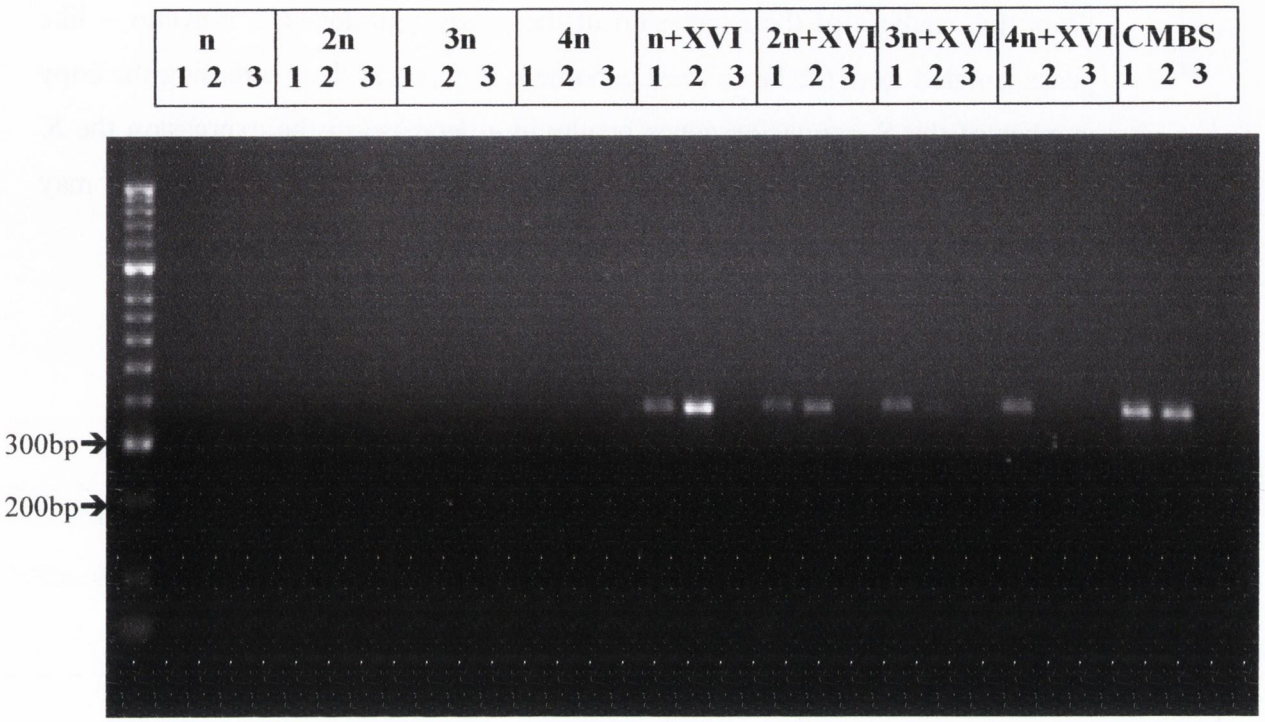


Figure 4.10: Experimental Primer set for ORF YPR160W.
n represents haploid *S. cerevisiae* strain increasing to tetraploid, 4n. n+XVI represents the haploid *S. cerevisiae* strain with an additional chromosome XVI, containing *S. bayanus*-like genes from the lager yeast genome, CMBS-33.

1: PCR reaction, (DNA only). 2: RT-PCR reaction, (RNA only). 3: Negative control (RT-PCR reaction minus reverse transciptase).

polymerase III promoter, however it appears that it is not until the triploid strain (3n + XVI) that the decrease in the level of gene expression become apparent.

From this analysis of the expression of the newly introduced *S. bayanus* – like genes isolated from the lager yeast genome it is apparent that increasing the copy number of the *S. cerevisiae* genes results in a decrease in the expression the *S. bayanus* – like homeologous genes. This indicates that gene dosage effect may prevent the over-expression of genes in polyploid strains of yeast.

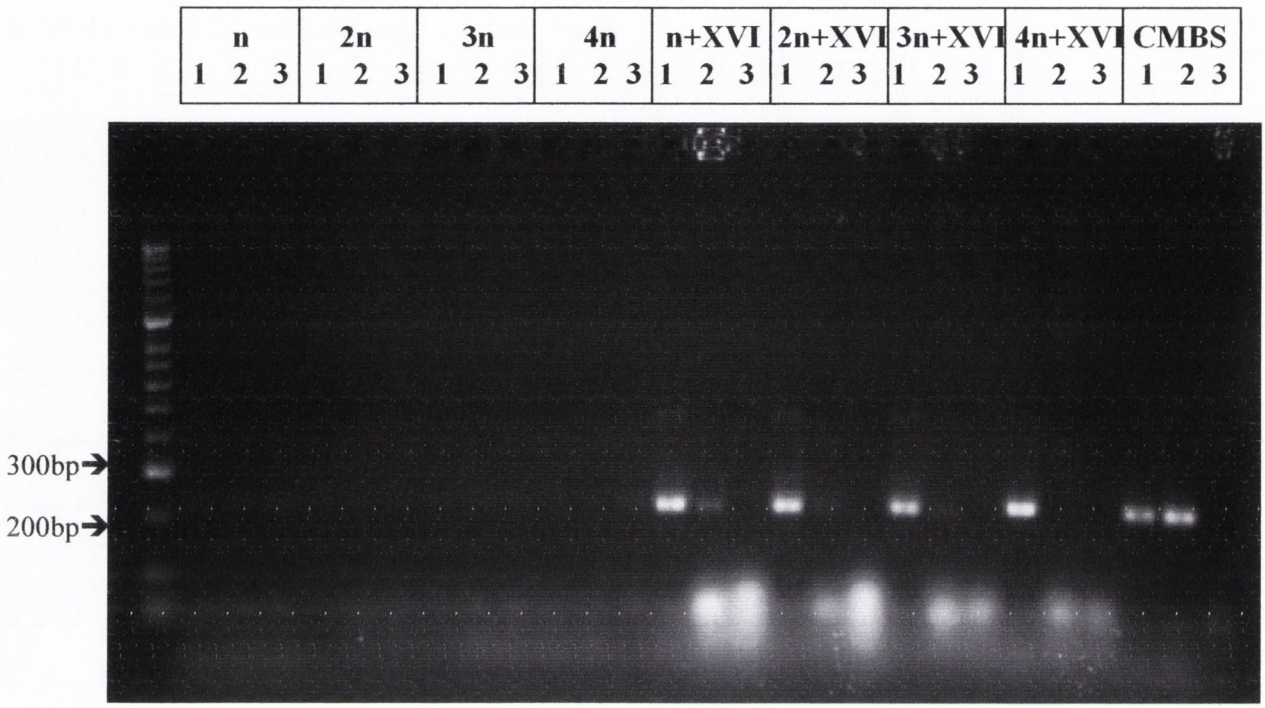


Figure 4.11: *S. bayanus*-like YPR161W primer set.
n represents haploid *S. cerevisiae* strain increasing to tetraploid, 4n. n+XVI represents the haploid *S. cerevisiae* strain with an additional chromosome XVI, containing *S. bayanus*-like genes from the lager yeast genome, CMBS-33.

1: PCR reaction, (DNA only). 2: RT-PCR reaction, (RNA only). 3: Negative control (RT-PCR reaction minus reverse transciptase).

4.3 Discussion

In examining the expression of homologeous genes, the gene YCR012C was chosen as a probe as it plays a major role in catalysing the transfer of high-energy phosphoryl groups from the acyl phosphate of 1,3-bisphosphoglycerate to ADP to produce ATP and is a key enzyme in glycolysis and gluconeogenesis. Gluconeogenesis is the process whereby glucose is synthesised from non-carbohydrate precursors, which enable the yeast cells to grow on non-sugar carbon sources such as ethanol and glycerol. The reactions of gluconeogenesis, mediates the conversion of pyruvate to glucose, which is the opposite of glycolysis. YCR012C (PGK1) is abundantly expressed in cells growing in glucose, and transcription is increased by heat shock. In contrast, mRNA levels are low in cells grown in pyruvate, acetate, or lactate, although the message stability is not affected by the carbon source. Transcription is activated by the transcription factors Rap1p, Abf1p and Reb1p, which each bind to sequences in the PGK1 promoter. As PGK1 is a highly expressed gene and its mRNA is relatively stable, an increasing expression of the gene is observed in the lager yeast strains during a fermentation. In contrast, there is no increase in gene expression in the laboratory strain S150. The level of mRNA was much higher in the CMBS-33 strain compared to the *S. cerevisiae* haploid strain, even though the same amount of total RNA was loaded on the gel. This suggests that both *S. cerevisiae* alleles in the lager strain are being expressed. Alternatively, other steps in mRNA biogenesis may have been altered to account for the increased level of expression.

The other probe from the left of the MAT locus YCR031C (RPS14A), a ribosomal protein of the small subunit and is required for ribosome assembly and 20S pre-rRNA processing. In rapidly growing yeast cells, approximately 60% of all transcription is devoted to ribosomal RNA biogenesis. The regulation of rRNA genes is affected by nutritional conditions and a number of signal transduction pathways that can induce or silence the ribosomal genes. These transcripts have a short lifetime, which leads to major implications for the expression of other genes. In the analysis performed, in the lager yeast strain there is a low level of

expression of this gene in days 1 and 3 of fermentations however, there is a significantly higher level of expression in day 8 in both the aerobic and anaerobic fermentations. However, in the laboratory strain, there is constitutive expression of the gene. Thus despite the fact that both strains were grown under identical environmental conditions the temporal pattern of expression differs. This suggests that biogenesis of this mRNA is differentially regulated in the two strains.

The probes from the right of the MAT locus were YCR057C (PWP2), which is a conserved 90S pre-ribosomal component essential for the proper cleavage of the 35S rRNA. The gene also interacts with YGR154C (GTO1) under oxidative stress. The second probe used was YCR077C (PAT1), which is required for faithful chromosome transmission, the maintenance of rDNA locus stability. In the DNA microarrays it was observed that the genes to the right of the MAT locus had a higher ratio of hybridisation in the lager strains of yeast, however this did not reveal any information about the level of gene expression. For the lager yeast strain, CMBS-33, it was estimated that there are two copies of *S. cerevisiae*-like genes to the left of the MAT locus and two copies of *S. bayanus*- like, while there are four copies of *S. cerevisiae*-like genes to the right of the MAT locus.

In this section, I wanted to determine if an increased copy number of a gene or series of genes also resulted in an increased level of gene expression. It was clearly seen in Figures 4.3 and 4.4, that the expression of the genes in the lager yeast were affected by the gene copy number as higher levels of expression were observed. It was also noted that the gene expression was different between the aerobic and anaerobic fermentations. However, the rationale used in this series of experiments may not have been ideal as in using the *S. cerevisiae* strain S150, which is a haploid strain, we are only observing how the expression of one copy of the gene is affected under the brewing conditions. We also do not observe the contribution from the *S. bayanus* genome in the gene expression.

Furthermore since the final steady state levels as depicted by Northern blots can be influenced by numerous variables such as environment, temporal expression, it is difficult to determine if the increased level of expression in CMBS-33 is solely

due to gene copy number. Nevertheless the significant increase in steady state levels of the RNAs in CMBS-33 suggests that all the *S. cerevisiae* alleles are expressed. To avoid the problems associated with the analysis of gene expression in the lager yeasts an alternative strategy was developed. A model system was established in which the lager gene sequences obtained from the BAC clone sequencing, as discussed in Chapter 3, were used to design specific primer sets for the lager yeast genome. The experimental set of primers were therefore specific to the lager yeast sequence obtained. Using this primer set it was possible to determine, if the YPR160W lager specific gene was firstly expressed in the CMBS lager strain and secondly by looking at the gene expression in increasing isogenic ploidy *S. cerevisiae* strains to determine if there was a gene dosage effect that would prevent the over-expression of genes in the lager yeast genome that contains mosaic chromosomes. From this analysis and the analysis of three other essential genes on chromosome XVI, in the region of low hybridisation to the *S. cerevisiae* DNA microarray chip, where the sequence has significantly diverged and is now *S. bayanus*-like, it was deduced that the increasing copy number of *S. cerevisiae* genes caused a decrease in *S. bayanus* – like gene expression.

Therefore it can be proposed that in the lager yeast genome there is a gene dosage affect that prevents the over-expression of genes that are present in both *S. cerevisiae*-like and *S. bayanus* – like copies in the genome. The decrease in expression of *S. bayanus*-like genes as the *S. cerevisiae* gene copy number increases may be due to the requirement for specific transcription factors contributed to the genome from *S. bayanus* that are not present in the *S. cerevisiae* genome. Therefore, when one copy of the lager yeast chromosome XVI was introduced into the isogenic polyploid *S. cerevisiae* strains, the genes did not have the specific transcription factors expression and perhaps compete poorly for the *S. cerevisiae* transcription factors. To obtain a definitive picture of gene expression in the lager yeast strains, it would be necessary to perform Northern blot analysis where one looks at the gene expression of the parental strains *S. cerevisiae* and *S. bayanus* in conjunction with the lager yeast strain.

Chapter 5
Generation of Stress Tolerant Lager Yeasts.

5.1 Introduction

A goal of the brewing industry is to pursue high gravity fermentation. The main reasons for using this technology include increasing the capacity of the brewery, a reduction in energy costs and improved microbiological stability and the production of more alcohol per unit of fermentable extracts. While performing high gravity fermentation, yeast encounters several stresses like high osmolarity, oxidative stress, ethanol stress, nutrient starvation and temperature stress (Jones and Greenfield, 1987; Jacobsen and Piper, 1989). As a result, poor yeast crop viability (Cahill *et al.*, 2000) and a stuck or sluggish fermentation are experienced (D'Amore, 1992; Patkova *et al.*, 2000). Another disadvantage of this method is an undesirable change in the flavour profile of the beer (Meilgaard, 2001). Lager beer strains are allopolyploids where at least two genomes co-exist as natural hybrids of *S. cerevisiae* and non-*S. cerevisiae* species. These industrial yeast strains sporulate poorly and as a result the breeding of industrial yeasts by crossing haploid strains is not common. Currently, no autotrophic mutants have been characterised in industrial yeast strains and the many dominant drug resistance markers designed for use in the absence of auxotrophic mutations cannot be used freely in industrial yeasts due to concerns of consumers about this aspect of genetically modified organisms.

One strategy towards improving high gravity brewing is the generation of yeast strains with increased stress tolerance. An initial strategy involved, EMS (Ethyl methanesulfonate) mutagenesis of the brewery yeast strain, CMBS-33, followed by the selection of strains with ability to withstand high gravity brewing conditions (James, Campbell & Bond, Unpublished data). The mutagenesis conditions were such that at least one hit per gene was expected. The exact gene copy number for the CMBS-33 parent strain was not known at the time and the assumption was made that it was at least a diploid strain. Since the traits that were to be selected may have been multigenic, at least 90% survivability following mutagenesis was required.

Initially, the selection involved fermentation of the mutagenised cells for 8 days in high gravity wort (20°P) followed by plating on to YEPM plates containing

15% alcohol. This selection turned out to be too harsh since there were no resistant colonies. This may have been because high gravity brewing combined with selection on 15% alcohol was too stressful for the yeast (James, Campbell and Bond, Unpublished data). One way these cells may survive in such harsh situations is by modifying their stress-response system. Usually, a specific acquired stress-tolerance (for example to heat shock stress) offers protection from other forms of stress (for example chemical stress), a phenomenon known as 'cross-tolerance' (Bond & Schlesinger, 1987). Usually the acquired thermotolerance is transient in nature, however stress tolerant human cell lines have been generated that maintain tolerance to the initial stress and cross tolerance to other stresses over many generations. Molecular analysis revealed that heat shock proteins (HSPs), in particular Hsp27 is constitutively expressed in these cell lines.

A similar rationale was used by James, Campbell and Bond to identify stress tolerant strains of lager yeasts. The hypothesis put forward was that stress tolerant lager yeasts may acquire increased viability and thus may survive the stressful conditions of high gravity brewing, therefore potentially allowing for higher fermentation rates.

The first generation of stress tolerant strains were selected by treatment with EMS. Following mutagenesis, the cells were allowed to recover and then were subjected to a heat stress treatment by placing the cells at 45°C, 50°C or 55°C for 10 minutes. The cells were then plated onto high wort agar plates (1.14g/ml). Surviving clones from the 55°C heat treatment were subjected to a second round of heat treatment and the resistant colonies were re-grown and the process repeated for a third time. Out of the 29 colonies that survived the initial heat shock only 6 showed repeated survival through the 55°C treatment. Three of these mutants designated C5B, C6B and C10B were analysed to determine if the increased thermotolerance had any effect on their ability to ferment in high gravity wort discussed in section 5.2.1. and at a molecular level to characterise the genomic differences between the parental and mutant strains. A complete

description of the first generation mutants is currently submitted for publication, (James, Usher, Campbell and Bond, Submitted).

In this chapter, characterisation of this first generation series of mutants and the isolation and characterisation of a second generation set of stress tolerant lager yeast strains is described. The purpose of these experiments were to test if stress tolerant strains could be generated in the absence of EMS and to determine if the new mutants are more tolerant to stresses and if they are better adapted to stress than the parent strains. The ability of the mutants to maintain the acquired tolerance to heat shock over many generations, was examined as was the ability of the mutants to develop any cross tolerance to other stress conditions.

5.2 Results.

5.2.1 Isolation of Second Generation Stress Tolerant Strains of Brewery Yeasts.

Analysis of the first generation of stress tolerant strains which had been generated using a combination of EMS treatment and selection for tolerance to exposure to high temperatures and high specific gravity wort, indicated that the isolated strains observed that they had undergone chromosomal rearrangements. Since, EMS treatment has been shown to induce point mutations it was proposed that the combined stress conditions used for selection may be responsible for induced gross chromosomal changes. Therefore to test if exposure to severe stress conditions such as high temperatures is sufficient to induce chromosomal rearrangements a second generation of stress tolerant mutants was generated using the following strategy. The first generation stress tolerant strains, C5, C6 and C10 along with the parent strain CMBS were grown on fresh YEPM plates and individual colonies picked. An aliquot of the cells was exposed to heat stresses of 45°C, 51°C and 55°C for 15 minutes. Following the heat stress the cells were plated onto YEPM agar plates and incubated at 30°C overnight, Table 5.1 shows the numbers of survivors from each round of heat shock treatment. One colony from each temperature class was picked and the stress selection repeated twice more. After the final round of heat stress the fermentation profiles of a number of the surviving clones were analysed under high gravity brewing conditions and compared to the profiles of their original parental strains.

5.2.2 Analysis of first generation stress - tolerant strains C5B C6B and C10B.

Firstly, the fermentation profiles of the three first generation stress tolerant strains, C5B, C6B and C10B were compared to the parental strain, CMBS-33 in 16°P and 20°P wort. The mutants C6B and C10B fermented as well the parental strain at high gravity fermentation, with C6B showing a faster sugar utilisation during the initial phase of fermentation. However the mutant C5B fermented slower than the parental strain and retained a higher sugar content than the

Table 5.1: Survivors from three rounds of heating shock treatment in the generation of the Second generation of stress mutants.

Round 1

CMBS	R.T.	45	51	55
10^6	T.M.T.C.	T.M.T.C.	T.M.T.C.	2
10^5	T.M.T.C.	T.M.T.C.	T.M.T.C.	1
10^4	T.M.T.C.	38	16	0
10^3	T.M.T.C.	14	4	0

C5B	R.T.	45	51	55
10^6	T.M.T.C.	T.M.T.C.	T.M.T.C.	0
10^5	T.M.T.C.	T.M.T.C.	T.M.T.C.	0
10^4	T.M.T.C.	36	18	1
10^3	35	21	1	0

C6B	R.T.	45	51	55
10^6	T.M.T.C.	T.M.T.C.	T.M.T.C.	2
10^5	T.M.T.C.	T.M.T.C.	39	1
10^4	T.M.T.C.	30	7	0
10^3	23	19	1	0

C10B	R.T.	45	51	55
10^6	T.M.T.C.	T.M.T.C.	T.M.T.C.	3
10^5	T.M.T.C.	T.M.T.C.	38	0
10^4	T.M.T.C.	35	5	0
10^3	21	17	1	0

Round 2

CMBS	R.T.	45	51	55
10^6	T.M.T.C.	T.M.T.C.	>1000	6
10^5	T.M.T.C.	>1000	>100	1
10^4	T.M.T.C.	>100	18	0
10^3	T.M.T.C.	14	2	0

C5B	R.T.	45	51	55
10^6	T.M.T.C.	>1000	>1000	8
10^5	T.M.T.C.	>100	>100	1
10^4	T.M.T.C.	54	14	0
10^3	T.M.T.C.	21	4	0

C6B	R.T.	45	51	55
10^6	T.M.T.C.	>1000	>100	19
10^5	T.M.T.C.	>100	>100	5
10^4	T.M.T.C.	29	5	0
10^3	32	19	1	0

C10B	R.T.	45	51	55
10^6	T.M.T.C.	>1000	>1000	9
10^5	T.M.T.C.	>100	68	1
10^4	T.M.T.C.	30	7	0
10^3	28	35	1	0

Round 3

CMBS	R.T.	45	51	55
10^6	T.M.T.C.	T.M.T.C.	24	0
10^5	T.M.T.C.	46	11	0
10^4	58	22	3	0
10^3	42	11	1	0

C5B	R.T.	45	51	55
10^6	T.M.T.C.	>100	36	0
10^5	T.M.T.C.	59	11	0
10^4	68	15	5	0
10^3	12	2	1	0

C6B	R.T.	45	51	55
10^6	T.M.T.C.	56	51	0
10^5	T.M.T.C.	12	25	0
10^4	56	2	1	0
10^3	38	0	0	0

C10B	R.T.	45	51	55
10^6	T.M.T.C.	11	12	0
10^5	T.M.T.C.	6	7	0
10^4	38	2	4	0
10^3	3	0	1	0

T.M.T.C.- too many to count, plates had a lawn of cells.

parental strain or the other mutant strains. The haploid *S. cerevisiae* (S-150) and *S. bayanus* strains showed poor fermentation profiles, indicating that individually these strains do not show an inherent high fermentative capacity. Thus the high fermentative capacity of the lager yeasts appears to arise from the polyploid nature resulting from the natural fusion of a *S. cerevisiae* and *S. bayanus* strain.

In fermentations carried out at 16°P wort, the mutant C10 performed consistently better than C5 and C6, and the parental strain as seen in Figures 5.1 and 5.2. Interestingly, the parental strain CMBS-33 showed good fermentation profiles in both high and low gravity fermentations. Taken together the data shows that the first generation mutant C10B had better fermentation profile than the parental strain CMBS-33, this was especially apparent when looking at the end-point values for the fermentations, (Table 5.2).

5.2.3 Analysis of second generation stress tolerant strains.

The fermentation profiles of the second generation of thermotolerant mutants were now compared to the first generation parental mutants under high gravity wort conditions. Thermotolerant mutants, CMBS-45 and CMBS-51 of the original parental strain CMBS-33 were also compared, (Fig.5.3). The nomenculture used refers to the temperature at which the strain was selected. From the graph it can be seen that after day 3 the thermotolerant strains perform better than the parental strain in high gravity wort. However all strains reach a similar end point with CMBS having a final brix value of 5.5 and an end point of 5.2 and 4.9 for CMBS-45 and CMBS-51 respectively.

For each of the original mutants, two thermotolerant strains were generated and their fermentation profiles compared with their parent at high gravity. The thermotolerant strain C5B-51 showed a faster initial rate of fermentation however at the end of fermentation there was no statistical difference between the strains; this was also the case for C6B and its thermotolerant strains, (Fig. 5.4 and 5.5 respectively). There is a more pronounced difference between the C10B strain and C10B45 and C10B51 the two new thermotolerant strains, (Fig.5.6). The end

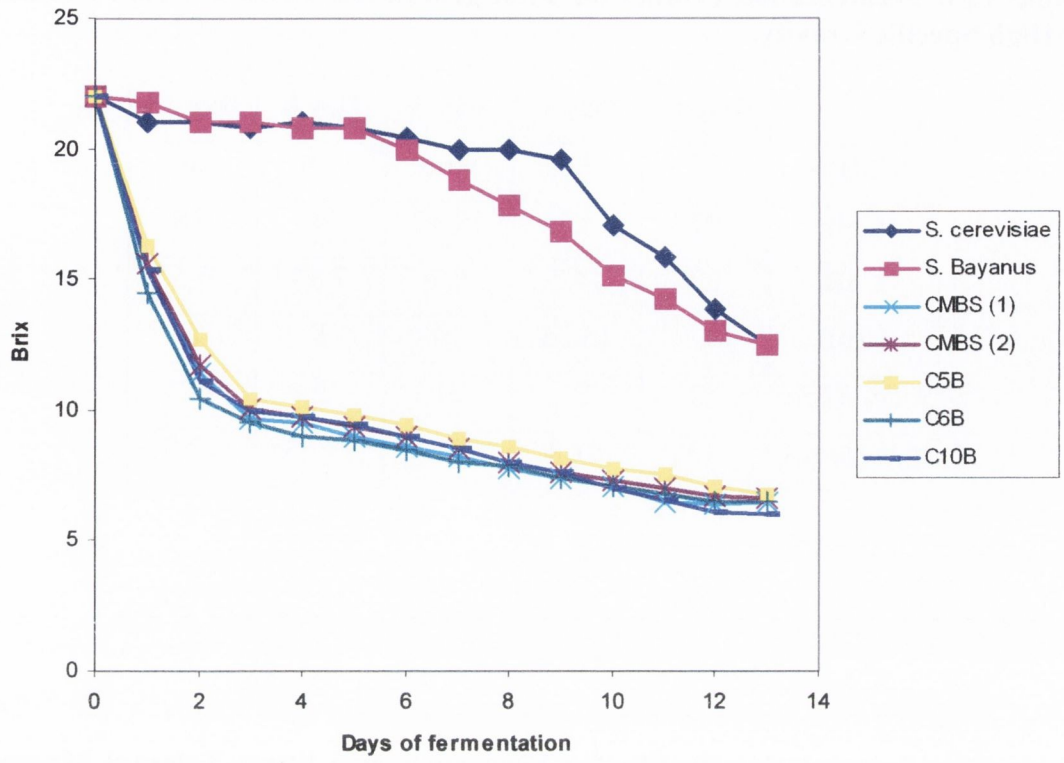


Figure 5.1 High gravity fermentations of parental strain CMBS and mutants C5, C6 and C10. *S. cerevisiae* and *S. bayanus* were used as non-lager strain controls. The fermentation progress was measured daily using a refractometer.

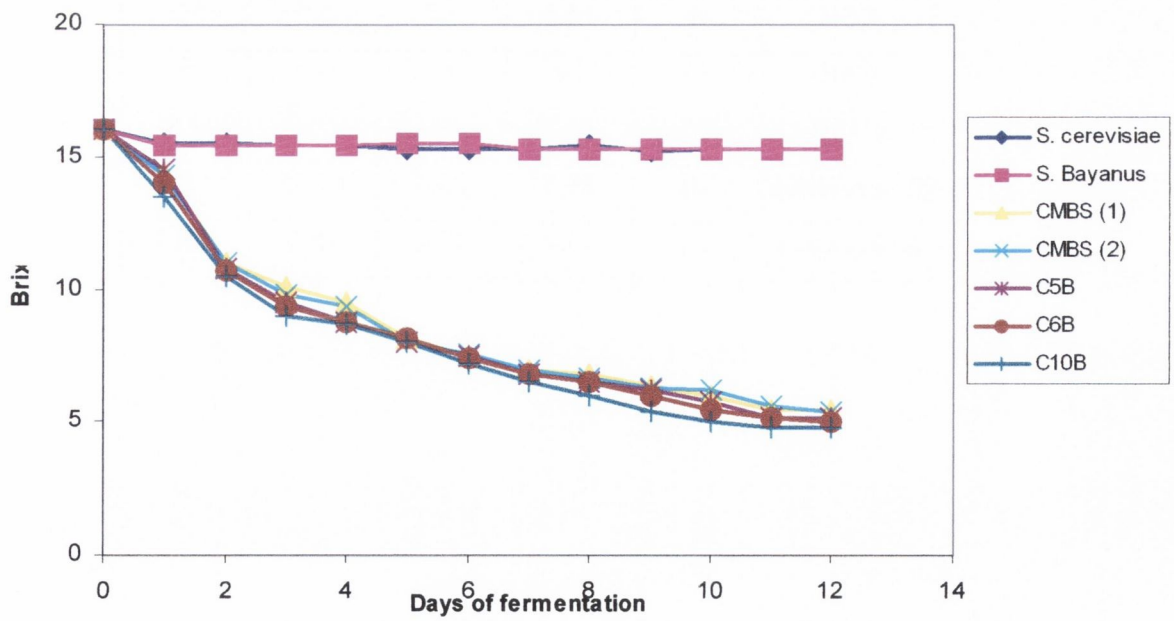


Figure 5.2 Low gravity fermentations of parental strain CMBS and mutants C5, C6 and C10. *S. cerevisiae* and *S. bayanus* were used as non-lager strain controls. The fermentation progress was measured daily using a refractometer.

Table 5.2a: Fermentation Profiles for First generation Stress Tolerant Mutants at High Specific Gravity.

	Day 0	Day 1	Day 3	Day 8	Day 13
CMBS	22	15.63	9.66	7.8	6.63
C5B	22	16.33	10.43	86	6.8
C6B	22	14.5	9.5	7.83	6.5
C10B	22	15.36	10	8	6
S. cerevisiae	22	21	21	20	12.5
S. bayanus	22	21.8	21	17.83	12.5

Table 5.2a: Fermentation Profiles for First generation Stress Tolerant Mutants at Low Specific Gravity.

	Day 0	Day 1	Day 3	Day 8	Day 14
CMBS	16	14.4	9.8	6.8	5.4
C5B	16	14.5	9.5	6.5	5.2
C6B	16	14	9.4	6.5	5
C10B	16	13.5	9	6	4.8
S. cerevisiae	16	15.5	15.4	15.35	15.3
S. bayanus	16	15.4	15.4	15.3	15.3

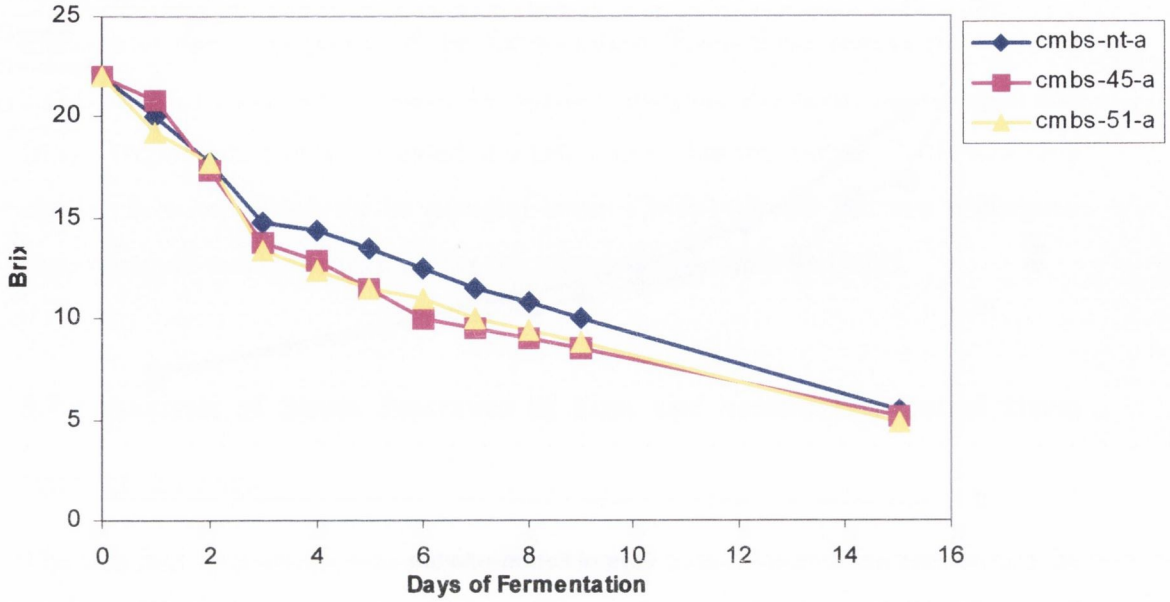


Figure 5.3 High gravity fermentation profiles of parental strain CMBS-33 and second generation thermo – tolerant survivors CMBS – 45 and CMBS – 51. Progress of fermentations was measured daily.

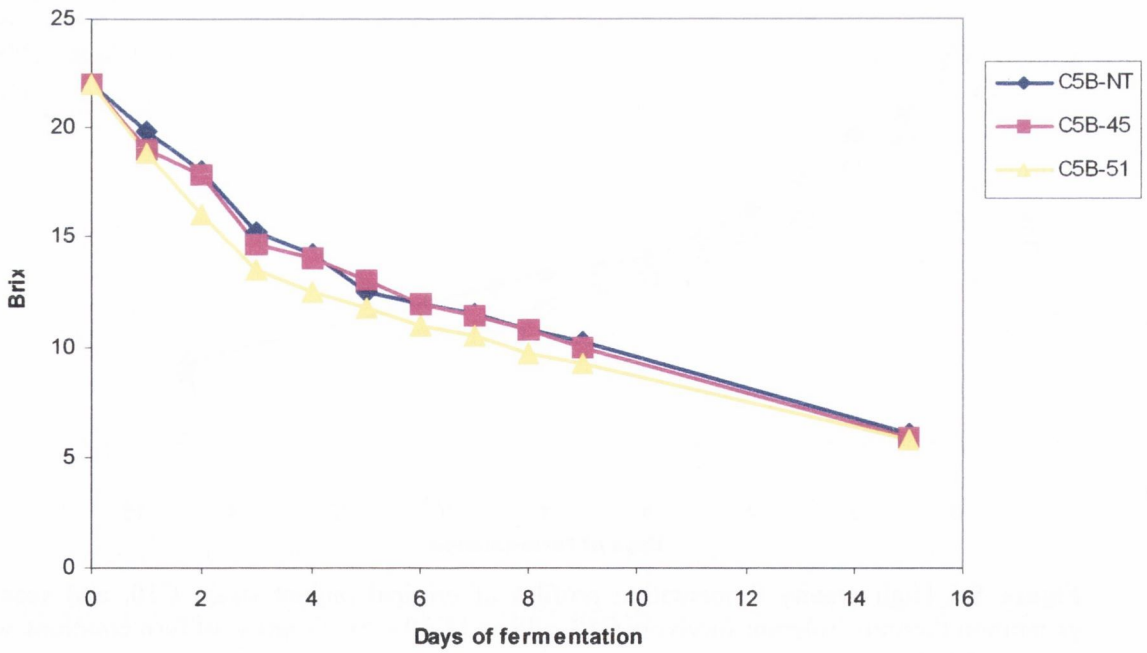


Figure 5.4 High gravity fermentation profiles of original mutant strain C5, and second generation thermo – tolerant survivors C5 – 45 and C5 – 51. Progress of fermentations was measured daily.

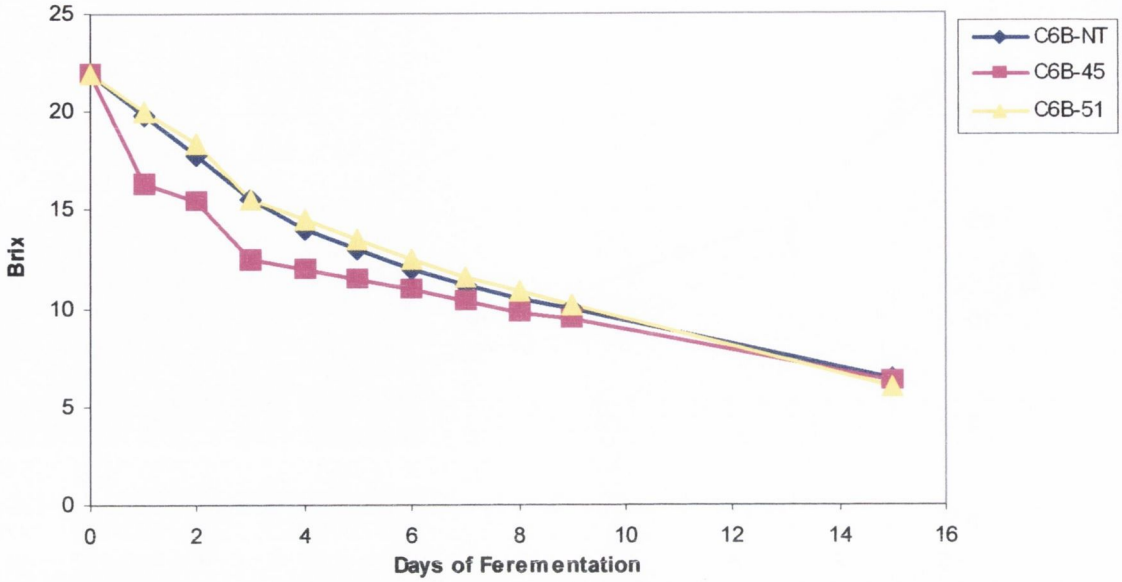


Figure 5.5 High gravity fermentation profiles of original mutant strain C6, and second generation thermo – tolerant survivors C6 – 45 and C6 – 51. Progress of fermentations was measured daily.

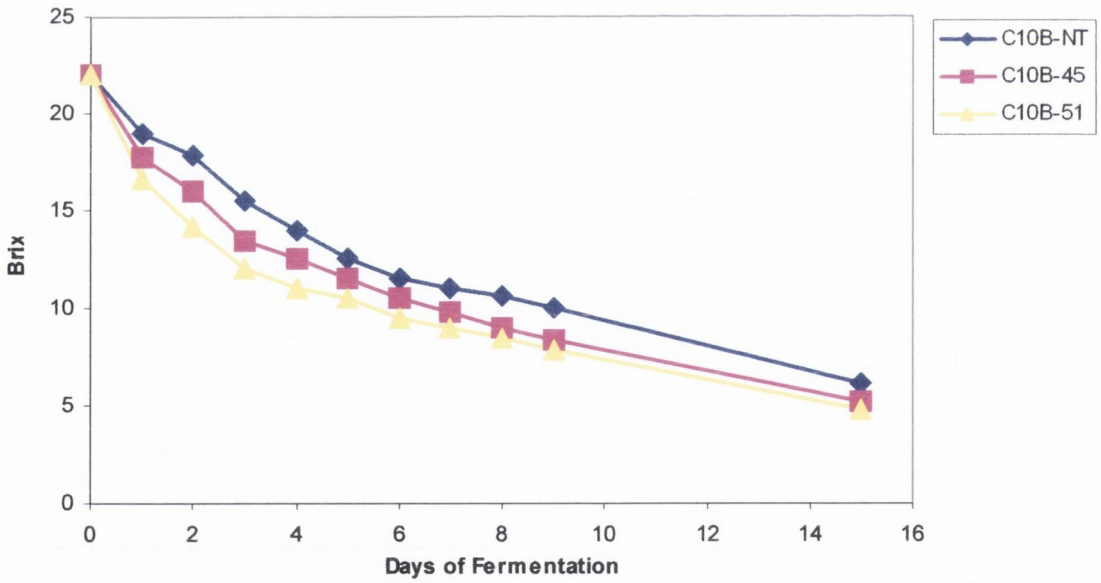


Figure 5.6 High gravity fermentation profiles of original mutant strain C10, and second generation thermo – tolerant survivors C10 – 45 and C10 – 51. Progress of fermentations was measured daily.

point brix values for the strains C10B, C10B45 and C10B51 were 6.1, 5.2 and 4.8 respectively. There is a marked increase in the fermentative capacity of the mutants in the early phase of the fermentation. From these results the mutants C10B and C10B51 were chosen for further analysis. Previous data (James and Bond, Unpublished data) revealed at a DNA level that the mutant C10B was very similar in composition to the parental strain CMBS-33 and had not undergone chromosomal rearrangement unlike the mutant strains C5B and C6B.

5.2.4 Analysis of Stress Tolerance of First and Second Generation stress tolerant mutants.

The original hypothesis was designed to test if stress tolerant strains would be cross tolerant to other forms of stress. Therefore we examined the ability of the heat tolerant strains to survive under oxidative stress conditions. The parental lager strain CMBS-33 was also tested as along with its second generation thermotolerant mutants. From the data presented in Figure 5.7 it can be seen that the parental strain (CMBS-33) is extremely tolerant to high concentrations of hydrogen peroxide (4 mM, 5 mM and 10 mM hydrogen peroxide) as is the thermotolerant strain CMBS-45. However, the strain CMBS-51 shows total inhibition of growth at just 4 mM hydrogen peroxide and at all higher concentrations.

Surprisingly, the first generation stress tolerant mutant C5B has lost its tolerance to oxidative stress and is completely inhibited at all concentrations of hydrogen peroxide, (Fig. 5.8). However, the second generation thermotolerant strains C5B45 and C5B51 have regained tolerance. The thermotolerant strains show a lesser degree of growth inhibition, with C5B45 being able to tolerate the stress better than C5B51, however at 10 mM hydrogen peroxide C5B51 is better able to tolerate the oxidative stress.

As with the mutant C5, the first generation stress tolerant mutant C6 mutant has also lost tolerance for oxidative stress, (Fig. 5.9). The second generation strains C6B45 has regained this tolerance, however the mutant C6B51 showed low

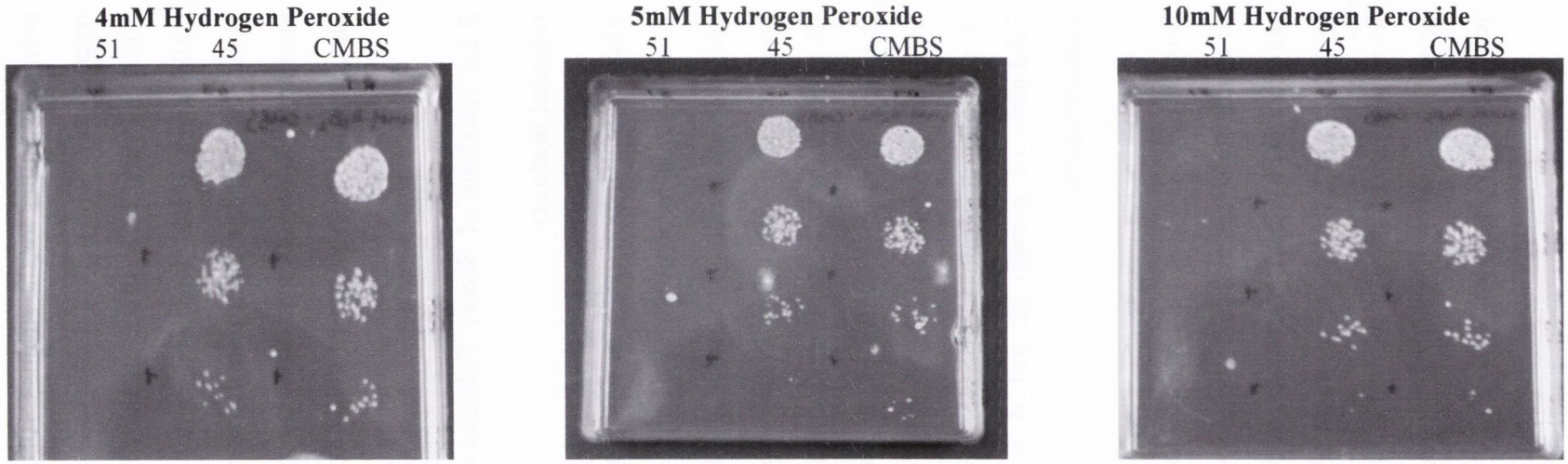


Figure 5.7 CMBS Hydrogen Peroxide Plates

Oxidative stress of parental strain CMBS and its thermo-tolerant mutants CMBS-45 and CMBS-51. Cells were exposed to media supplemented with increasing concentrations of hydrogen peroxide and incubated at 30°C overnight.

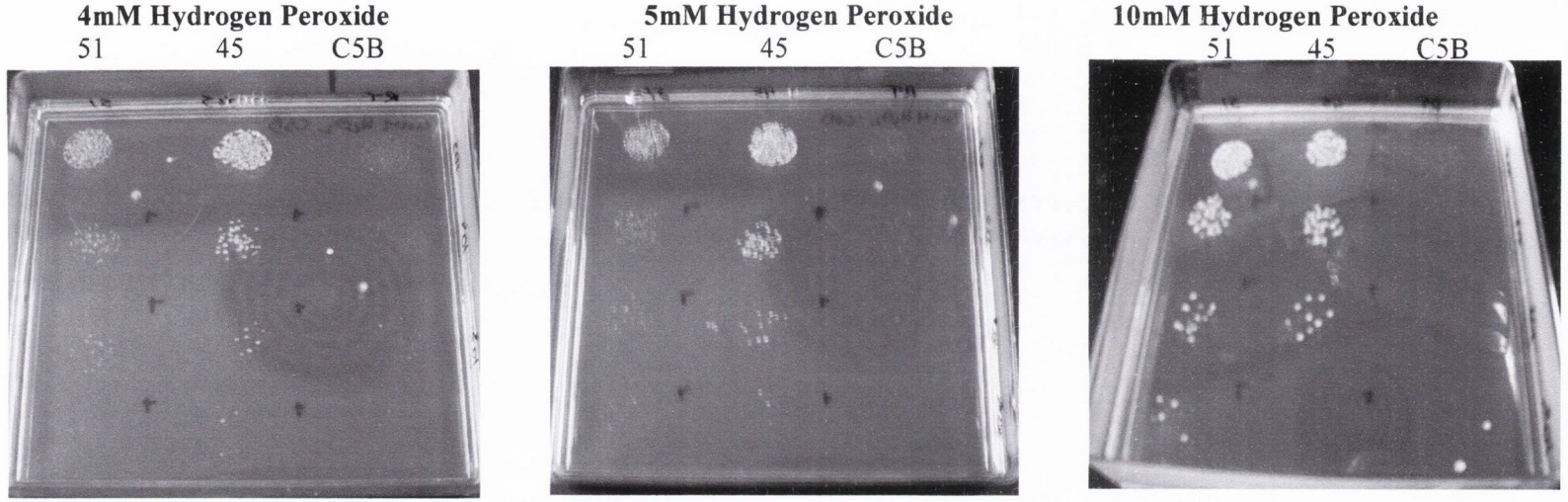


Figure 5.8 C5B Hydrogen peroxide plates

Oxidative stress for first generation stress tolerant mutant C5B and its second generation thermo-tolerant mutants C5B-45 and C5B-51. Cells were exposed to media supplemented with increasing concentrations of hydrogen peroxide and incubated at 30°C overnight.

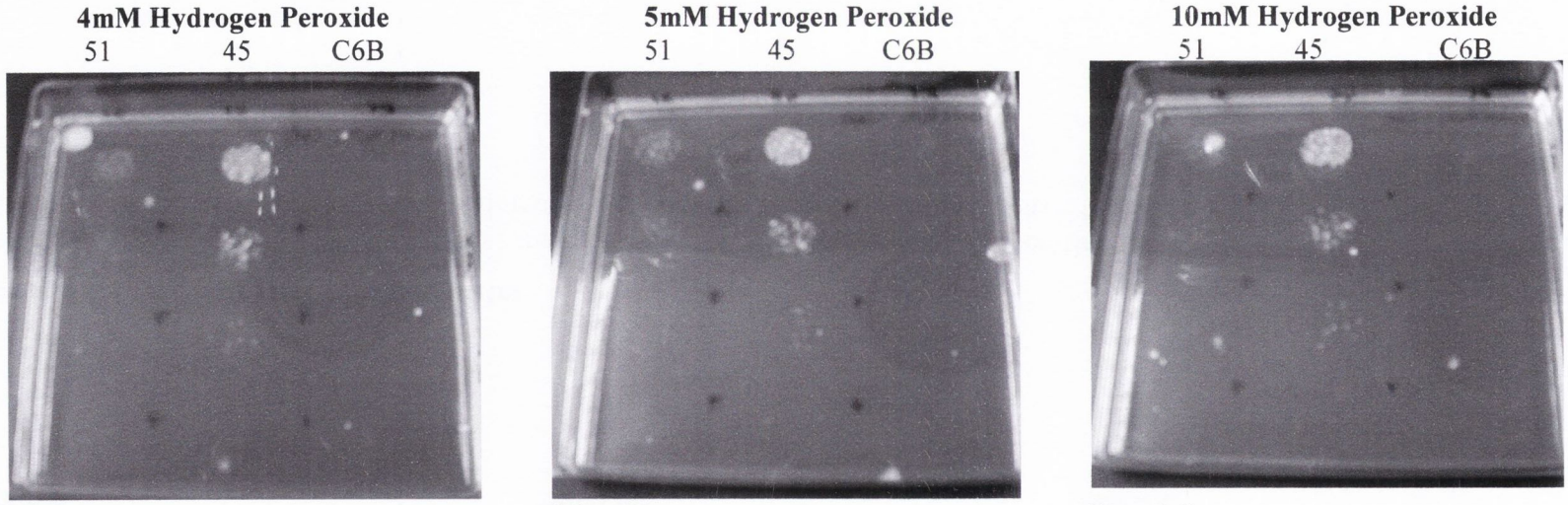


Figure 5.9 C6B Hydrogen Peroxide plates.

Oxidative stress for first generation stress tolerant mutant C6B and its second generation thermo-tolerant mutants C6B-45 and C6B-51. Cells were exposed to media supplemented with increasing concentrations of hydrogen peroxide and incubated at 30°C overnight.

tolerance to hydrogen peroxide. The mutant C6 shows strong inhibition on the 4 mM H_2O_2 plate and no growth was visible on any other concentration. The thermotolerant strain C6B45 shows no growth inhibition at 4 mM or 5 mM, however at 10 mM H_2O_2 there is some evidence of inhibition of growth. C6B51 shows some inhibition of growth on the 4 mM plate, and there is a gradual increase in inhibition as the concentration of H_2O_2 is increased with no growth on the 10 mM plate.

The first generation mutant C10 has lost tolerance for oxidative stress, however the second generation strains C10B45 and C10B51 have regained this tolerance, (Fig.5.10). C10B was poorly able to tolerate the 4 mM H_2O_2. C10B45 shows no inhibition of growth at 4 mM or 5 mM hydrogen peroxide supplemented plates, with some inhibition visible at 10 mM. The thermotolerant strain C10B51 shows no inhibition of growth at any of the hydrogen peroxide concentrations.

5.2.5 Analysis of the Thermotolerant Strain C10B51.

Further analysis was carried out on the strains C10B and C10B51 as these strains had been shown to have a better fermentation profile than the other mutant strains and the newly derived mutant C10B51 had an increased tolerance to oxidative stress.

5.2.5.1 Thermotolerant strain C10B51 is tolerant to oxidative stress.

The mutant C10B51 showed robust growth on the hydrogen peroxide plates. To verify the cross-resistance to oxidative stress, the mutant and its parent (C10B) were grown on menadione plates. The oxidative stress agent was changed from hydrogen peroxide to menadione for this series of experiments as menadione has a higher melting point (102°C) than hydrogen peroxide (2°C), which lessens the chance of evaporation from media when pouring plates. Menadione is also classed as a superoxide. The biological toxicity of superoxide is due to its capacity to inactivate ion sulphur cluster containing enzymes, which are critical in a wide variety of metabolic pathways, thereby liberating free iron in the cell,

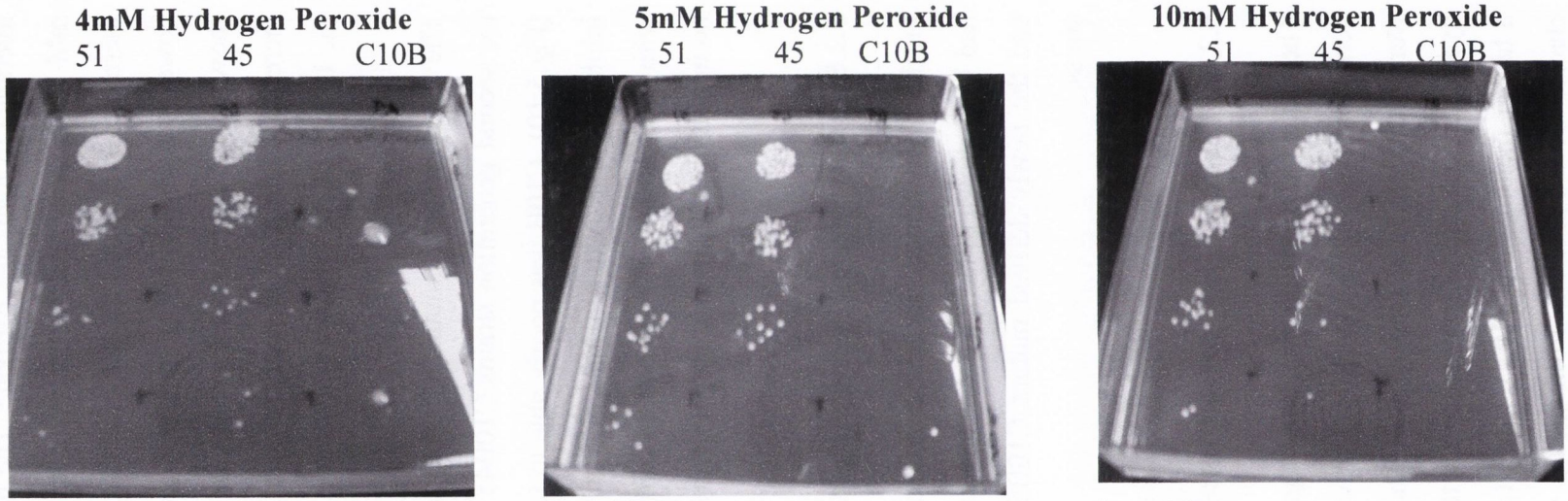


Figure 5.10 C10B Hydrogen Peroxide.

Oxidative stress first generation stress tolerant mutant C10B and its second generation thermo-tolerant mutants C10B-45 and C10B-51. Cells were exposed to media supplemented with increasing concentrations of hydrogen peroxide and incubated at 30°C overnight.

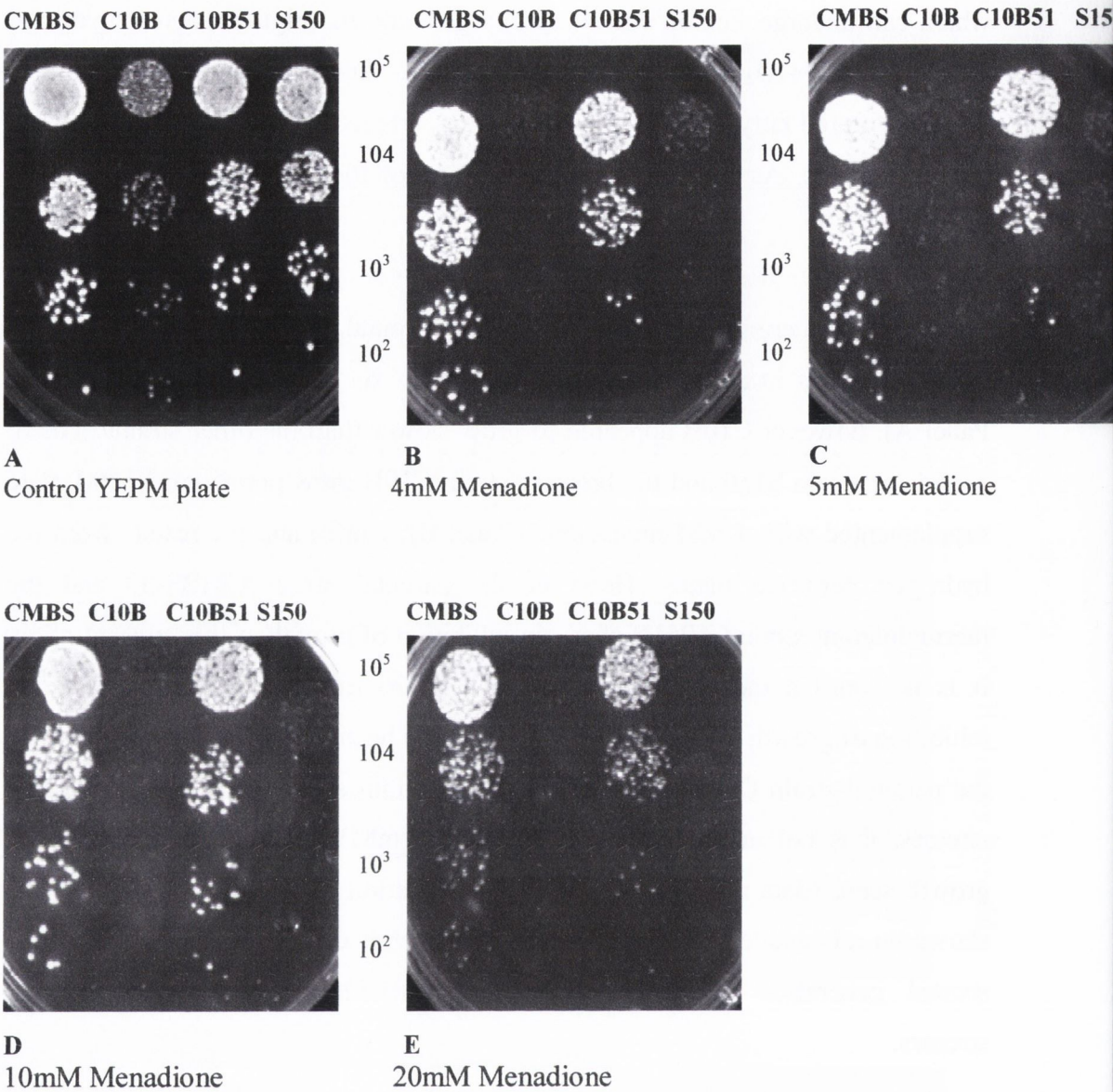
which can undergo Fenton chemistry and generate the highly reactive hydroxyl radical. In its HO_2 form, superoxide can also initiate lipid peroxidation of polyunsaturated fatty acids. It also reacts with carbonyl compounds to create toxic peroxy radicals. As such, superoxides are one of the main causes of oxidative stress.

The haploid *S. cerevisiae* strain S150 and the parental lager strain CMBS-33 were used as controls in this analysis. All strains grew on the control plate, (Fig.5.11, Panel A), however C10B appeared to grow slower than the other strains. The *S. cerevisiae* strain S150 and the brewery strain C10B grew poorly on YEPM plate supplemented with 4 mM menadione (Panel B), confirming the results from the hydrogen peroxide plates. However the parental strain CMBS-33 and the thermotolerant strain C1051B show no inhibition of growth at this concentration. It is not until a menadione concentration of 20 mM that one can see some inhibition of growth in C1051B, (Panel E). It can been seen from these results that the parental strain CMBS-33 is a very robust strain able to with stand oxidative stresses, it is not until a concentration of 100 mM menadione is inhibition of growth seen, (data not shown). The first generation stress tolerant strain C10B shows no advantage to oxidative stress however it can clearly be seen that the second generation thermotolerant strain C10B-51 can withstand oxidative stresses.

5.2.5.2 Molecular Analysis of C10B and C10B51 under high specific gravity conditions.

Since the stress tolerant mutant C10B51 shows cross tolerance to heat stress and oxidative stress and an increased fermentative capacity, the strain was further characterised at a genomic level. (A full characterisation of the first generation parental strain C10B stress tolerant strain is currently submitted for publication).

The lager yeast strains are aneuploidy in nature possessing unequal numbers of both *S. cerevisiae*-like and *S. bayanus*-like chromosomes. Recombination has occurred between the homeologous chromosomes resulting in a number of mosaic



A
Control YEPM plate

B
4mM Menadione

C
5mM Menadione

D
10mM Menadione

E
20mM Menadione

Figure 5.11: Menadione oxidative stress of lager yeast strains.

chromosomes consisting of parts of *S. cerevisiae* and *S. bayanus* genes (Bond *et al.*, 2004., Kodama *et al.*, 2005). Using the technique of competitive genomic hybridisation (CGH) to DNA microarray, Bond *et al.*, (2004) had previously characterised the mosaic nature of the lager yeast genome and then estimated the copy number of *S. cerevisiae*-like genes in two lager years strains, as discussed in chapter 1. In this study CGH analysis was performed, firstly to compare C10B51 to its parent C10B to determine if there are any genetic differences between the two strains. DNA from both strains taken at day 1 of a 20°P fermentation were differentially labelled with Cy5 and Cy3. The labelled DNA was mixed and CGH carried out as described in Chapter 2. As a control, each strain was differentially labelled with Cy5 and Cy3 to determine the background scatter in the experiment. Secondly, arrays were performed to determine if the strains were stable during a single round of high gravity fermentation by carrying out CGH analysis of DNA isolated at the beginning (day 1) and the end (day 8) of a fermentation.

The average ratios of hybridisations for the ORFs on each chromosome obtained from competitive hybridisation using the parental strain C10 and the thermotolerant strain C10B51 are shown in Table 5.3. YAL refers to the left arm of chromosome I, while YAR refers to the right arm of chromosome I. The same paradigm is used for the other chromosomes. When DNA from C10B isolated from day 1 of a fermentation is differentially labelled with Cy5 and Cy3 and competitively hybridised, each chromosome yields a mean ratio of hybridisation of 1.0±0.2. A similar result was observed with differentially labelled C10B51 DNA isolated from day 1 of the fermentation, (Table 5.3, panels 1 and 2). These control experiments establish the criteria for distinguishing changes between the two strains. Competitive hybridisation using DNA from both C10B and C10B51 isolated on Day 1 shows a much higher degree of variability in ratio of hybridisation for, (Table 5.3, panel 5) all chromosomes. For all chromosomes with the exception of YCL, YDR, the ratio of hybridisation is greater than 1.0, which indicates that the C10B51 strain has a higher *S. cerevisiae* gene content, than C10B.

Table 5.3 Average Ratio of Hybridisations

CHR	C10B Day 1 VS C10B Day 1	C10B51 Day1 VS C10B51 Day1	C10B Day1 VS C10B Day8	C10B51 Day1 VS C10B51 Day8	C10B Day1 VS C10B51 Day1
YAL	1.0±0.2	1.0±0.2	0.6±0.2	0.5±0.2	1.2±0.4
YAR	1.0±0.2	1.0±0.2	0.7±0.2	0.6±0.2	1.5±0.4
YBL	1.0±0.2	1.0±0.4	1.0±0.3	0.7±0.3	1.8±0.4
YBR	1.0±0.2	1.0±0.4	1.3±0.3	0.9±0.3	1.7±0.4
YCL	1.0±0.2	1.0±0.2	0.7±0.3	0.7±0.5	0.9±0.4
YCR	1.0±0.2	1.0±0.2	0.8±0.5	0.8±0.5	1.3±0.5
YDL	1.0±0.2	1.0±0.2	1.2±0.4	1.2±0.6	1.0±0.3
YDR	1.0±0.2	1.0±0.2	1.4±0.4	1.6±0.6	0.9±0.3
YEL	1.0±0.2	1.0±0.2	0.9±0.3	0.6±0.3	1.9±0.4
YER	1.0±0.2	1.0±0.2	1.0±0.3	0.7±0.3	1.8±0.3
YFL	1.0±0.2	1.0±0.2	0.8±0.3	0.8±0.7	2.0±0.6
YFR	1.0±0.2	1.0±0.2	0.7±0.3	0.4±0.1	2.3±0.5
YGL	1.0±0.2	1.0±0.2	1.2±0.3	1.1±0.5	1.1±0.3
YGR	1.0±0.2	1.0±0.2	1.2±0.3	1.1±0.5	1.0±0.2
YHL	1.0±0.2	1.0±0.3	1.0±0.3	0.8±0.6	1.9±0.4
YHR	1.0±0.2	1.0±0.3	1.0±0.3	0.7±0.2	2.1±0.8
YIL	1.0±0.	1.0±0.2	0.9±0.4	0.6±0.6	2.3±0.5
YIR	1.0±0.2	1.0±0.2	0.8±0.4	0.5±0.2	2.4±0.5
YJL	1.0±0.2	1.0±0.2	1.2±0.4	0.8±0.3	1.8±0.4
YJR	1.0±0.2	1.0±0.4	0.8±0.4	0.9±0.7	1.4±0.4
YKL	1.0±0.2	1.0±0.2	1.0±0.3	0.8±0.2	1.3±0.4
YKR	1.0±0.2	1.0±0.2	1.0±0.3	0.9±0.7	1.3±0.2
YLL	1.0±0.2	1.0±0.2	0.9±0.3	0.7±0.2	1.3±0.3
YLR	1.0±0.2	1.0±0.2	1.4±0.5	1.9±1.3	1.0±0.3
YML	1.0±0.2	1.0±0.2	1.0±0.4	0.7±0.3	1.8±0.3
YMR	1.0±0.2	1.0±0.2	1.3±0.4	1.0±0.3	1.6±0.3
YNL	1.0±0.2	1.0±0.4	1.4±0.4	1.0±0.3	1.2±0.3
YNR	1.0±0.2	1.0±0.2	1.2±0.4	0.7±0.3	1.3±0.3
YOL	1.0±0.2	1.0±0.2	1.0±0.4	0.9±0.4	1.7±0.3
YOR	1.0±0.2	1.0±0.2	1.4±0.4	1.2±0.4	1.5±0.6
YPL	1.0±0.1	1.0±0.2	1.3±0.3	1.0±0.3	1.5±0.3
YPR	1.0±0.2	1.0±0.2	1.2±0.3	1.1±0.6	1.6±0.4

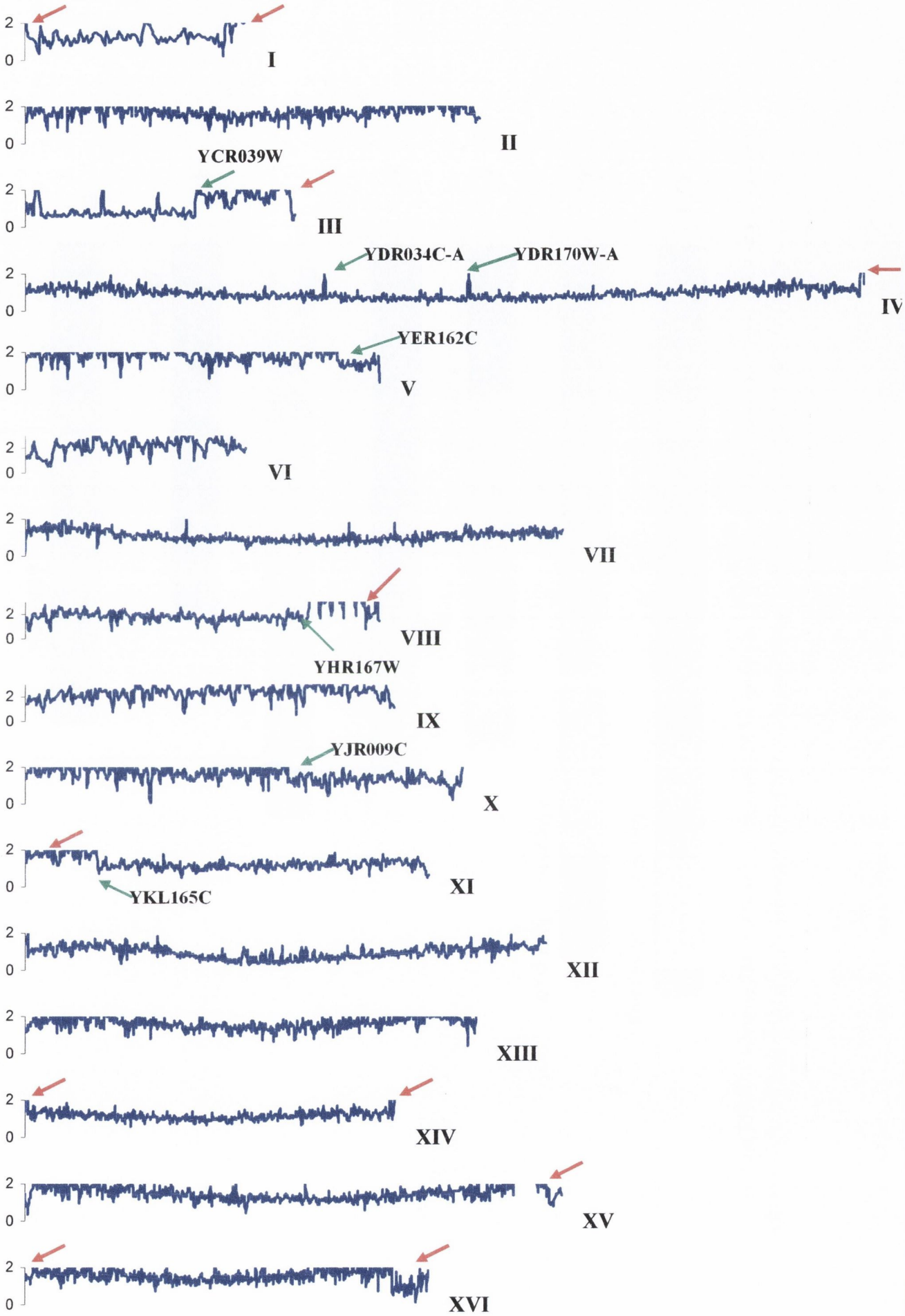
To examine the chromosomal changes existing between C10B and C10B51, the ratio of hybridisation for each ORF was plotted against its chromosomal position, (according to the *S. cerevisiae* alignment). Eight distinct changes in the ratio of hybridisation are observed upon analysis of the data. On chromosome III there is a 'jump' in the ratio of hybridisation at YCR039C, which is the MAT locus, all genes to the right of this loci have an increased ratio of hybridisation, (Fig. 5.12). This site of recombination location has also been previously observed in the lager brewery strain CMBS (Bond *et al.,* 2004). Chromosome V shows a very high ratio of hybridisation and at ORF YER161C there is a drop in the ratio of hybridisation, implying that the strain C10B51 has undergone a recombination event at this site. On chromosome VI, the ratio of hybridisation 'jumps' at the ORF YFL051C and ORFs to the right of this loci show an increased ratio of hybridisation. YFL051C is an uncharacterised ORF but is downstream of two autonomously replicating sequences that function as chromosomal replicating origins and in chromosome maintenance. In the case of chromosome X, a 'jump' in the ratio of hybridisation is observed at ORF YJR009C in both C10B and C10B51 resulting in a decrease in the ratio of hybridisation. Jumps in the ratio of hybridisation are also observed on chromosomes VIII, XI, XV and XVI. The location of all major rearrangements are illustrated in Fig. 5.13. Additionally, changes in ratio of hybridisation are evident at the telomeres or in the subtelomeric regions of the chromosomes.

To determine if the genomes of these lager yeast strains are stable under high gravity fermentation conditions the microarray DNA profiles of the parent (C10B) and thermotolerant strain (C10B51) were compared on days 1 and day 8. Within the time frame of a single fermentation, changes in the ratio of hybridisations are observed as shown in Table 5.3, (panels 3 and 4). The details of the changes are best illustrated by plotting the ratios of hybridisations against the ORF position as available from *Sacchromyces* Genome Database (www.yeastgenome.org), as seen in Figures 5.14 – 5.15.

In general chromosomes are stable with fewer recombination events than that observed when comparing C10B and C10B51. Most noted differences in C10B

Figure 5.12 Plot of ratio of hybridisations for Chromosomes I – XVI for the DNA microarray **C10B Day1 vs. C10B51 Day1**. The sizes of the plots are proportional to the chromosome size. The **red arrows** indicate regions of telomere amplification. The **green arrows** indicate specific gene amplification, discussed in the text.

C10B Day 1 vs. C10B51 Day 1



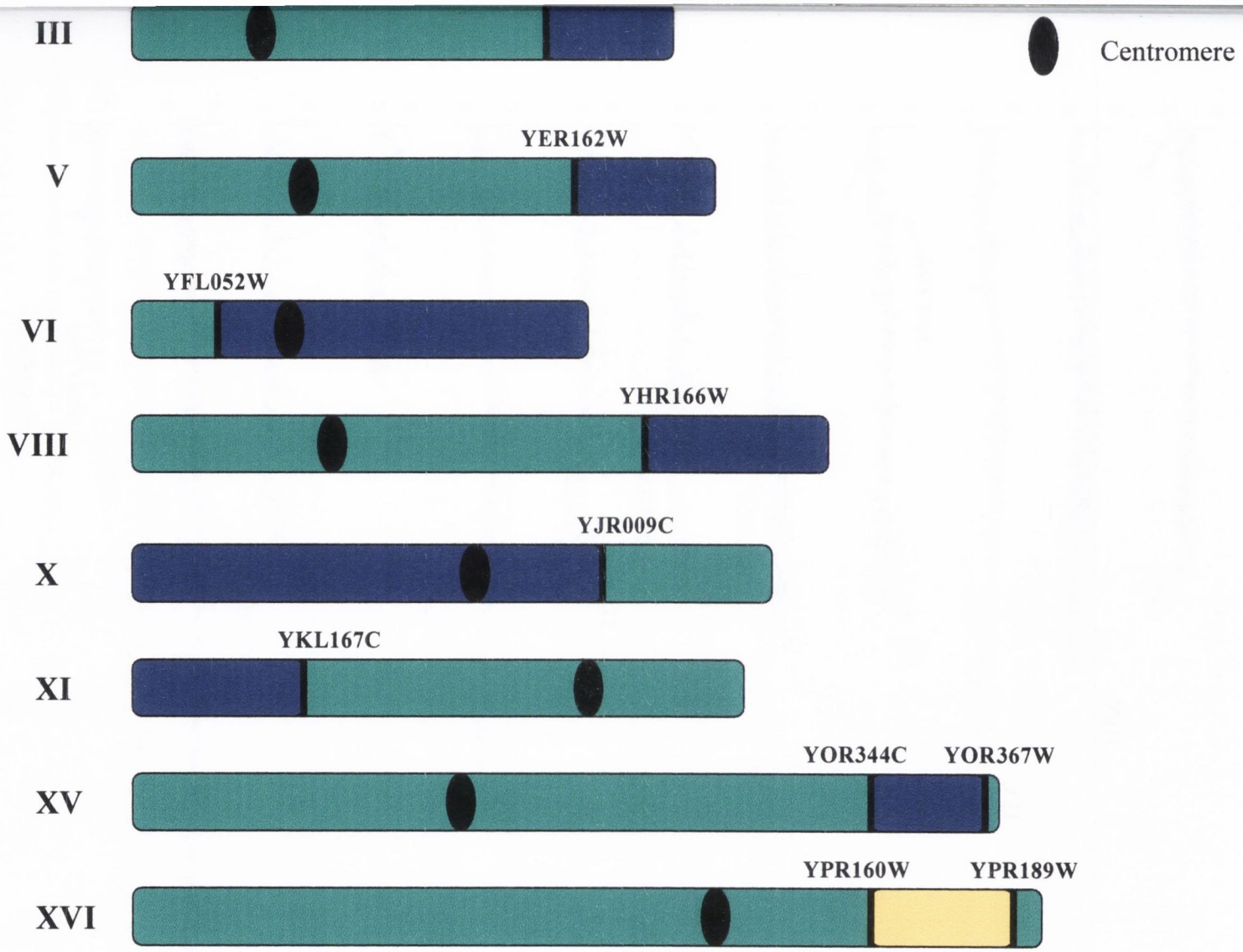
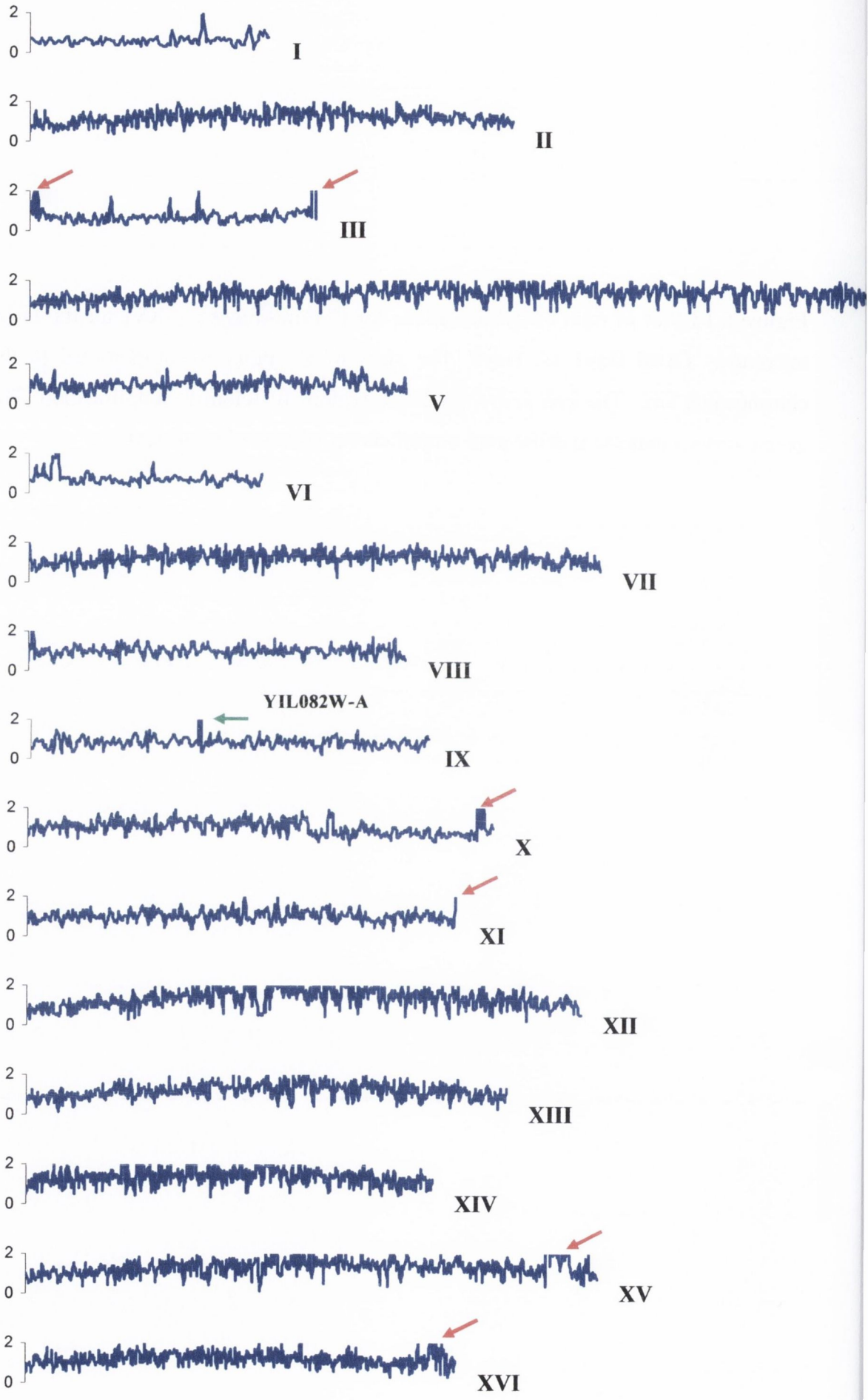


Figure 5.13: Schematic of major rearrangements in the ratio of hybridisation for DNA Microarray C10B Day1 vs. C10B51 Day1. The location of the 'jumps' in ratio of hybridisation are shown by | and the closest ORF.

Figure 5.14 Plot of ratio of hybridisations for Chromosomes I – XVI for the DNA microarray **C10B Day1 vs. Day8**. The sizes of the plots are proportional to the chromosome size. The **red arrows** indicate regions of telomere amplification. The **green arrows** indicate specific gene amplification, discussed in the text.

C10B Day 1 vs. C10B Day 8



day 1 vs. day 8 are telomere amplifications, evident in chromosomes III, VIII, X, XI, XV and XVI, (Fig5.14). The ratios of hybridisation for chromosome VI, shows that the ratio of hybridisation for the right arm (ROH=0.4) is significantly lower for C10B51 compared to the parental strain C10B (ROH=0.7) in day 1 vs. day 8, (Table 5.3, panles 2 and 3). This result implies that there has been a recombination event resulting in partial loss of the *S. cerevisiae* copy of these genes in the second generation of thermotolerant strains during the fermentation (Compare panel 2 to 5, Table 5.3). On chromosome IX there is also specific gene amplification observed at YIL082W-A, a transposable element gene. Individual spot amplifications and decreases were also observed in the arrays, (Fig 5.14, green arrows).

When comparing the genome of C10B51 during a high gravity fermentation, (Fig 5.15), a generalised amplification of chromosome IV was observed. The amplification of telomere regions was also observed, similar to the pattern seen in the C10B CGH analysis. There is localised gene amplification on chromosome V at YER060W-A which is involved in purine-cytosine permease and on chromosome IX at YIL082W-A, as previously seen in the analysis of C10B CGH data, (Fig. 5.14). In both the microarrays for C10B Day 1 vs. Day 8 and C10B51 Day 1 vs. Day 8 on chromosome XII, there is localised region of amplification at YLR154W. This region is a known major rRNA locus.

The genome of C10 appears to be much more stable overall during the fermentations with an average ratio of hybridisation of 1.4, whereas the average ratio of hybridisation for C10B51 is 0.8.

To determine if regions of amplification might be associated with specific genes associated with stress tolerance such as heat shock genes or oxidative stress genes. The ratio of hybridisation for these genes was examined as shown in Tables 5.4 and 5.5. It was observed that few of the Hsp genes had an increased ratio of hybridisation at a DNA level. There appears to be no correlation between acquired stress tolerance (eg. oxidative stress) and the amplification of specific sets of genes, (Table 5.5).

Figure 5.15 Plot of ratio of hybridisations for Chromosomes I – XVI for the DNA microarray **C10B51 Day1 vs. Day8**. The sizes of the plots are proportional to the chromosome size. The **red arrows** indicate regions of telomere amplification. The **green arrows** indicate specific gene amplification, discussed in the text.

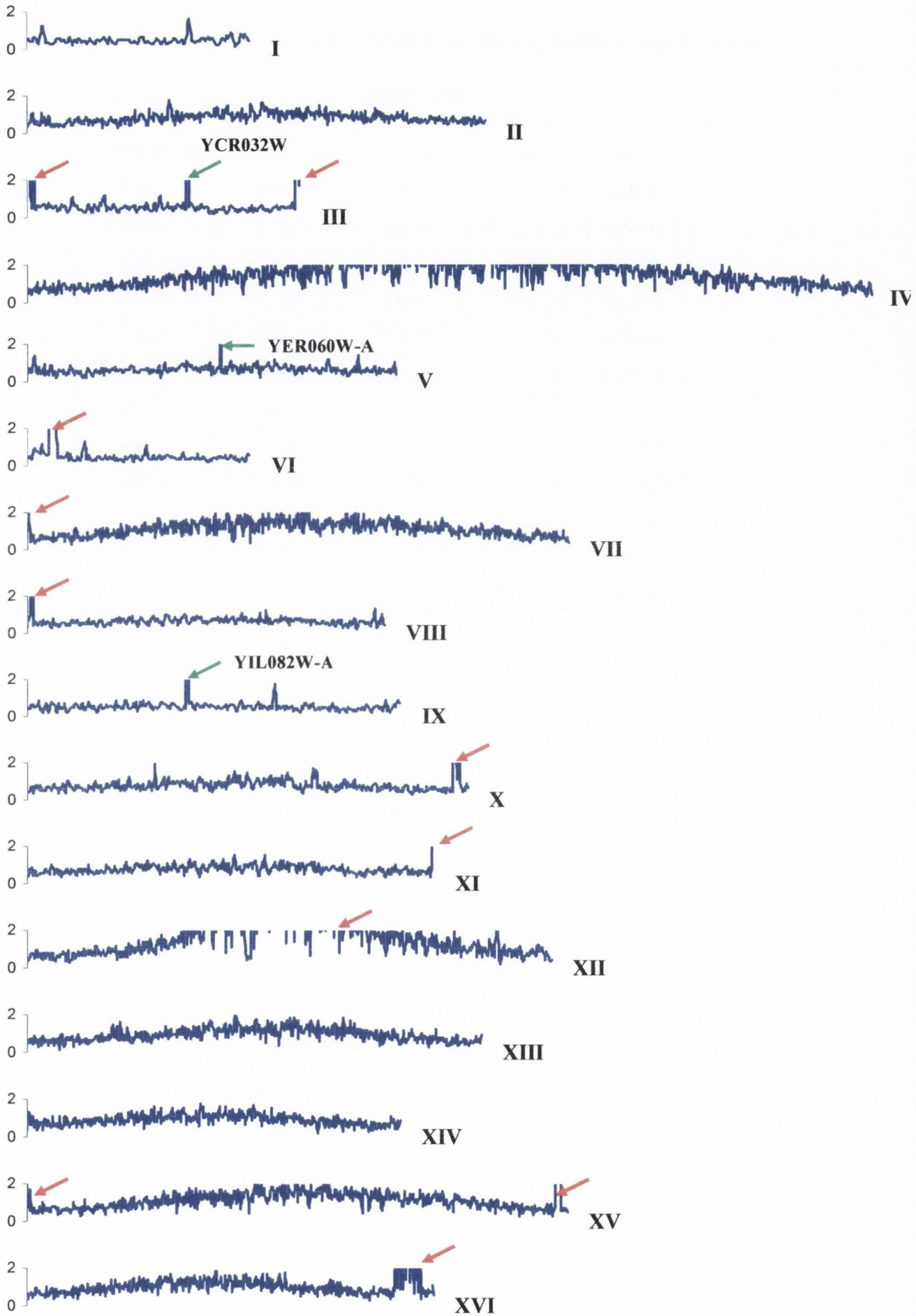

C10B51 Day 1 vs. C10B51 Day 8.
2
0
I
II
YCR032W
III
IV
YER060W-A
V
VI
VII
VIII
YIL082W-A
IX
X
XI
XII
XIII
XIV
XV
XVI

Table 5.4 Ratio of Hybridisation for Hsp Genes.

		Microarrays		
Gene Name	**Systematic Name**	**C10 Day1 vs. Day8**	**C10B51 Day1 vs. Day 8**	**C10 Day1 vs. C10B51 Day1**
HSP10	YOR020C	1.073	1.164	1.208
HSP12	YFL072W	0.402	0.241	1.965
HSP26	YBR072W	1.14	0.843	1.114
HSP30	YCR021C	0.51	0.533	0.788
HSP31	YDR533C	0.805	0.633	1.144
HSP32	YPL280W	0.895	0.609	1.516
HSP33	YOR391C	1.557	1.502	1.544
HSP34	YMR322C	0.781	0.57	1.448
HSP35,36	YGR192C	0.786	0.741	1.165
HSP40	YNL064C	1.518	1.045	1.417
HSP42	YDR171W	1.795	2.552	0.728
HSP48	YGR254W	0.833	0.5	1.506
HSP60	YLR259C	1.391	2.177	0.86
HSP78	YDR258C	0.912	1.21	0.729
HSP82	YPL240C	1.081	0.742	1.046
HSP90	YMR186W	1.207	0.869	1.126
HSP104	YLL026W	1.037	0.998	1.491
HSP150	YJL159W	0.652	0.443	1.465

Table 5.5 Ratio of Hybridisation of Oxidative Stress Related Genes.

Microarrays

Gene Name	Systematic Name	C10 Day1 vs. Day8	C10B51 Day1 vs. Day 8	C10 Day1 vs. C10B51 Day1
YLR043C	TRX1	1.199	1.139	1.202
YLR109W	AHP1	0.9	1.358	0.712
YML028W	TSA1	1.05	0.787	1.541
YMR038C	LYS7	1.02	0.83	1.17
YNL259C	ATX1	0.679	0.813	0.934
YOR079C	ATX2	2.072	1.78	1.487
YBR006W	UGA2	0.806	0.548	1.511
YCL033C		0.309	0.326	0.88
YCL035C	GRX1	0.532	0.478	0.694
YCR083W	TRX3	0.478	0.337	1.463
YDL166C	FAP7	0.989	0.87	1.139
YDR099W	GRX3	1.065	1.891	0.892
YDR513W	TTR1	0.957	0.702	0.98
YER043C	SAH1	1.027	0.673	1.954
YER174C	GRX4	0.567	0.393	1.045
YGR209C	TRX2	1.042	0.898	1.23
YHR106W	TRR2	0.83	0.554	1.675
YJR048W	CYC1	0.857	0.797	1.553

5.2.5.3 Confirmation of amplified ORFs in stress tolerant strains C10B and C10B51 by Real-time PCR.

To validate the CGH data, a selection of ORFs that showed a high ratios of hybridisation were selected for real-time PCR analysis. This quantitative approach involves comparing the Ct values of the samples of interest with that of a control ORF, as described in Chapter 2. The Ct vales of the control and sample are then normalised to an endogenous gene, NADH, (YDL171C). The values of the ratio of hybridisation and comparative Ct values for five ORFs are shown in Table 5.6. In general there is a very good correlation between the Ct values and the ratios of hybridisation (Fig. 5.16). Taking all the data points into consideration, a correlation coefficient of 0.95 is obtained indicating that the CGH data accurately reflects the changes in the DNA content in the lager yeast genome.

Table 5.6 Real Time PCR Analysis.

ORF	Arrays	C10B Day1 vs. C10B Day8	C10B51 Day1 vs. C10B51 Day8	C10B Day1 vs. C10B51 Day1
YBR134W	Ratio Of Hybridisation	0.518	1.021	7.443
	dd Ct Values	1.641	0.877	5.549
YHL047C	Ratio Of Hybridisation	2.953	10.782	0.72
	dd Ct Values	3.524	15.635	1.07
YGR097W	Ratio Of Hybridisation	1.082	1.018	0.894
	dd Ct Values	1.236	1.002	1.004
YJR115W	Ratio Of Hybridisation	0.35	0.709	5.324
	dd Ct Values	1.007	0.848	8.19
YPL056C	Ratio Of Hybridisation	0.711	1.363	2.625
	dd Ct Values	0.848	1.563	3.452

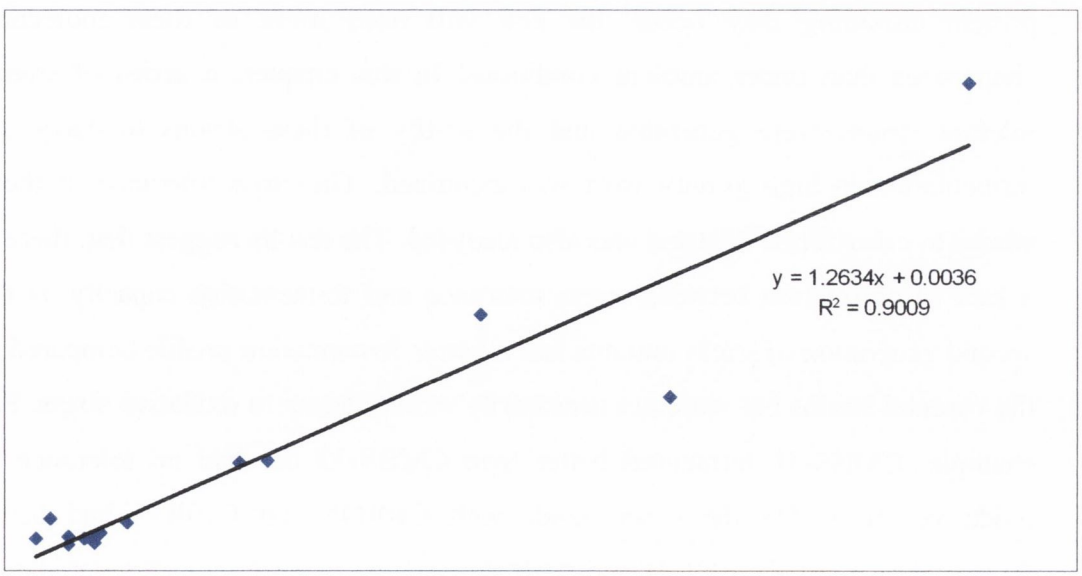


Figure 5.16: Plot of the Ct values obtained from Real-time PCR vs CGH ratios of hybridisation with a fitted linear line showing the correlation of the data.

5.3 Discussion

The cellular response to stress is obviously aimed at protecting cells from the detrimental effects of stress and at repairing possible damage (Estruch, 2000). Protective responses of living cells have initially been identified in studies of the heat shock response (Parsell *et al.,* 1991., Lindquist *et al.,* 1998). Cells exposed to elevated temperature increase the synthesis of Hsps, many of which function as molecular chaperones. They control the conformation of other proteins or keep protein complexes in a functionally competent state. Classical heat shock response studies have revealed two fundamental features, firstly the response leads to the acquisition of stress tolerance. Once the cells have been challenged with a mild stress they become resistant to severe stress, as was seen in this study in the generation of the thermotolerant strains. The acquisition of stress tolerance holds for almost all stress conditions and is considered to be one of the main purposes of the cellular stress response. In several cases the exposure to one type of stress has been demonstrated to lead to tolerance to other types of stresses as well. This phenomenon of cross-tolerance suggests that different stress conditions require common cellular responses such as the adjustment of energy metabolism and the production of protective proteins (e.g. Hps). The second fundamental aspect of the stress response is that stress genes play an important role in normal unstressed cells such as Hsps. Under heat stress conditions when the risk of protein unfolding may occur, the cell will need more of these molecular chaperones than under ambient conditions. In this chapter, a series of stress-tolerant strains were generated and the ability of these strains to carry out fermentation in high gravity wort was examined. The cross tolerance of these strains to other forms of stress was also analysed. The results suggest that, there is a lack of correlation between stress tolerance and fermentative capacity as the second generation of stress mutants had a better fermentation profile compared to the parental strains but were not necessarily more tolerant to oxidative stress. For example, CMBS-51 fermented better than CMBS-33 but had no tolerance to oxidative stress. On the other hand, both C10B45 and C10B51 had better fermentation rates than C10B and both showed increased tolerance to oxidative stress. It was also observed that the parental strain CMBS-33, is an extremely robust strain, able to withstand high levels of oxidative stress.

Oxygen is known for being both essential and dangerous for aerobic life. The toxicity is averted by various cellular systems acting by imposing a tight control on the intracellular concentration of O_2-derived oxidants, the cellular thiol redox state, the metabolism of iron and copper by repairing oxidative damage. The oxidative stress response mediating transcription factor Yap1p is cytoplasmic and upon stress exposure is translocated to the nucleus. A large set of genes are also responsive via ARE's – API-Responsive elements. Skn7p is a transcription factor controlling a set of genes overlapping with the Yap1-regulon. Skn7p is a response regulator protein controlled by the Sln1p osmosensing histindine kinase and it contains a DNA binding domain similar to the heat shock transcription factor with which it has been shown to interact. Skn7p interacts with and supports the function of different transcription factors as Yap1p and Hsf1p. In this study it was interesting to note that few of the Hsp genes or oxidative stress response genes had an increased ratio of hybridisation at a DNA level in the stress tolerant mutants. This shows that there is no correlation between the amplification of Hsp genes and oxidative stress and stress tolerance. However transcription analysis would be necessary to determine if there was an increased expression of these genes in the thermotolerant strains.

During the isolation of the first generation stress tolerant mutants it was observed that the mutants had undergone a number of chromosomal rearrangements. These mutants had been selected following treatment with EMS and then selection for heat tolerance and growth in high gravity wort. The second generation of thermotolerant mutants were selected in the absence of EMS but these also showed chromosomal rearrangements. Therefore the rearrangements result from the exposure to stress rather than resulting from EMS treatment. Chromosomal rearrangements were observed at known sites if recombination (Fig. 5.13). Telomeric amplification was also observed as previously highlighted, (Fig. 5.14 and 5.15). We also examined the chromosomal stability during a fermentation and observed that no recombination was evident. However, comparing day 1 vs. day 8, telomeric amplification was observed as was specific gene amplification, most notably genes surrounding the rRNA locus on chromosome XII.

Storchova *et al.*, (2006) have previously identified 39 genes that are required for survival of polyploid strains. The majority of these genes are involved in the repair of double stranded breaks in DNA via homologous recombination and includes the RAD52 epistasis group, members of this group include RAD50, RAD51, RAD52, RAD54, RAD59, MRE11 and XRS2. Interestingly none of these genes show increased ratio of hybridisation under conditions where chromosomal rearrangements were shown to occur such as in thermotolerant strain C10B51, (data not shown).

From the microarrays performed, it can be deduced that the chromosomes in the parental strain C10 and the thermotolerant strain C10B51 are not stable during an aerobic high gravity fermentation and that new sites of recombination unique to the stress tolerant strains can be identified. To date 217 ORFs have been identified that show a change in the ratio of hybridisation greater than 2.5 on the linear scale, (see appendix I for a complete list).

Chapter 6
Chromosome Stability in Lager yeast: Does environment affect the chromosome stability?

6.1 Introduction

From the previous chapter, we saw that the stress tolerant mutants have undergone genome rearrangements and gene loss suggesting that exposure to stress can induce chromosomal changes. The first generation mutants were selected following EMS treatment for both heat tolerance and growth on high gravity wort, while the second generation mutants were selected for thermo-tolerance only. Thus exposure to heat stress and/or high gravity fermentation can result in chromosomal rearrangements. Since, modern day brewers are routinely moving to high gravity brewing, it is of importance to determine if exposure to high gravity brewing conditions will induce chromosome instability.

To examine this, the lager yeast strain CMBS-33 was fermented under a variety of conditions to observe how the strain performed under different fermentation conditions and further to observe the stability of the lager yeast chromosomes. Fermentations were carried out under high gravity (20°P) and 'normal' gravity (16°P) conditions as well as under aerobic and anaerobic conditions. Each fermentation was performed independently and in duplicate.

6.2 Results.

6.2.1 Fermentation of strain CMBS-33.

To examine the effects of high gravity fermentation on chromosome stability, fermentations using the lager strain CMBS-33 were performed under two different specific gravities and either in aerobic or anaerobic conditions, as described in section 2.20. The specific conditions for each fermentation are listed in Table 6.1.

	Specific Gravity	Aerobic	Anaerobic
M1	High (20°P)	✓	
M2	High (20°P)	✓	
M3	High (20°P)		✓
M4	High (20°P)		✓
M5	Normal (16°P)	✓	
M6	Normal (16°P)	✓	
M7	Normal (16°P)		✓
M8	Normal (16°P)		✓

Table 6.1: Fermentations performed using lager yeast strain CMBS-33.

The fermentation profiles for the high gravity fermentations (M1 – M4) are shown in Fig.6.1. From the graph it can be observed that the two aerobic fermentations (M1 and M2), performed identically under high gravity conditions. It was not until day 3 that one can observe any difference in the fermentation rate between the aerobic and anaerobic fermentations. After this point the aerobic fermentations begin to show an increased fermentation rate. All of the fermentations reached an attenuation level of approximately 1.02 by day 10 of the fermentation. The specific gravity values were used to calculate the concentration of alcohol produced as described in the methods section, (Fig.6.2). From this graph it is interesting to observe that there is a higher percentage alcohol in the aerobic fermentations (9.2%) compared to the anaerobic fermentations (~8%).

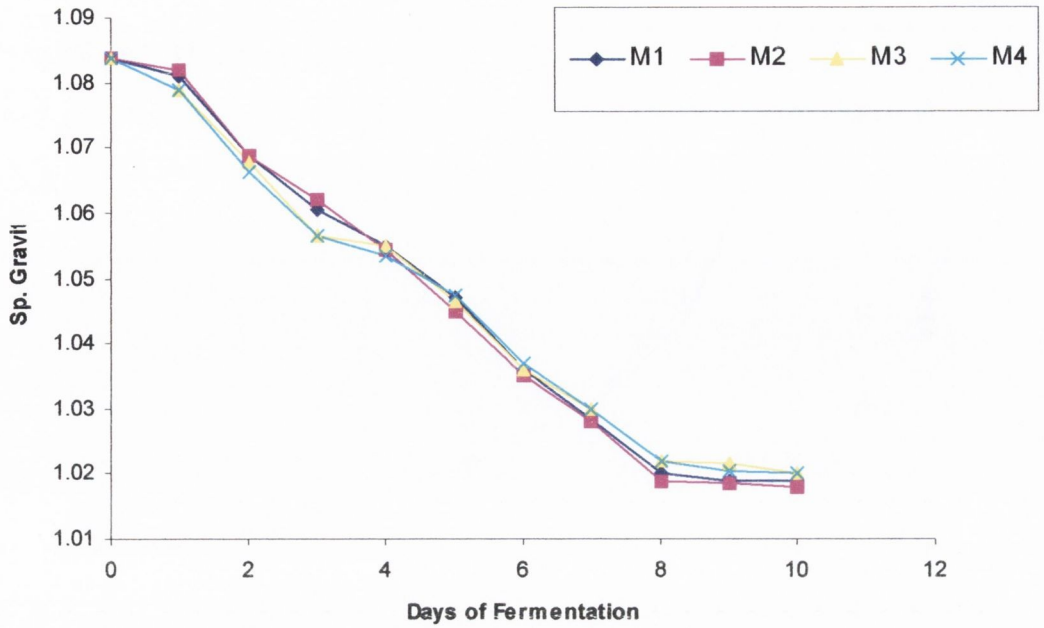


Figure 6.1 High Gravity Fermentation of Lager Brewery Strain CMBS-33. Fermentations were performed in duplicate. M1 and M2 aerobic high gravity. M3 and M4 anaerobic high gravity. The specific gravity of the brews was measured daily with cells collected on days 1 and 8 for DNA extraction and CGH analysis.

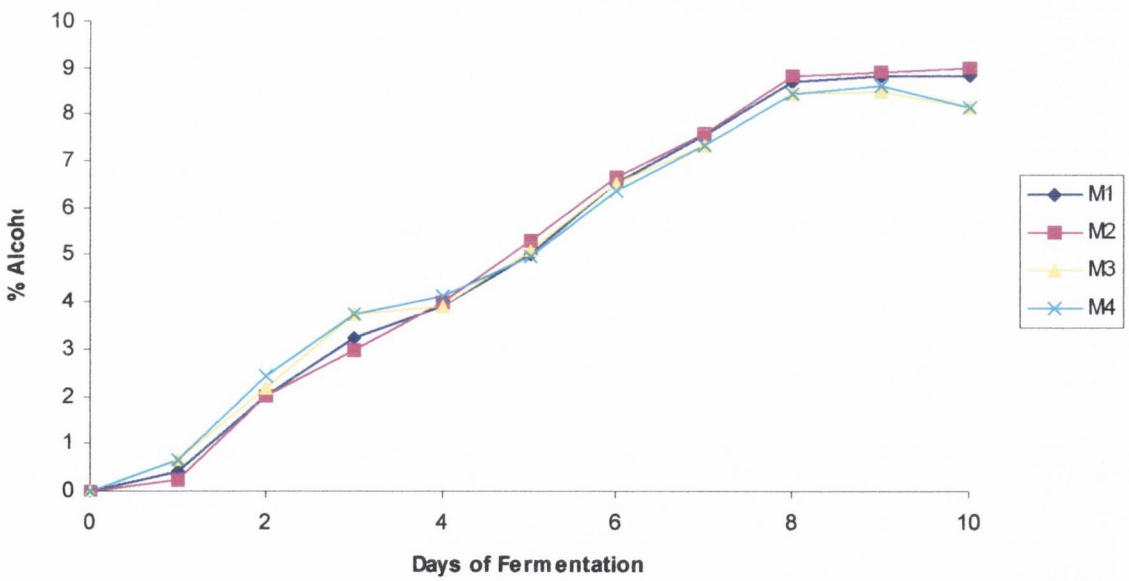


Figure 6.2 Percentage alcohol for each of the high gravity brews. Yeast was pitched at 1.5 x 10^7 cells/ml into 20°P wort.

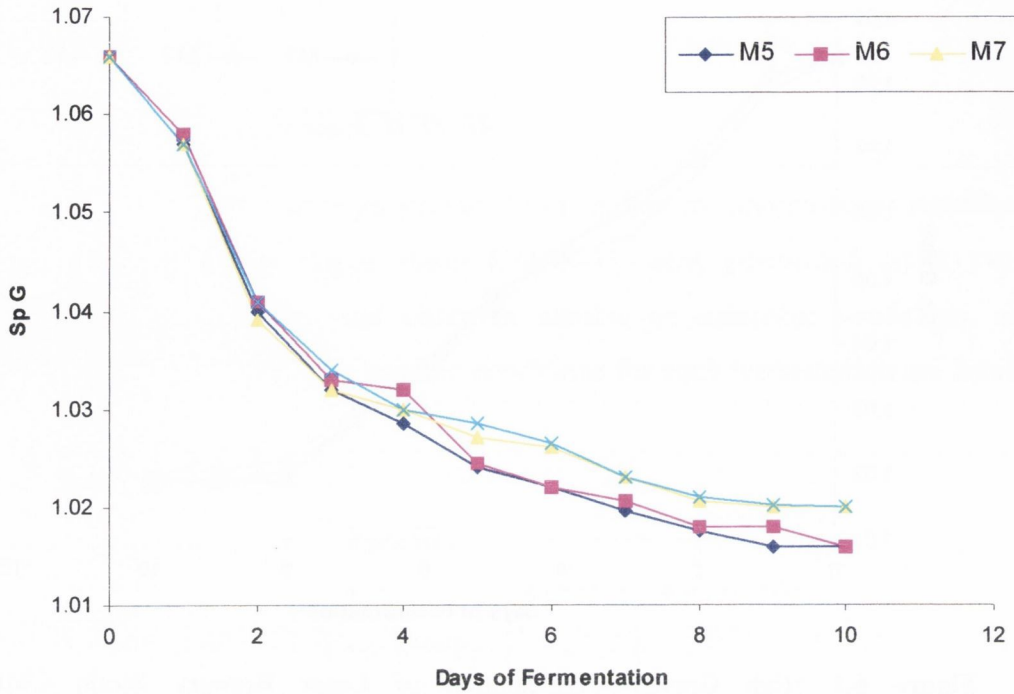


Figure 6.3 Low Gravity Fermentation of Lager Brewery Strain CMBS-33. Fermentations were performed in duplicate. M1 and M2 aerobic low gravity. M3 and M4 anaerobic low gravity. The specific gravity of the brews was measured daily with cells collected on days 1 and 8 for DNA extraction and CGH analysis.

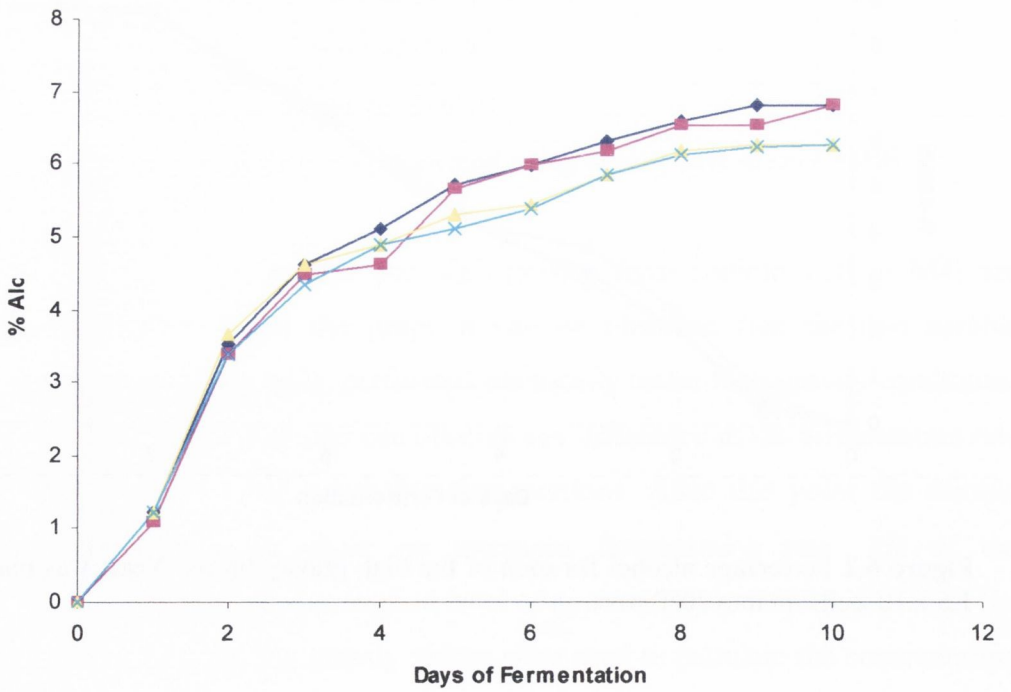


Figure 6.4 Percentage alcohol for each of the low gravity brews. Yeast was pitched at 1.5 x 10^7 cells/ml into 20°P wort.

This result is slightly unexpected as in industrial fermentations, which are normally performed under anaerobic conditions, one would expect anaerobic fermentations to result in a higher alcohol content.

The fermentation profiles for the low gravity fermentations (M5 – M8) are shown in Figure 6.3. There is a noticeable difference between the aerobic and anaerobic fermentations after day 4, where the fermentation rate of the aerobic brews is significantly greater than the anaerobic brews. The aerobic fermentations reach a final attenuation of 1.016 compared to 1.02 for the anaerobic fermentations. The percentage alcohol for each fermentation was plotted as seen in Figure 6.4. There is a clear difference between the aerobic and anaerobic fermentations observed after day 4, with the aerobic brews achieving a final percentage alcohol of 6.7% compared to the anaerobic brews that produced 6.25%, (Fig. 6.4). As with the high gravity fermentations this result was unexpected.

From this data, it is observed that CMBS-33 can survive and perform quite well in wort concentrations ranging from low to very high gravity and in aerobic and anaerobic conditions. Following on from these fermentation experiments, DNA was extracted from cells harvested on days 1 and 8 for all samples M1 – M8. The samples were differentially labelled with Cy3 and Cy5 as described in the Chapter 2, Sections 2.8 and 2.9 and CGH performed to determine if the genome of CMBS-33 is stable during fermentation, specifically comparing high vs. low gravity and aerobic and anaerobic conditions.

6.2.2 Effects of high gravity vs. low gravity fermentations on the CMBS-33 genome stability.

The data obtained from the DNA microarrays was normalised using the data normalising software Varan (www.bionet.espci.fr/varan/), as described in chapter 2. After the initial analysis was performed, the data from the microarray M2 was discounted as there was poor incorporation of the labels into the samples and a smudge was also visible of the array. A summary table of the average ratios of hybridisation for each chromosome is shown in Table 6.2. From the data in the

Table 6.2 Average Ratios of Hybridisation for DNA microarrays

		Arrays						
CHR	Number of Genes	M1	M3	M4	M5	M6	M7	M8
I	108	1.8±0.7	1.2±0.2	1.2±0.2	1.0±0.1	1±0.2	1.0±0.2	1.0±0.1
II	426	0.9±0.8	0.9±0.4	0.9±0.5	0.9±0.2	0.9±0.2	0.9±0.2	0.9±0.1
III	176	1.3±0.6	1.0±0.3	1.1±0.3	1.0±0.2	1.1±0.2	1.0±0.2	1.0±0.2
IV	806	0.9±0.4	0.8±0.3	0.9±0.2	0.9±0.2	0.8±0.2	1.0±0.2	0.9±0.2
V	286	1.1±0.6	0.8±0.2	1.0±0.3	1.0±0.2	1.0±0.2	1.0±0.2	0.9±0.2
VI	135	1.5±0.6	1.0±0.2	1.0±0.2	0.9±0.1	1.0±0.1	1.0±0.2	0.9±0.2
VII	563	0.9±0.4	0.8±0.2	1.0±0.2	1.0±0.2	0.8±0.3	1.0±0.2	0.9±0.2
VIII	280	1.1±0.7	0.8±0.2	1.0±0.3	1.0±0.1	1.0±0.1	1.0±0.2	1.0±0.2
IX	225	1.1±0.6	0.8±0.2	0.9±0.2	0.9±0.2	1.1±0.2	1.0±0.2	1.0±0.2
X	389	1.0±0.6	0.8±0.3	0.9±0.3	0.9±0.2	0.8±0.2	0.9±0.2	0.9±0.2
XI	338	0.9±0.4	0.8±0.3	1.0±0.3	1.0±0.2	1.0±0.3	1.1±0.3	1.0±0.2
XII	540	1.4±0.7	1.0±0.4	1.1±0.3	1.1±0.3	0.8±0.2	1.1±0.2	1.0±0.2
XIII	493	1.0±0.6	0.8±0.2	1.0±0.3	1.1±0.3	0.9±0.3	1.1±0.3	1.0±0.2
XIV	419	1.0±0.6	0.8±0.2	1.0±0.4	0.9±0.2	0.8±0.2	1.0±0.2	0.9±0.1
XV	567	0.9±0.5	0.8±0.2	0.9±0.2	0.9±0.2	0.8±0.2	1.0±0.2	0.9±0.2
XVI	491	1.0±0.5	0.8±0.3	0.9±0.3	0.9±0.2	0.8±0.2	1.0±0.2	0.9±0.2

Total number of genes 6242.

table it can be seen that there is a greater variability in the arrays M1, M3 and M4, than arrays M5 – M8, thus implying that a high specific gravity of a fermentation has an affect on the genome stability. This is most notable on Chromosomes I, III, V, VI, IX and XII, especially in array M1. In the case of the low gravity fermentations (M5 – M8), there were no significant changes observed in the summary table. The data in this table also shows that the results for duplicate arrays, (e.g. M3 and M4, M5 and M6) is very similar, thus allowing for comparisons between groups.

The data was then further analysed using the program ArrayTools, as described in Chapter 2, to determine the effect of wort gravity on chromosomal stability. Initial analysis performed was to determine if the specific gravity of the wort used in fermentation has any effect on the genome stability. A total of 658 genes, (11% of total genes analysed) were identified as being significantly affected at an alpha level of 0.001. It was observed that 50% of genes affected by a change in specific gravity are located within 20 ORFs of the end of the chromosomes and were defined as subtelomeric and telomeric genes as shown in Table 6.3. It is interesting to note that Chromosome I and VI show that 87 and 90% of genes affected are located in the telomere region respectively. The mean ratio of hybridisation of all ORFs for high gravity vs. low gravity arrays were plotted against the ORF position for each chromosome, (Fig. 6.5A – 6.5P). It can be clearly seen that the high gravity fermentation has a more profound effect on the chromosome stability (black columns) whereas the low gravity (pink line) fermentations are more stable with an average ratio of hybridisation of 0.99. A table listing the 658 genes found to be significantly different between the two conditions can be found in appendix II.

The remaining genes identified where the ratio of hybridisation was affected by changes in wort gravity during fermentation were observed in small ORF clusters and/or were evenly distributed amongst the chromosomes. One interesting gene cluster is on chromosome 12 encompassing the region YLR152C to YLR180W, as seen in Figure 6.5L. This region flanks a major ribosomal RNA gene cluster. This cluster covers approximately 1.2 Mb region consisting of tRNA elements

Table 6.3 Number of genes affected by change in Specific Gravity during Fermentation.

Chromosome	Number of genes affected by high gravity	Percentage located at telomere
I	33	87
II	35	43
III	16	25
IV	74	36
V	25	68
VI	20	90
VII	49	37
VIII	35	51
IX	30	33
X	46	51
XI	24	46
XII	62	37
XIII	69	23
XIV	44	28
XV	45	56
XVI	51	53

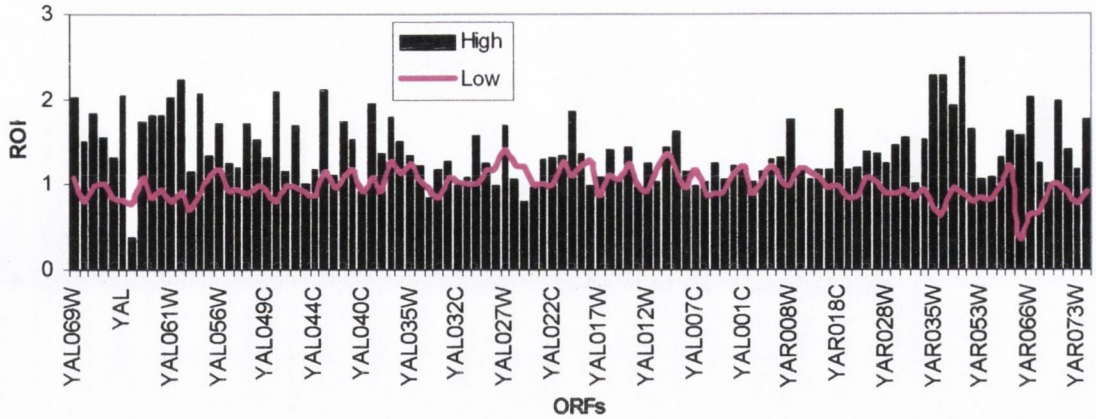


Figure 6.5A DNA microarray for chromosome I. The blue columns represent the average ratios of hybridisation for high gravity fermentations. The pink line represents the average ratio of hybridisation for low gravity fermentations.

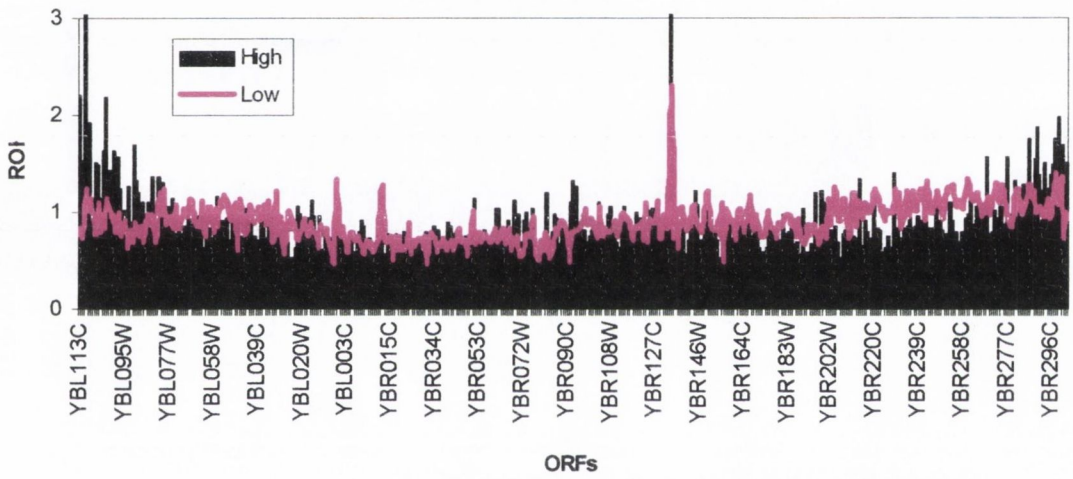


Figure 6.5B DNA microarray for chromosome II.

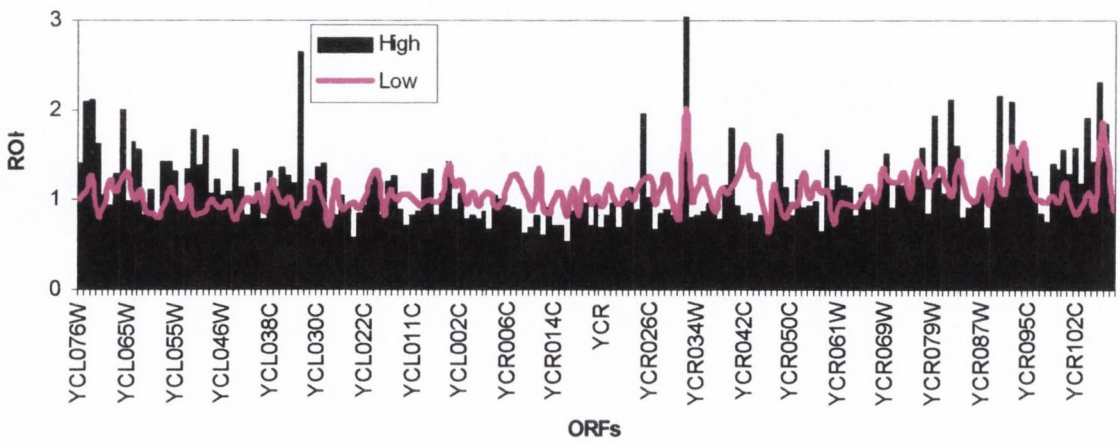


Figure6.5C: DNA Microarray for Chromosome III.

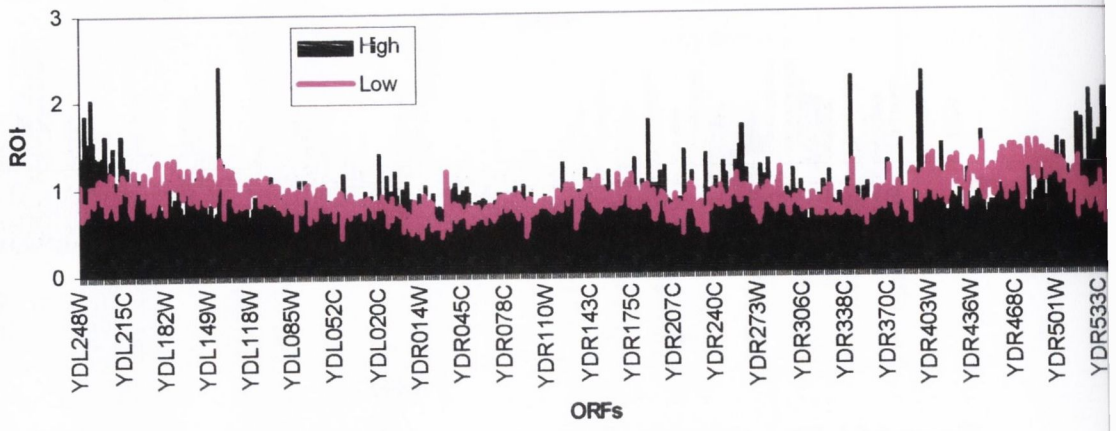


Figure6.5D: DNA microarray for chromosome IV.

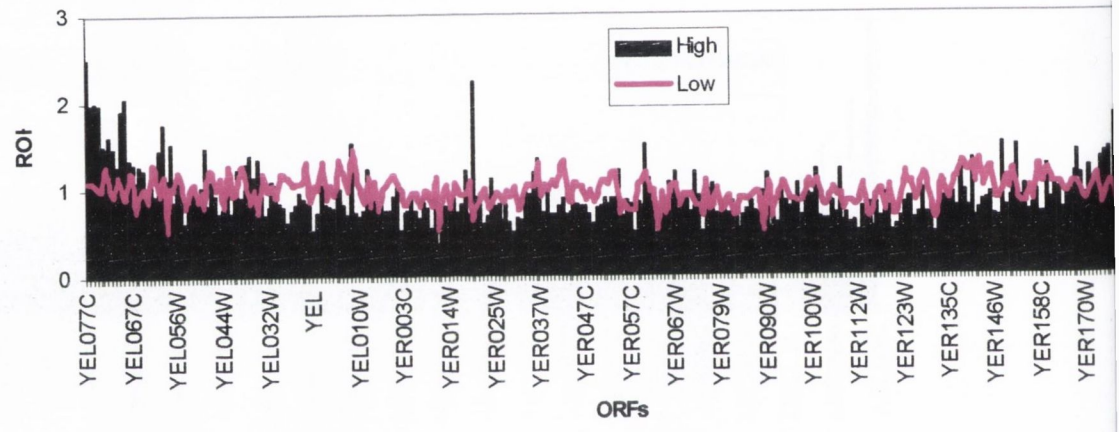


Figure6.5E: DNA Microarray for chromosome V.

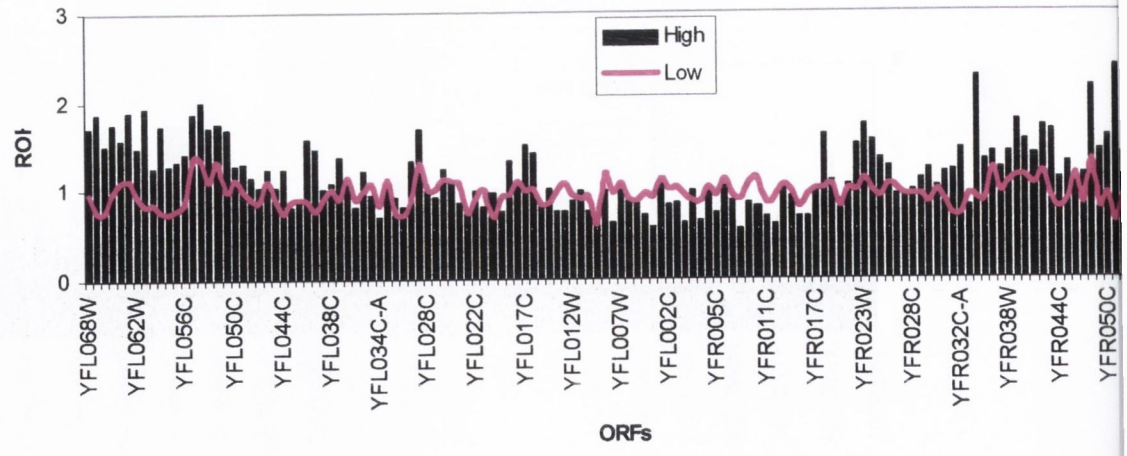


Figure 6.5F DNA microarray for chromosome VI.

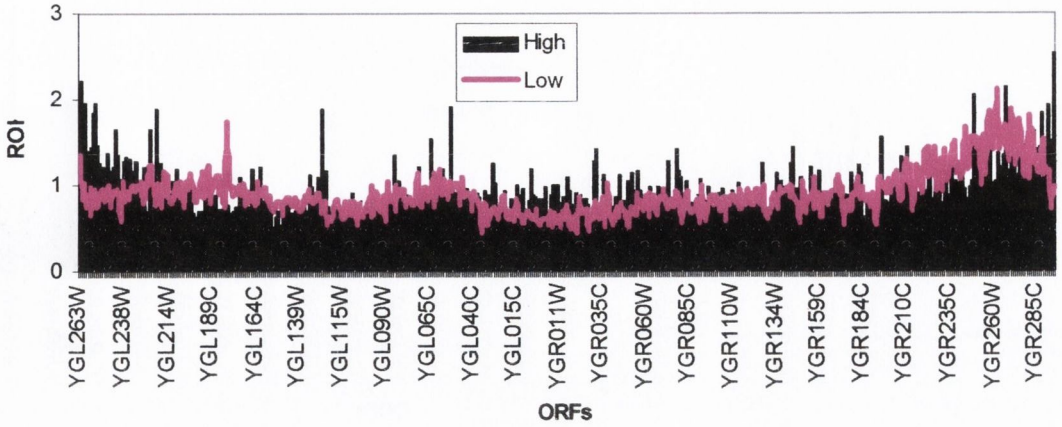


Figure6.5G: DNA Microarray for Chromosome VII.

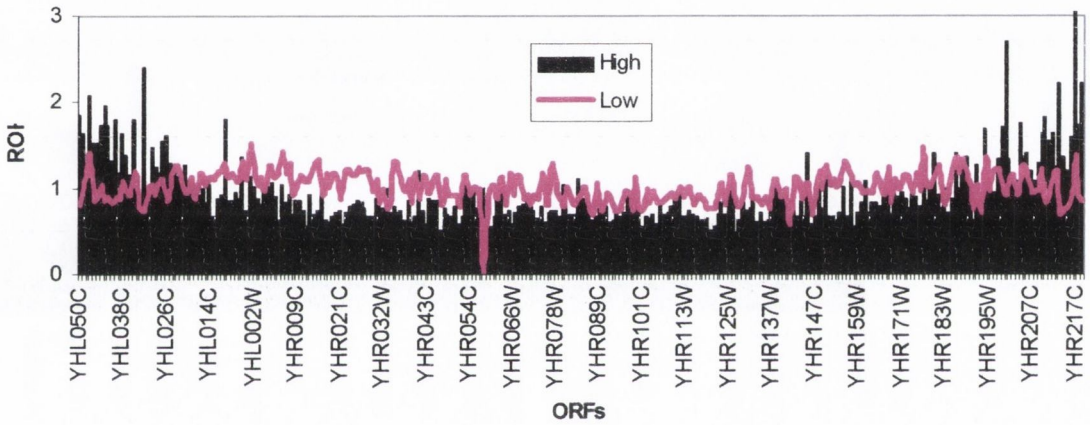


Figure6.5H: DNA Microarray for Chromosome VIII.

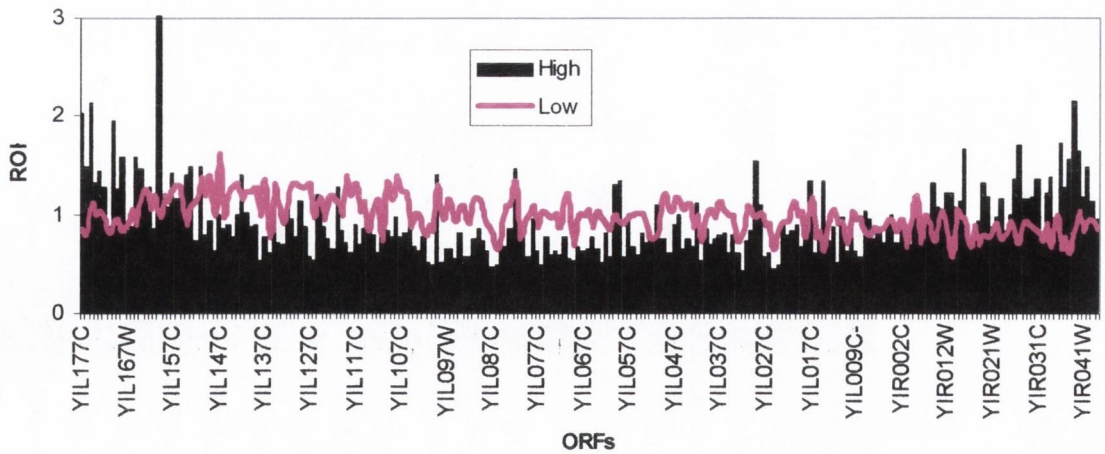


Figure 6.5I DNA microarray for chromosome IX.

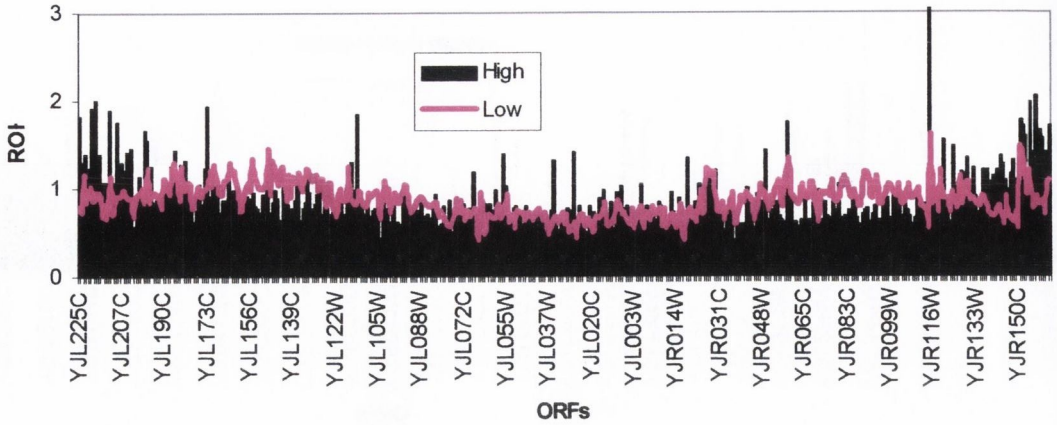


Figure 6.5J: DNA Microarray for Chromosome X.

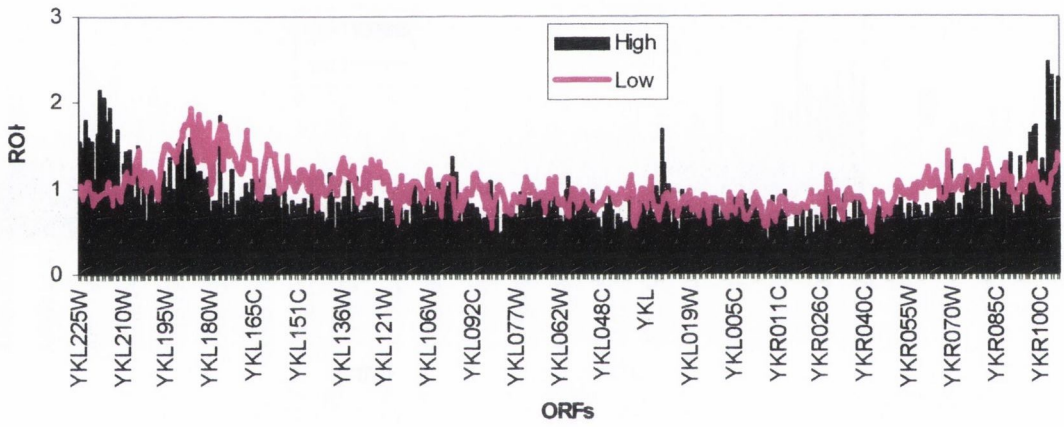


Figure 6.5K: DNA Microarray for Chromosome XI.

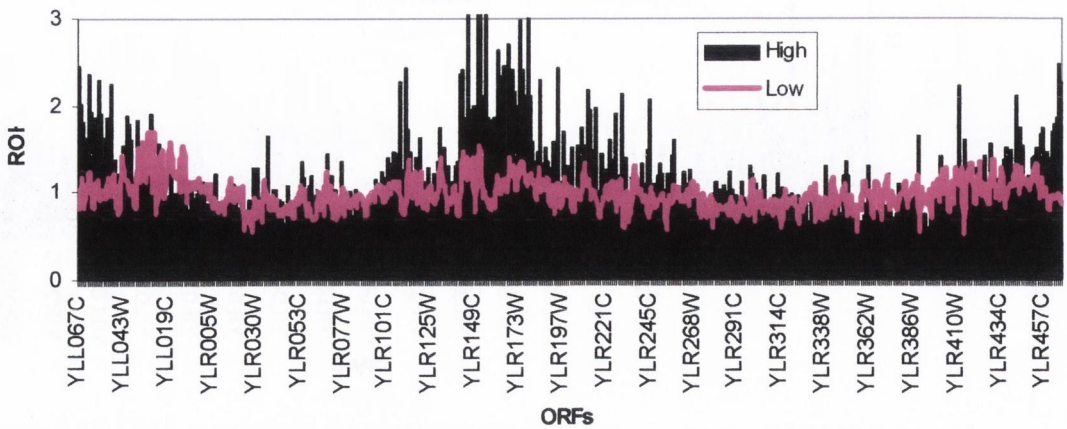


Figure6.5L: DNA Microarray for Chromosome XII

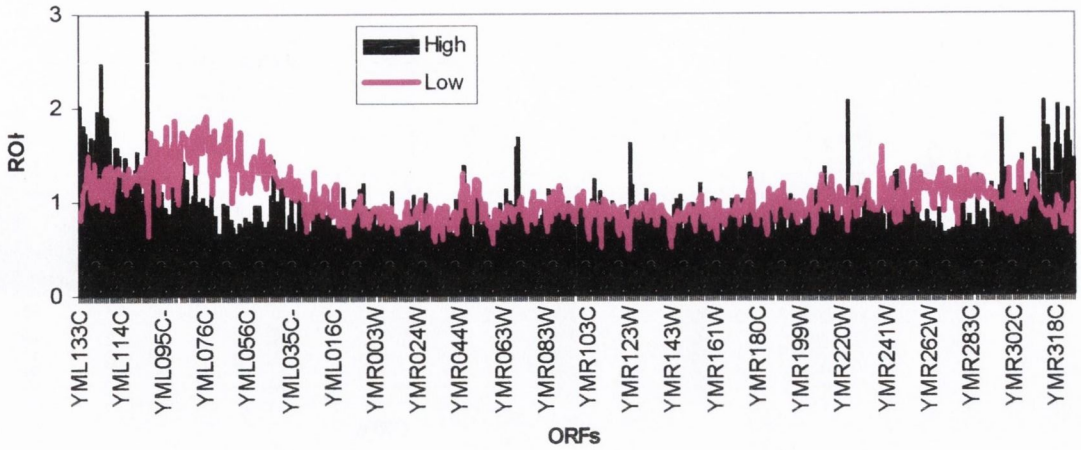


Figure 6.5M DNA microarray for chromosome XIII.

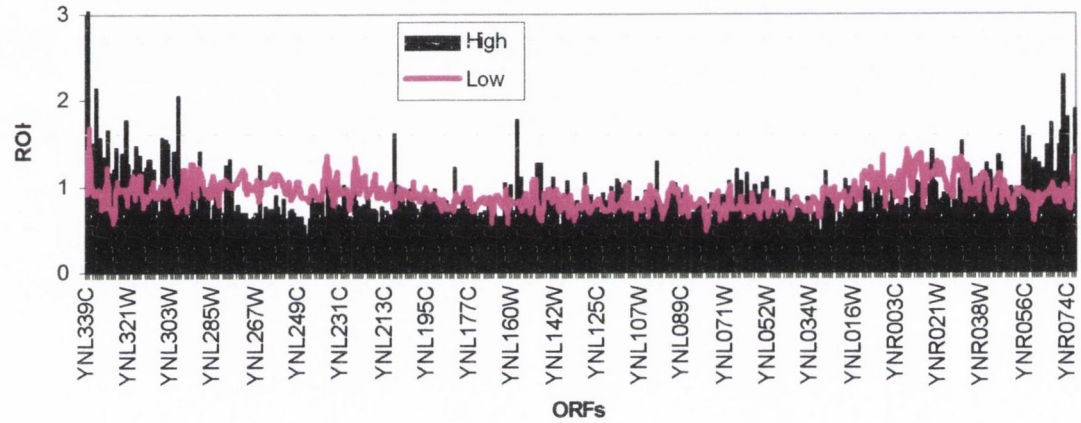


Figure6.5N: DNA microarray for chromosome XIV.

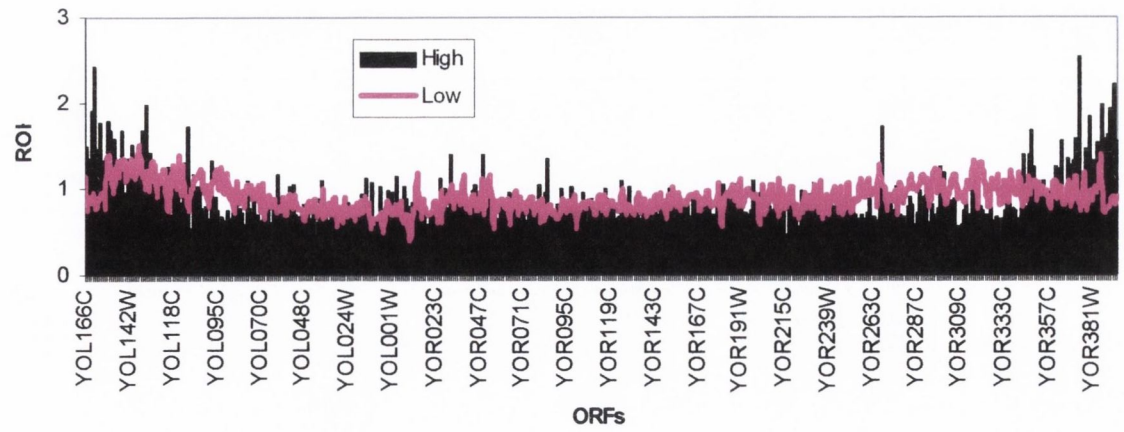


Figure6.5O: DNA microarray for chromosome XV.

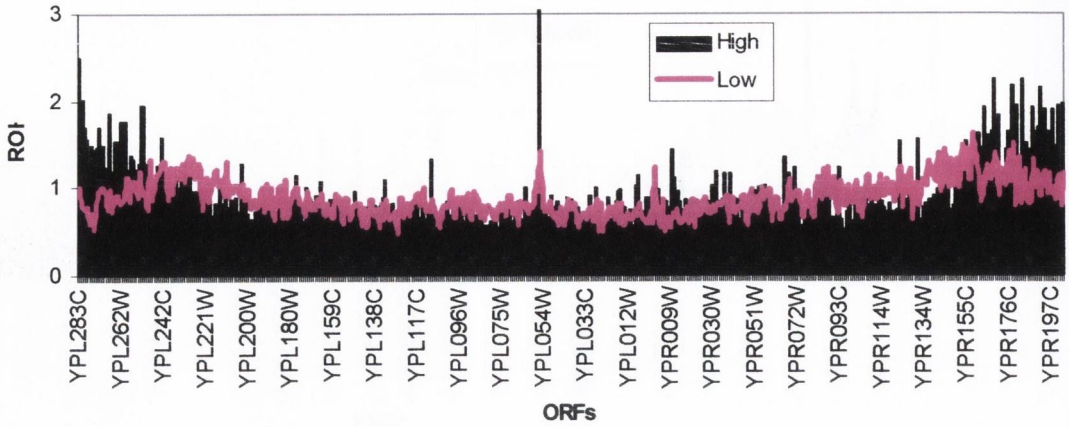


Figure6.5P: DNA microarray for chromosome XVI.

and 100 – 150 tandem copies of a 9.1kb repeat which contains the genes for 5S, 5.8S, 25S and 18S rRNAs.

However, it is not just high specific gravity that has an effect on genome stability. In Figure 6.5K representing high vs. low gravity for chromosome XI it can be seen that ORFs on the left arm of the chromosome have been affected at low gravity conditions. This effect is more pronounced in Figure 6.5M, which shows the average ratios of hybridisation plotted against the ORFs for chromosome XIII. A cluster of genes from YML024W to YML095C show an increase in the ratio of hybridisation in the low gravity fermentation. Through this study three major classes of changes have been identified under this analysis, telomeric changes, rRNA flanking genes and genes amplified in low gravity but not in high gravity.

It was also observed from the data of 658 genes affected by specific gravity that one group of genes involved in helicase activity, the YRF1 genes, show a lower ratio of hybridisation in the low gravity fermentations (average ratio of 0.94) compared to the high gravity fermentations (average ratio of 2.43). This group of genes is composed of seven genes, YDR545W, YER190W, YGR296W, YLR466W, YLR467W, YNL339C, YPL283C, YRF1-1 to YRF1-7 respectively. The ratio of hybridisation value for these genes under high and low gravity conditions are listed in Table 6.4. It may be coincidental that this group of genes show a higher ratio of hybridisation as they are flanked by Ty elements and an ARS element that contains a 36bp repeat sequence. Another group of telomeric genes that showed a higher ratio of hybridisation in the high gravity fermentations are the flocculation genes; YAR050W (FLO1), YHR211W (FLO5) and YAL063C (FLO9). Flocculation is the calcium – dependent, non-sexual aggregation of yeast cells into 'flocs' and is stimulated by nutrient limitation and is greatly important in brewery yeast strains (Teunissen and Steensma, 1995). Also noted was that the MAL group of genes involved in maltose transport and maltose permease show an increase ratio of hybridisation in high gravity fermentations, (Table 6.4B).

Table 6.4: Average Ratio of Hybridisation for YRF1 genes.

	Average Ratio of Hybridisation	
Gene	**High Gravity**	**Low Gravity**
YDR545W	2.4	0.9
YER190W	2.2	0.9
YGR296W	2.5	1
YLR466W	2.5	1
YLR467W	2.3	0.9
YNL339C	2.6	0.9
YPL283C	2.5	1

Table 6.4B: Average Ratio of Hybridisation for MAL genes.

		Average Ratio of Hybridisation	
Gene	**Systematic Name**	**High Gravity**	**Low Gravity**
MAL11	**YGR289C**	1.8	1.5
MAL12	**YGR292W**	1.9	1.1
MAL 13	**YGR288W**	1.4	1.3
MAL31	**YBR298C**	1.3	1.1
MAL32	**YBR299W**	1.9	1.1
MAL33	**YBR297W**	1.7	1.4

Of the 53 known genes involved in oxidative stress responses only 4 genes, YCL033C, YCR083W, YJR144W and YLR109W are shown to have a higher ratio of hybridisation in the high gravity fermentations, (data not shown). In the high gravity fermentations there is an average ratio of 2.2 compared to an average ratio of 0.8 in the low gravity fermentations. It can be deduced that the gravity of the wort used in fermentation does have an affect on the chromosomal stability of the lager brewing yeast. The effect in most notable in high gravity fermentations and the telomere and sub-telomeric regions of the chromosomes appear to be most affected. There is also an affect on chromosome stability in the low gravity fermentations but these changes do not occur at the telomeres but at clusters of genes in the chromosome.

However to be able to draw a stronger conclusion from this work the ratio of hybridisation values would need to be correlated with mRNA expression data.

6.2.3. Effects of aerobic vs. anaerobic fermentation on the CMBS-33 genome stability.

A second analysis was performed on the microarray data to determine if the fermentations performed in the presence or absence of oxygen affected the chromosomal stability in the lager strain CMBS-33. When the data was screened at a p-value of 0.001, no genes were significantly affected by the presence or absence of oxygen during the fermentation. This result is also observed when the data is plotted as ratio of hybridisation vs. ORFs for individual chromosome, as shown in Figures 6.6A and B representing data for chromosomes VI and X respectively.

Further analysis was performed by subdividing the data into high gravity aerobic and high gravity anaerobic fermentations. This analysis was performed at an alpha level of 0.001, no genes were found to significantly affect by the presence or absence of oxygen at this specific gravity. The same analysis was performed using the low gravity data sets. At a p-value of 0.001, 25 genes were found to be significantly affected by the presence of oxygen in low gravity fermentation.

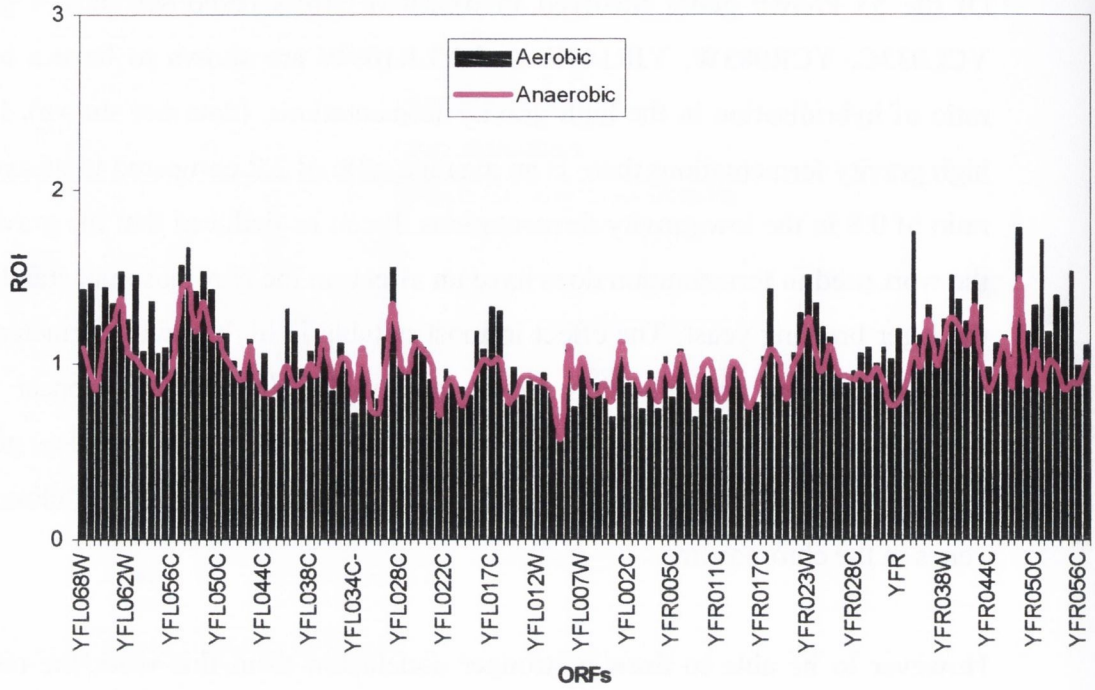


Figure 6.6.A DNA microarray for chromosome VI. The blue columns represent the average ratios of hybridisation for aerobic fermentations. The pink line represents the average ratio of hybridisation for anaerobic fermentations. As can be seen there is no significant difference between the two fermentation conditions.

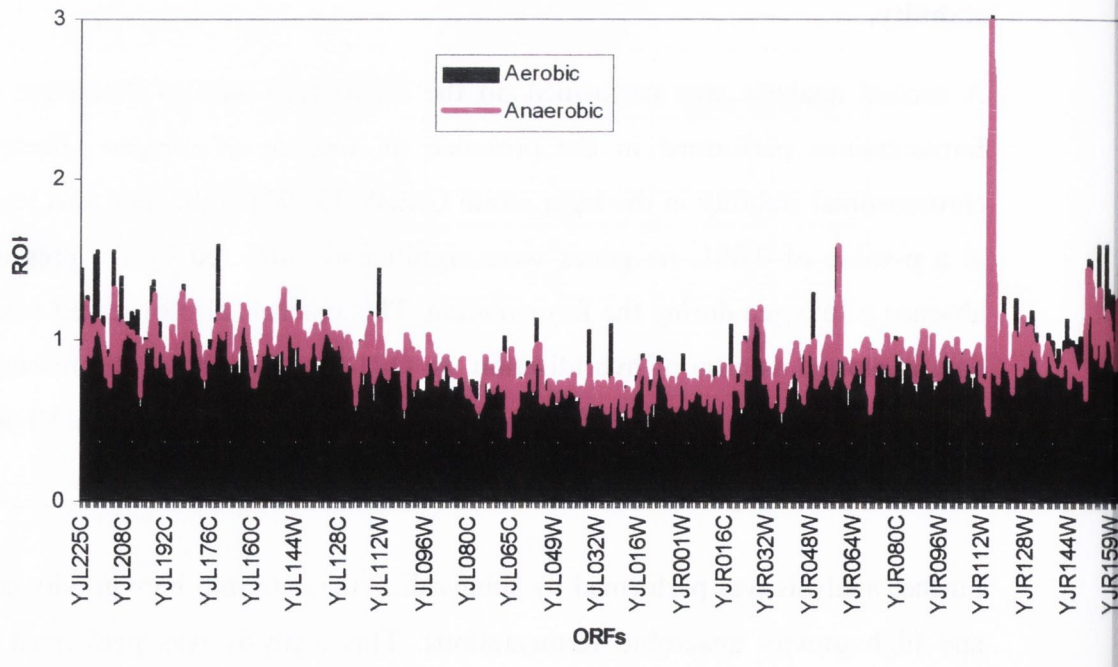


Figure 6.6B DNA microarray for chromosome X. The blue columns represent the average ratios of hybridisation for aerobic fermentations. The pink line represents the average ratio of hybridisation for anaerobic fermentations. As can be seen there is no significant difference between the two fermentation conditions with the average ratio of hybridisation for both conditions 0.87.

Table 6.5 list these genes along with their ratio of hybridisations. As can be seen from the table, generally a higher ratio of hybridisation was observed in the aerobic fermentations compared to the anaerobic condition. It is also interesting to note that none of the genes affected in this analysis are located at the telomeres of the chromosomes. It is possible that these may be random changes in the DNA. However further work would be to look at the transcription levels of the affected ORFs in the brewing strains compared to a haploid *S. cerevisiae.*

Table 6.5. Genes affected by aerobic or anaerobic conditions at low gravity fermentation conditions.

Unique id	Ratio of geom means	Geom mean of ratios in class 1 : Aerobic	Geom mean of ratios in class 2 : Anaerobic	M5 (Aerobic)	M6 (Aerobic)	M7 (Anaerobic)	M8 (Anaerobic)	Parametric p-value
YCL008C	1.532	1.342	0.876	0.146		0.604	1.6	0.0005952
YCL009C	1.57	1.32	0.841	0.941	0.925	0.348	0.301	0.000377
YCR046C	1.597	0.885	0.554	-0.161	-0.26	0.297	0.447	0.0001599
YCR048W	1.622	1.312	0.809	-0.01	-0.243	-0.874	-0.703	0.0003266
YCR051W	1.499	1.274	0.85	-0.387	-0.488	-1.252	-0.874	0.0006151
YCR059C	1.511	1.47	0.973	-0.312	-0.092	-1.197	-0.684	0.0006028
YCR061W	1.548	1.28	0.827	-0.135	-0.208	-1.411	-0.548	0.0003643
YDR014W	0.679	0.686	1.011	0.241	0.544	-0.558	-0.055	0.000897
YDR042C	0.669	0.686	1.026	-0.33	-0.024	-0.883	-0.82	0.0006927
YDR388W	1.541	0.738	0.479	0.001	-0.044	-0.639	-0.509	0.0004696
YER019W	1.668	0.869	0.521	-0.038	-0.103	0.482	0.763	0.000155
YGL180W	0.516	1.107	2.146	0.44	0.671	-0.138	0.06	0.0009937
YGR069W	0.641	0.713	1.113	0.245	0.453	-0.285	-0.183	0.0009166
YHR033W	1.466	0.985	0.672	0.337	0.375	-0.439	-0.108	0.0008853
YIL015C-A	1.583	0.861	0.544	-0.357	-0.075	-1.1	-0.655	0.000444
YLR415C	1.539	0.711	0.462	-0.245	-0.73	-0.044	0.354	0.0005678
YML083C	1.526	1.91	1.252	-0.19	0.167	-0.868	-0.425	0.0003028
YML102C-A	1.751	0.888	0.507	0.12	0.48	-0.708	-0.14	0.0002789
YMR122C	1.525	0.651	0.427	-0.436	-0.653	0.054	-0.022	0.0008606
YMR222C	1.582	0.916	0.579	-0.401	-0.475	-1.14	-0.948	0.0001829
YMR276W	1.552	0.992	0.639	-0.415	-0.671	0.055	0.017	0.0007967
YOL110W	1.652	1.231	0.745	-0.425	-0.815	-1.361	-1.091	0.0003351
YOR181W	1.522	0.738	0.485	-0.584	-0.399	-1.308	-0.92	0.0003925
YOR384W	0.668	0.864	1.294	0.213	0.588	-0.366	-0.134	0.0005592
YPR161C	0.618	0.952	1.54	0.31	0.54	-0.36	-0.021	0.0001086

6.3 Discussion

To examine if environmental conditions during fermentation affect the chromosome stability, a series of fermentations were performed under a variety of conditions. Firstly four high gravity fermentations (20°P) were performed, two under aerobic conditions and two under anaerobic conditions, where the brew was over - layered with sterile mineral oil. The second set of fermentations was performed under normal to low gravity conditions (16°P) with duplicates in aerobic and anaerobic conditions.

From the fermentation data obtained, the lager brewery strain CMBS-33 can survive and perform relatively well in wort concentrations ranging from low gravity to high gravity. A better fermentation profile for CMBS-33 was observed under aerobic conditions, this may reflect the need for oxygen for fatty acids and sterol synthesis, required for membrane maintenance. Using this initial data, cells were collected at days 1 and day 8 of the fermentation process and CCGH analysis performed on the isolated DNA to determine if these environmental conditions affect the genome stability and can play a role in promoting genetic flexibility and adaptive evolution to the stressful conditions the yeast cells experience during fermentation. Genome instability has been documented in laboratory yeast strains grown under nutritional selection (Dunham *et al.*, 2002). Using *S. cerevisiae* microarray chips it was possible to compare the genome of CMBS-33 at the two time points of day 1 and day 8. A downfall of this experimental design is that this type of analysis can give a lower estimate for the copy number of each of the chromosomes. This is due to the sequence divergence of the *S. cerevisiae*-like and non-*S. cerevisiae*-like genome homeologues in the lager yeast strain. Thus this can affect the degree of hybridisation between the target and the probe. However, the differential labelling of the lager yeast DNA with the two dyes Cy3 and Cy5 allows one to set the limits of homologous hybridisation. Divergence from these limits allows detection of differences between the *S. cerevisiae* and by deduction, the *S. bayanus* components of the two DNA samples under investigation. Following the performance of the DNA microarrays the data obtained from the fermentation M2 was not used in the

further analysis as analysis using the Microarray Tools program identified that it did not meet the criteria for being classified as belonging to the 'high gravity' class. Subsequent investigation of the data revealed low hybridisation signals and additionally uneven labelling across the microarray chip. Therefore this data was discarded.

Initial examination of the microarray data revealed that there is some variability between each duplicate fermentation. This is most likely because each fermentation was performed independently of one another from an individual colony. These small variations may represent sporadic genome changes that occur independent of the physiological conditions. However this variability was not seen to be statistically significant.

The initial examination of the microarray data was to investigate the effect of wort specific gravity on the genome stability. When comparing the classes of high vs. low gravity at the stringent p-value of 0.001, 658 genes were observed to be statistically significantly different between the two classes. One family of genes that were observed to be affect by the change in specific gravity were the YRF1 genes, which are a family of genes found in the telomeric Y' elements and encode a DNA helicase known as Y'-Help1 (Y'-HELicase Protein 1), which is induced in strains deficient for telomerase activity and expressed during meiosis. DNA helicase temporarily allows for the separation of the two strands of the DNA double helix therefore allowing DNA synthesis to take place at a replication fork. Y' is located adjacent to the telomeric repeats, either as a single copy or as a tandem repeat of two to four copies, at many but not all telomeres. Two types of Y' elements are known, Y'-S elements are 5.2 kb[1] and Y'-L elements are 6.7 KB in length, respectively (Yamada *et al.,* 1998). Any particular tandem array of Y' elements consists of either Y'-S or Y'-L, but not a combination of both elements. In the study by Yamada *et al.,* (1998) it was observed that expression of native Y' elements in wild-type cells is repressed probably by the telomere silencing effect. However, when telomeres have shortened in telomerase-defective mutant cells and all telomeric repeats have been lost, the silencing effect is not effective. The Y' element may then be de-repressed to produce Y'-Help1. Y'-Help1 then may

enhance homologous DNA recombination among Y' elements and, as a consequence, may induce Y' amplification to prevent chromosomal loss and cell death. It appears that Y'-Help1 can only be effective in certain circumstances, such as when the telomere and subtelomeric structures are altered due to telomere shortening. Since telomere amplification is a noted effect in high gravity fermentation, it is possible that this class of helicases are active under these conditions. Transcription analysis of these genes should verify this.

Another group of telomeric genes that showed a higher ratio of hybridisation in the high gravity fermentations were the flocculation genes. Two classes of flocculation phenotypes have been characterised, the FLO1 – type is inhibited by mannose sugars and the NewFLO – type is inhibited by the sugars mannose, maltose, glucose and sucrose (Stratford, 1989). There are three dominant flocculation genes, FLO1, FLO5 and FLO8, two of which were found to be significantly affected by changes in specific gravity in this study, namely FLO1 and FLO5. Floc forming ability conferred by FLO1 is chymotrypsin sensitive and heat resistant. Flo1p is a lectin like cell surface protein, which aggregates, cells into flocs by binding to mannose sugar chains on the surfaces of other cells. The sequence similarity between FLO5 and FLO1 suggests that Flo5p is also a mannose binding lectin like cell surface protein. Sequence analysis suggests that the flocculation genes are a multi-gene family localized to telomeric sequences (Teunissen and Steesma, 1995). The FLO9 gene is 94% similar to the FLO1 gene and may be the source of a 4.2Kb transcript whose presence is not affected by the deletion of FLO1 or FLO5.

The data obtained in this study indicates that the fermentation of yeast cells in high specific gravity wort results in a greater degree of chromosomal instability most notably in the telomeres. There were some specific regions of localised amplification observed in the low gravity fermentations that were specific to this environment. Therefore combining high gravity fermentation along with aerobiosis stresses increase the instability of the lager yeasts chromosomes. High levels of chromosome instability has previously been observed in polyploid strains of *S. cerevisiae* (Mayer & Aguilera, 1990). It has previously been shown

that tetraploid strains of *S. cerevisiae* display an increase in chromosome loss compared to diploid strains (Andalis *et al.*, 2004), an increase in chromosomal rearrangement had also been observed in polyploid strains (Huang & Koshland, 2003). The loss of chromosomes along with genetic rearrangements are not directly related to an increase in mitoitic frequency however Storchova *et al.*, (2006), have observed that polyploids show an increased sensitivity to double strand break inducing agents. Storchovas study also showed that tetraploids require homologous recombination for survival, thus implying that there is a high degree of spontaneous DNA lesions. Polyploid cells also display defects in microtubule – kinetochore attachment which can lead to chromosome non-disjunction and therefore chromosome loss.

The chromosomal rearrangements and gene amplifications seen in the lager yeast strains may be explained by models of non-homologous DNA repair mechanisms. Spontaneous replication-induced double strand breaks in the DNA are likely to initiate chromosomal rearrangements and amplification of DNA. The presence of repeat sequences such as Ty elements, multicopy genes, ARS sequences, flanking double strand breaks can invoke intra – and intermolecular recombination events resulting in the formation of dicentric chromosomes. At anaphase, as the centromeres are pulled to opposite poles the breaking of the dicentric dimmers can lead to gross chromosomal rearrangements and chromosome loss (VanHulle *et al.*, 2007). Further rounds of replication results in the DNA amplification of genes in the vicinity of the repeat sequences (Butler *et al.*, 2004) as was also observed in this study. Additionally, specific regions of DNA amplification were also noted under high gravity conditions. This may result from double stranded breaks, followed by non-homologous recombination and subsequent amplification of DNA in the vicinity. This was most evident for genes of the rRNA cluster on chromosome XII and on chromosome IV.

These studies on polyploid strains of *S. cerevisiae* may aid in the understanding of the mechanisms responsible for chromosome loss and rearrangements observed in the lager yeast strains. Chromosome loss and rearrangements in a polyploid genome consisting of mosaic chromosomes appears to confer a selective

advantage to these strains of yeasts which is notable in their fermentative capacity and ability to adapt and survive extreme environmental conditions.

When looking at the affect of aerobic vs. anaerobic conditions on genome stability at the stringent p-value of 0.001 no significant genes were detected. Therefore one can conclude from this study that the genome stability of the lager yeast strain CMBS-33 is not affected by the presence of oxygen wort used in fermentation. However, some cumulative effects were observed when the stresses high gravity and aerobiosis were combined. The lack of effect by oxygen is not unexpected as fermentations quickly achieve anaerobic conditions due to the high level of carbon dioxide produced. Therefore actively preventing additional oxygen entering the system may not be necessary. This work may also have implications in the maintenance of pure breed yeast stocks and the usage of high specific gravity in brewing.

Chapter 7
General Discussion.

7.1 In Summary.

Yeast is important for systems biology not only as an excellent model species but also from an industrial point of view. A wealth of biological information such as genome wide data and metabolic pathways are available on the yeast *Saccharomyces cerevisiae*, allowing it to serve as a basis for the characterisation of the more complex industrial strains of yeast. The lager yeasts arose from the natural genome fusion of two yeast strains *S. cerevisiae* and a *S. bayanus* – like strain. Classical genetic studies using the technique of single chromosome transfer from lager strains to the haploid *S. cerevisiae* have concluded that lager yeast genomes consist of *S. cerevisiae* chromosomes, non-*S. cerevisiae* and mosaic chromosomes consisting in part of *S. cerevisiae* and non-*S. cerevisiae* genes as discussed in detail in Chapter 1. The analysis of individual genes in lager strains of yeast has revealed at least two or more copies of most genes, one closely related to the equivalent gene in *S. cerevisiae* with the other showing a higher degree of divergence, i.e. Sc- and non-Sc type chromosomes. The overall analysis of the lager yeast genome indicates that the genomes are polyploidy. However individual chromosomes or chromosome regions appear to be aneuoploid in nature.

Initial CGH studies (Bond *et al.,* 2004), of the lager yeast genome provided information on the gene copy number and also highlighted eight chromosomes in which recombination events have occurred between the parental strains *S. cerevisiae* and *S. bayanus*, (Table 1.1). Using this data, a goal of this work was to contribute to the current knowledge of the genomic organisation in the lager brewery strains. Through the utilisation of well established techniques PFGE and Southern blot analysis, it has been possible to ascertain a gross picture of the chromosome composition in the lager yeast strains. In this study chromosomes III and XVI were focused on as both chromosomes show unique regions of chromosomal recombination.

On chromosome III, the ratio of hybridisation of genes on either side of the MAT locus varies, genes to the left of the MAT locus have a ratio of hybridisation of

between 0.5 and 1.0, which was strain dependent, whereas the genes to the right of the MAT locus have an increased ratio of hybridisation of 1.7. Thus indicating that there is a higher copy number of *S. cerevisiae*-like genes to the right of the MAT locus in the lager yeast strains as a result of a recombination event between homeologus chromosome III's or the recombination of genes to the right of the MAT locus with another chromosome. The results obtained (Fig. 3.1 A, B, C) confirmed the CGH data and showed that the lager yeast strains chromosome III has the same electrophoretic pattern as the *S. cerevisiae* chromosome III and has not undergone any gross chromosomal rearrangement indicating that recombination has occurred between homeologous chromosome pairs at the MAT locus to generate a series of mosaic chromosomes.

The unique CGH data obtained for chromosome XVI revealed that the region from YPR160W to YPR190C sequence has significantly diverged from that of *S. cerevisiae* sequence for this region resulting in a ratio of hybridisation of 0.2. To gain an initial gross picture of this region, PFGE and Southern blot analysis was used to confirm the CGH data and determine if there had been any inter-chromosomal rearrangement within the lager yeast genome. The resulting analysis revealed that the region is lacking *S. cerevisiae* like genes, thus confirming the CGH data and again revealed that chromosome XVI has not undergone any gross chromosomal rearrangement. A total genomic library of the lager strain CMBS-33 was constructed to allow the characterisation of the area of recombination at the ORFs YPR159W - YPR160W at a nucleotide level, as discussed in Chapter 3, section 3.2.1.4. From the sequence obtained it was observed that the YPR159W lager yeast sequence had not diverged from that of the available *S. cerevisiae* YPR159W sequence. It was also interesting to note that the intergenic region was highly conserved which shows that the recombination between the parental strains did not occur in this region. The sequence obtained for the lager yeast YPR160W shows that the recombination between the parental strains occurs within the beginning of the ORF resulting in a gene that appears to be non-functional. As YPR160W is a non-essential gene involved in glycogen phosphorylation, null strains are viable and cellular glycogen is also rapidly catabolized to glucose by the Sga1p glucoamylase, (YIL099W). The most likely

cause for the recombination to occur with an ORF is the presence of repeat sequence in the form of a dubious ORF within the YPR160W gene.

The next aim was to examine the expression levels of genes in the lager yeast. Firstly the expression levels of homologous genes was examined under standard fermentation conditions. A caveat of the experimental design was that only the expression level of the *S. cerevisiae*-like genes was examined. The contribution of *S. bayanus* genes in the lager yeast was not included in this study. Chromosome III was chosen for this initial study due to the significant difference in ratio of hybridisation observed in the CGH analysis as discussed in Chapters 1 and 3. It was observed that the overall level of hybridisation in the lager strain CMBS-33 was higher for all four probes used, (Chapter 4, section 4.2.1). This most likely reflects the increase in copy number in *S. cerevisiae*-like genes in the lager yeast and indicated that all alleles are contributing to the expression profiles. The hybridisation levels were dependent on the probe homology, the gene copy number and the level of expression for each gene and if the expression of the specific gene being investigated is influenced by the environment in which the cells were grown.

To determine if the copy number of *S. cerevisiae* genes influences the expression profile of *S. bayanus*-like genes in the lager strains, a model system was developed where a section of the mosaic chromosome XVI was introduced into isogenic polyploid *S. cerevisiae* strains. Using this rationale, it was possible to examine the expression of the *S. bayanus* component of the lager genome. The resultant analysis of the expression of YPR160W RNA by RT-PCR indicated that this gene can produce a mature mRNA. Furthermore, as the *S. cerevisiae* gene copy number increases, the expression of the *S. bayanus*-like gene decreased. This data when taken together with the sequencing data proposes that in the lager yeast the ORF YPR160W is transcribed into an RNA sequence but when the mRNA is exported to the cytosol it is not translated into a functional protein.

The experiment was repeated for three other ORFs in the region of low hybridisation on chromosome XVI, YPR161W, YPR175W and YPR186C. The

same result was obtained for these ORFs indicating that as the ploidy of the *S. cerevisiae* genome increased the level of expression of the *S. bayanus*-like genes decreased. The apparent decrease in expression of the *S. bayanus*-like genes may be due to the lack of a lager yeast specific transcription factor in the isogenic polyploid *S. cerevisiae* strains used and the inability of the lager gene to compete for the *S. cerevisiae* transcription factors. Since all of the lager yeast genes showed the same response to increased *S. cerevisiae* gene copy number, it is possible to draw the general conclusion that the copy number of homeologous genes can influence the expression of other homeologous alleles, suggesting that gene dosage may occur in lager yeasts. Exploring this phenomenon in more detail will provide greater insights into the complex expression patterns in polyploid cells. In the lager yeast the complexity of gene regulation is unknown and it is essential to know the nature of the regions promoter for a given gene and how many transcriptional factors bind the promoter.

In the second section of this project, the aim was to generate stress tolerant strains of the lager yeast strain CMBS-33. These stress tolerant strains would be beneficial to the brewing industries as industrial yeast strains experience increasing exposure to environmental stresses during the fermentation process. While there is vast knowledge about the cells responses to exposure to environmental stresses in haploid strains of yeast, the knowledge base for stress responses in polyploid yeast strains and other industrial yeast strains has been less well examined. In general, the polyploid lager yeast strains appear to be inherently stress tolerant, withstanding environmental conditions not tolerated by haploid *S. cerevisiae* strains, as discussed in Chapter 5, section 5.2.4.

The protection to various forms of stresses can be induced by a prior exposure of cells to any stress condition that is capable of inducing Hsps. This phenomenon is referred to as cross – tolerance or in the case of this work thermotolerance as this was the basis of the method used to isolate stress tolerant strains of lager yeasts. Therefore the acquired thermotolerance in cells should render cells resistant to many other forms of stress including hydrostatic pressure, osmotic and oxidative stress (Castro *et al.,* 2007, Hohmann, 2002). It was not previously known if such

cross – tolerance would enhance the fermentative capacity of lager yeast strains and more importantly whether the acquired resistance to stress was a heritable characteristic. Heritable stress tolerance has previously been demonstrated in *S. cerevisiae* and has been associated with genetic alterations in regulators of the stress response (Lindquist *et al.,* 1995, Van Dijck *et al.,* 2000), although stress resistance may be acquired by other mechanisms independent of the known stress factors, including trehalose, Hsp104 and transcription factors. Previous stress tolerant strains generated involved using a combination of EMS mutagenesis and selection of cells surviving exposure to high temperatures and high specific gravity wort. From this study three stress – tolerant strains were identified. Each of these strains retained their fermentative capacity and one mutant C10B was found to show increased fermentation rates compared to the parental strain CMBS-33. These mutants retained their thermotolerance over many generations they did not show cross tolerance to other forms of stress such as oxidative stress as discussed in chapter 5. Therefore from this it can be concluded that inherited thermotolerance does not necessarily confer increased fermentative capacity to the mutants or cross – tolerance to other forms of stress.

Genomic analysis of the first generation of stress mutants indicated that the strains had undergone gross chromosomal rearrangements and/or chromosomal loss. This posed the question: if these gross changes were the result of EMS mutagenesis or from the exposure to the stress conditions or if both scenarios had an affect? A second generation of stress- tolerant mutants were generated by exposure to heat and high specific gravity, in the absence of EMS treatment, as discussed in Chapter 5. Chromosomal rearrangements were also observed in these strains. Therefore it can be deduced that exposure to extreme stress conditions results in chromosomal changes in the lager yeast genomes. A further analysis of the strains using DNA microarray technology revealed that such changes can occur during a single round of high gravity fermentation. Under these conditions, localised regional amplification rather than chromosome rearrangements are more apparent. However, specific chromosome rearrangements were observed in individual fermentations indicating that the genomes of the lager brewing yeasts

highly dynamic and can undergo chromosome alterations during the brewing process.

To determine if the chromosomal alterations were influenced by environmental stresses, the chromosomal stability of the parental strain CMBS-33 was analysed in fermentations performed in high or low gravity and in the presence or absence of oxygen. The data obtained from seven independent experiments indicated that the fermentation of yeast cells in high gravity wort resulted in a greater degree of chromosomal instability most notably in the telomeric regions. Some specific regional amplifications were also observed noted in cells fermented in low gravity wort that were not apparent in cells fermented in high gravity wort. Thus suggesting that specific chromosomal changes may relate to specific environmental conditions. Further to this, combining stresses such as exposure to high specific gravity and aerobiosis may exacerbate the effects of chromosomal instability.

A key observation in this study was the presence of localised regions of gene amplification, as evident for genes flanking the major rRNA gene locus on chromosome XII. This locus encompasses a region of approximately 900kbp and contains 100 – 180 repeats of rRNA genes. Another such cluster is found on chromosome I in the region YAR028W – YAR040W. These localised gene amplification events are most often associated with the presence of a Ty element and/or an ARS sequence in the flanking regions. However, genes showing the highest levels of amplification YBR134W and YJR115W, are not associated with such elements. Both genes are classified as 'dubious ORFs' in the Saccharomyces Genome Database, however in the lager yeast genome they may represent 'hot spots' for chromosome rearrangements or there may be uncharacterised Ty elements associated with the genes in the lager yeasts.

High levels of chromosome instability have previously been shown in polyploid strains of *S. cerevisiae* (Mayer & Aguilera, 1990). Using isogenic polyploid strains of *S. cerevisiae*, Andalis *et al.*, (2004), showed that tetraploid strains of *S. cerevisiae* displayed an approximate 400 – fold increase in chromosome loss

compared to isogenic diploid strains. Additionally small but significant increases in chromosomal rearrangements were observed in polyploid strains (Huang & Koshland, 2003). Such chromosomal loss and rearrangements are not directly related to increases in mitotic frequency, rather polyploid strains display an increased sensitivity to double strand break inducing agents (Storchova *et al.*, 2006).

The chromosomal rearrangements and gene amplifications observed in the lager yeast strains may be best explained by models of non – homologous DNA repair mechanisms. Chromosomal rearrangements and gene amplification most likely initiate from the spontaneous replication induced double strand breaks (DSBs) in DNA. The presence of palindromic sequences in the vicinity of DSBs invokes intra – or inter – molecular recombination events that result in the formation of dicentric chromosomes. The subsequent rounds of replication, leads to the amplification of DNA in the genes surrounding the palindromic sequences. At anaphase, as the centromeres are pulled apart to the opposite poles, breakage of the dicentric chromosomes can lead to gross chromosomal changes and chromosome non – disjunction (VanHulle *et al.*, 2007).

The defects observed in isogenic *S. cerevisiae* strains along with the emerging picture of mechanisms involved in the repair of DSBs in *S. cerevisiae* will aid in the understanding of underlying mechanisms that may be responsible for gene amplification and rearrangements observed in aneuploidy lager yeasts. While chromosomal rearrangements have been shown to reduce the fitness in haploid *S. cerevisiae* strains (Anadalis *et al.*, 2004), the existence of a polyploid genome consisting of mosaic chromosomes appears to confer a certain degree of selective advantages to the lager yeasts in respect to the capacity to ferment and adapt to extreme environmental conditions. This implies that the lager yeasts have either an increased ability to repair DNA lesions or can withstand DNA damage due to the redundant nature of their genome. These findings that the exposure to environmental stress can influence stability of chromosomes may lead to implications for the brewing industry in the maintenance of pure breed yeast stocks and in the utilisation of high specific gravity brewing.

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Appendix I

Name	F635 Median	F635 Mean	F635SD	B635 Median	B635 Mean	B635SD	F532 Median	F532 Mean	F532SD	B532 Median	B532 Mean	B532SD	Ratio of Medians	LogRatio	F635MedianMnB635	F532 MedianMnB532
YAR061W	797	817	173	103	111	134	618	618	97	348	365	276	2.57	1.362	694	270
YAR062W	3143	3061	719	105	107	24	1579	1530	287	405	408	59	2.588	1.372	3038	1174
YAR069C	3883	3961	789	103	106	24	1713	1762	342	380	381	57	2.836	1.504	3780	1333
YBL016W	14561	14254	2221	108	110	26	5875	5712	932	363	365	61	2.622	1.391	14453	5512
YBL109W	51159	47853	10753	113	113	24	11478	10893	2619	408	409	56	4.611	2.205	51046	11070
YBR134W	15964	16146	5336	103	105	25	2543	2448	667	412	412	62	7.443	2.896	15861	2131
YBR246W	13086	12523	3137	104	106	23	5336	5103	1204	397	400	64	2.628	1.394	12982	4939
YBR261C	16550	14913	5038	112	114	23	6459	5859	1946	418	420	58	2.721	1.444	16438	6041
YCR041W	9411	9401	2063	102	103	23	4045	3998	824	465	464	66	2.6	1.379	9309	3580
YCR098C	15867	15826	2176	114	114	22	6317	6237	786	421	422	58	2.672	1.418	15753	5896
YDR544C	39780	40365	10420	108	112	30	10853	10778	2862	367	370	56	3.783	1.92	39672	10486
YEL008W	10672	10663	2284	105	106	23	4096	4092	832	374	377	53	2.839	1.505	10567	3722
YEL012W	16785	16473	3324	113	119	124	5633	5482	1079	405	415	161	3.189	1.673	16672	5228
YEL016C	8757	8304	2283	105	111	65	3410	3216	849	384	388	76	2.859	1.516	8652	3026
YEL019C	12193	12156	2594	115	117	26	4439	4357	906	399	404	63	2.99	1.58	12078	4040
YEL068C	6841	6809	1645	106	109	40	3018	2941	657	402	407	71	2.575	1.364	6735	2616
YER010C	9596	9049	3778	108	109	29	3686	3469	1293	410	416	68	2.896	1.534	9488	3276
YER140W	12606	12420	3175	115	116	25	5226	5046	1247	400	399	59	2.588	1.372	12491	4826
YFL001W	7393	7442	1554	117	137	285	3188	3261	592	448	475	400	2.655	1.409	7276	2740
YFL007W	9910	10000	2506	109	112	29	3451	3435	728	435	442	64	3.25	1.7	9801	3016
YFL009W	10309	9713	2523	105	107	26	3907	3676	865	376	380	58	2.89	1.531	10204	3531
YFL013C	11681	10974	3155	118	127	134	4941	4742	1179	441	449	83	2.57	1.362	11563	4500
YFL016C	8467	8716	2689	109	112	45	3535	3511	1017	357	358	63	2.63	1.395	8358	3178
YFL017W-A	5046	4977	1392	107	118	73	2023	1982	445	402	402	62	3.047	1.607	4939	1621
YFL025C	7115	6863	2267	116	124	118	3019	2859	863	403	409	85	2.675	1.42	6999	2616
YFL026W	11895	12118	3406	109	117	80	4256	4463	1212	378	385	89	3.039	1.604	11786	3878
YFL027C	6595	6615	1417	99	100	22	2893	2873	516	423	427	62	2.63	1.395	6496	2470
YFL028C	9873	9247	2419	114	116	25	3681	3442	843	400	403	57	2.974	1.573	9759	3281
YFL029C	9988	8645	4204	113	116	27	3196	2883	1293	430	431	63	3.57	1.836	9875	2766
YFL033C	9905	9275	2627	109	110	23	3878	3635	953	381	386	55	2.801	1.486	9796	3497
YFL035C	14445	13892	2964	105	106	23	5832	5578	1208	383	384	55	2.632	1.396	14340	5449
YFL036W	6121	6053	1055	110	112	38	2661	2620	438	406	409	69	2.666	1.414	6011	2255
YFL050C	6576	6843	1624	111	114	31	2761	2783	553	400	406	69	2.738	1.453	6465	2361
YFR004W	16193	16057	2838	113	114	25	6400	6339	1123	407	410	58	2.683	1.424	16080	5993
YFR006W	9998	9081	3458	116	124	121	3785	3457	1205	405	409	84	2.924	1.548	9882	3380
YFR013W	8872	9264	2603	105	106	23	3421	3505	900	418	419	59	2.919	1.546	8767	3003
YFR018C	7106	6885	3600	105	106	21	2640	2528	1115	402	403	57	3.128	1.645	7001	2238
YFR019W	6426	6421	1578	112	141	183	2836	2781	606	413	430	146	2.606	1.382	6314	2423
YFR021W	16943	16855	3504	122	122	24	6220	6103	1172	438	440	59	2.909	1.541	16821	5782
YFR022W	12979	12729	6380	111	135	394	4781	4606	2122	416	434	246	2.948	1.56	12868	4365
YFR023W	8929	9663	3045	112	113	23	3546	3722	1007	424	425	60	2.824	1.498	8817	3122
YFR024C	10721	10682	2280	108	114	120	4372	4363	844	353	360	142	2.641	1.401	10613	4019
YFR024C-A	11170	10924	3021	105	108	32	4680	4552	1219	400	401	56	2.585	1.37	11065	4280
YFR025C	11322	10802	2494	106	120	199	3964	3772	854	379	388	150	3.129	1.646	11216	3585
YFR026C	3025	3029	848	103	105	26	1129	1091	222	365	367	58	3.825	1.935	2922	764
YFR029W	15289	15129	2214	116	118	29	6178	6046	899	432	432	58	2.641	1.401	15173	5746
YFR034C	16657	17235	4823	116	119	24	6789	6889	1795	416	417	58	2.595	1.376	16541	6373
YFR035C	7367	7314	2221	108	111	43	3070	3097	861	415	421	101	2.734	1.451	7259	2655
YFR036W	9812	9629	2188	107	116	97	4006	3934	895	378	381	68	2.675	1.42	9705	3628
YFR039C	10461	10659	2791	109	111	24	3714	3780	855	387	387	55	3.112	1.638	10352	3327
YFR042W	5287	5370	1144	108	109	28	2101	2091	407	414	415	62	3.07	1.618	5179	1687
YFR048W	5317	5400	1551	108	111	36	2160	2217	582	381	381	58	2.928	1.55	5209	1779
YFR050C	14340	13656	3192	99	102	23	5711	5453	1242	368	369	56	2.665	1.414	14241	5343
YHL005C	4169	4316	1231	105	107	41	1929	1912	472	391	393	76	2.642	1.402	4064	1538
YHL024W	11141	11127	1860	108	109	26	4563	4495	784	365	368	55	2.628	1.394	11033	4198
YHL035C	4882	5003	1288	117	118	24	2174	2206	498	458	460	62	2.777	1.473	4765	1716
YHR005C-A	2192	2137	1233	103	108	39	1176	1177	514	365	373	68	2.576	1.365	2089	811
YHR067W	4299	4391	1626	109	124	105	2037	2097	642	404	410	76	2.566	1.359	4190	1633
YHR166C	1246	1372	743	108	119	104	854	903	286	410	416	65	2.563	1.358	1138	444
YHR167W	3702	3789	606	110	114	42	1365	1363	189	399	401	66	3.718	1.895	3592	966
YHR168W	9210	9340	1965	105	105	22	3209	3317	653	437	441	64	3.285	1.716	9105	2772
YHR169W	11948	11824	2620	123	139	306	3502	3483	705	425	429	102	3.843	1.942	11825	3077
YHR170W	7088	7037	2047	111	116	43	2656	2727	690	378	382	84	3.063	1.615	6977	2278
YHR171W	7644	7520	2442	106	110	39	2660	2679	768	380	386	82	3.306	1.725	7538	2280
YHR172W	9257	8904	2098	121	123	25	2822	2819	571	425	425	58	3.811	1.93	9136	2397
YHR173C	3448	3264	1068	102	103	23	1478	1396	371	367	370	56	3.012	1.591	3346	1111
YHR175W	10418	10374	1792	105	107	29	2809	2814	472	402	405	57	4.285	2.099	10313	2407
YHR176W	10149	9431	3189	109	111	23	3471	3263	1001	392	393	57	3.261	1.705	10040	3079
YHR177W	15381	15394	3172	112	114	28	3912	3884	762	444	446	66	4.403	2.138	15269	3468
YHR178W	4885	4949	1554	106	109	26	2022	2012	533	353	355	53	2.863	1.518	4779	1669
YHR179W	13368	13218	2912	107	109	23	4825	4750	1072	376	379	61	2.981	1.576	13261	4449
YHR180W	5046	4961	1511	105	107	24	1889	1831	542	367	369	55	3.246	1.699	4941	1522
YHR181W	7472	7197	2033	105	107	22	2607	2515	654	379	381	57	3.307	1.725	7367	2228
YHR182W	13882	13233	5144	110	112	25	4473	4263	1578	425	428	62	3.402	1.766	13772	4048
YHR184W	5098	5399	1343	111	120	102	2348	2468	559	423	428	76	2.591	1.373	4987	1925
YHR185C	6877	6511	2075	107	109	25	2178	2077	601	377	380	58	3.759	1.91	6770	1801
YHR186C	10294	10236	1522	110	116	110	2998	2946	422	406	409	62	3.929	1.974	10184	2592
YHR187W	8973	8739	1919	108	109	23	2786	2759	540	371	376	55	3.671	1.876	8865	2415
YHR188C	12038	11869	2194	131	272	988	3049	3053	528	454	489	256	4.588	2.198	11907	2595
YHR189W	25376	24744	4942	106	109	40	7262	7008	1331	384	388	75	3.674	1.877	25270	6878
YHR190W	6146	6060	1753	102	102	23	2446	2387	607	384	387	57	2.931	1.551	6044	2062
YHR191C	7597	7758	2223	105	106	34	2927	2967	733	436	438	63	3.008	1.589	7492	2491
YHR192W	12123	12216	2070	106	108	24	3595	3556	543	451	453	63	3.822	1.934	12017	3144
YHR194W	17192	17142	3336	107	109	25	3801	3727	653	399	401	58	5.022	2.328	17085	3402
YHR195W	13750	13888	2115	104	105	26	4559	4624	633	431	435	69	3.306	1.725	13646	4128
YHR196W	4955	4553	1928	113	119	98	1985	1894	652	414	413	72	3.082	1.624	4842	1571
YHR197W	4327	4304	1174	112	118	68	1674	1631	380	409	414	71	3.332	1.736	4215	1265
YHR198C	13519	13234	2444	121	123	28	4132	4002	686	427	433	68	3.616	1.854	13398	3705
YHR199C	17040	15133	5354	107	123	154	4920	4426	1495	380	408	304	3.73	1.899	16933	4540
YHR200W	30124	29983	3230	110	113	39	8893	8733	889	376	380	56	3.524	1.817	30014	8517
YHR201C	14639	14230	3306	116	118	33	5007	4822	1076	411	414	63	3.16	1.66	14523	4596
YHR202W	9398	9302	1108	118	119	24	3039	3057	355	445	447	61	3.577	1.839	9280	2594
YHR204W	8435	8412	3399	112	167	389	2437	2350	807	397	411	116	4.08	2.029	8323	2040
YHR205W	10444	10222	2667	125	381	2981	3081	3033	722	451	707	3214	3.924	1.972	10319	2630
YHR206W	13963	13583	3069	110	125	231	4408	4299	982	386	398	174	3.444	1.784	13853	4022
YHR207C	10192	10080	2039	103	104	21	3651	3561	674	416	417	56	3.119	1.641	10089	3235
YHR208W	8131	7666	2061	111	114	37	3381	3230	837	387	394	78	2.679	1.422	8020	2994

YHR209W	6425	5998	1669	100	101	23	2451	2301	584	363	365	58	3.029	1.599	6325	2088
YHR210C	14040	13329	3017	114	117	69	3640	3504	714	418	427	93	4.322	2.112	13926	3222
YHR213W	2626	2543	705	101	128	218	1310	1282	302	369	379	99	2.683	1.424	2525	941
YHR217C	65535	61936	11523	126	131	35	13099	12299	2775	380	380	58	5.143	2.363	65409	12719
YIL001W	8888	8111	3062	104	106	40	3593	3287	1125	366	370	67	2.722	1.445	8784	3227
YIL002C	9773	10043	3754	107	107	25	3755	3835	1299	380	383	60	2.864	1.518	9666	3375
YIL003W	10934	10337	2376	112	148	806	3769	3581	759	378	428	1127	3.191	1.674	10822	3391
YIL005W	7443	7173	2016	108	111	24	2768	2678	668	375	381	58	3.065	1.616	7335	2393
YIL006W	18183	17401	5444	111	112	23	7134	6765	2086	386	387	56	2.678	1.421	18072	6748
YIL007C	17248	16706	3729	112	114	22	6570	6367	1379	413	414	58	2.783	1.477	17136	6157
YIL008W	4190	4335	728	106	107	23	1763	1783	262	366	369	56	2.923	1.548	4084	1397
YIL009C-A	7858	7862	1243	111	112	24	3209	3146	465	382	383	53	2.74	1.454	7747	2827
YIL009W	7259	6346	2850	102	103	23	2596	2285	887	369	372	55	3.214	1.684	7157	2227
YIL012W	3152	3243	1065	108	115	47	1562	1507	403	374	376	56	2.562	1.357	3044	1188
YIL013C	9040	8969	1818	122	123	25	3809	3735	722	432	435	59	2.641	1.401	8918	3377
YIL014W	10217	9118	3576	112	130	166	3564	3272	1147	420	431	105	3.214	1.684	10105	3144
YIL017C	13667	12456	5359	123	128	65	4524	4146	1657	442	450	107	3.318	1.73	13544	4082
YIL019W	13640	13539	2297	117	119	25	5053	4975	786	408	411	60	2.911	1.542	13523	4645
YIL020C	23550	21841	5848	116	122	84	7772	7186	1908	435	446	149	3.194	1.675	23434	7337
YIL021W	6728	7379	2744	108	124	211	2669	2818	968	351	356	87	2.856	1.514	6620	2318
YIL023C	17406	17091	5177	111	120	156	7109	6855	2036	405	409	113	2.58	1.367	17295	6704
YIL028W	8764	8396	3046	107	109	24	3089	2950	948	364	368	56	3.177	1.668	8657	2725
YIL030C	8348	8802	3047	110	118	128	3405	3458	1047	397	404	83	2.739	1.453	8238	3008
YIL034C	14199	13931	6361	122	127	35	5430	5380	2349	445	447	83	2.824	1.498	14077	4985
YIL035C	10591	10596	2088	110	117	64	4531	4533	824	440	444	68	2.562	1.357	10481	4091
YIL042C	24928	24801	4051	110	111	26	8795	8606	1369	375	380	56	2.948	1.559	24818	8420
YIL043C	1524	1524	554	110	114	47	930	927	227	439	445	68	2.88	1.526	1414	491
YIL044C	8704	8658	990	111	113	25	3399	3367	403	409	408	57	2.874	1.523	8593	2990
YIL045W	4695	5275	2613	108	113	42	2106	2282	981	413	417	68	2.709	1.438	4587	1693
YIL046W	26439	26334	3829	119	121	37	8789	8678	1192	420	422	61	3.145	1.653	26320	8369
YIL047C	7672	7818	3586	110	152	277	2706	2861	1126	406	430	221	3.288	1.717	7562	2300
YIL049W	6866	6490	1445	110	113	36	2407	2339	552	424	429	71	3.407	1.768	6756	1983
YIL050W	12400	11825	2532	108	111	47	4953	4766	960	389	395	88	2.693	1.429	12292	4564
YIL054W	8567	8113	2343	102	102	22	3186	3038	797	461	463	64	3.106	1.635	8465	2725
YIL056W	7817	7557	1506	106	108	23	3362	3293	663	370	372	58	2.577	1.366	7711	2992
YIL059C	9859	9099	3464	103	104	22	3632	3356	1148	382	382	58	3.002	1.586	9756	3250
YIL060W	8639	8380	2210	111	165	397	3734	3627	893	404	425	197	2.561	1.357	8528	3330
YIL064W	12525	11543	4693	109	113	78	4859	4522	1698	388	388	78	2.777	1.474	12416	4471
YIL066C	14246	14315	2950	111	115	66	5468	5468	1113	402	405	62	2.79	1.48	14135	5066
YIL067C	16833	16646	2239	106	107	23	6061	6016	824	445	446	63	2.978	1.575	16727	5616
YIL068C	4397	4325	1423	102	104	23	2008	1999	544	400	400	59	2.671	1.417	4295	1608
YIL071C	5673	5623	1829	111	123	107	2520	2432	684	436	448	155	2.669	1.416	5562	2084
YIL078W	10002	9628	2214	107	113	97	4044	3943	860	361	371	111	2.687	1.426	9895	3683
YIL089W	6126	5977	1487	111	112	23	2693	2629	577	411	413	59	2.636	1.398	6015	2282
YIL090W	4444	4817	1512	108	109	22	1972	1991	519	387	391	54	2.736	1.452	4336	1585
YIL091C	9935	9753	3219	108	110	24	3680	3508	1032	370	371	55	2.969	1.57	9827	3310
YIL094C	12988	12699	2363	123	165	420	5340	5176	984	446	460	146	2.629	1.394	12865	4894
YIL097W	6065	5790	1659	119	131	99	2699	2629	620	441	452	114	2.633	1.397	5946	2258
YIL099W	17662	16704	4646	113	123	147	5469	5106	1418	366	373	109	3.439	1.782	17549	5103
YIL106W	9132	8361	3021	108	110	24	3817	3496	1143	378	379	57	2.624	1.392	9024	3439
YIL107C	11498	11204	2284	114	125	137	4767	4644	927	435	443	96	2.628	1.394	11384	4332
YIL108W	6675	7048	3789	121	130	140	2374	2503	1160	427	430	71	3.366	1.751	6554	1947
YIL109C	11019	10786	2283	112	140	153	4519	4326	872	395	402	73	2.645	1.403	10907	4124
YIL110W	9661	9451	2065	112	121	106	3522	3482	719	419	423	66	3.077	1.622	9549	3103
YIL112W	5444	5294	1575	118	120	25	2533	2477	601	457	455	60	2.566	1.359	5326	2076
YIL117C	7519	7355	2286	111	114	34	3292	3213	873	418	423	76	2.578	1.366	7408	2874
YIL118W	5646	5724	1103	103	106	53	2471	2457	454	371	377	72	2.64	1.4	5543	2100
YIL122W	9013	9008	2122	116	118	24	3909	3938	820	457	459	64	2.577	1.366	8897	3452
YIL131C	15500	13709	4910	104	106	22	6214	5589	1898	386	386	55	2.642	1.401	15396	5828
YIL132C	11460	11302	1825	107	108	21	4554	4459	725	363	367	56	2.709	1.438	11353	4191
YIL135C	17143	16806	2718	107	110	23	6828	6669	1069	362	366	50	2.635	1.398	17036	6466
YIL139C	9639	9320	2070	111	113	24	4063	3932	835	378	379	58	2.586	1.371	9528	3685
YIL151C	6648	6676	2012	101	103	25	2684	2658	714	383	386	59	2.845	1.509	6547	2301
YIR002C	7040	7245	2323	105	115	105	2715	2787	715	380	384	70	2.97	1.57	6935	2335
YIR005W	2051	2051	549	106	108	26	1024	1026	170	402	404	64	3.127	1.645	1945	622
YIR007W	13918	14224	3088	103	106	25	4729	4750	981	445	446	63	3.225	1.689	13815	4284
YIR011C	8218	7774	2416	108	113	69	3309	3108	921	359	363	66	2.749	1.459	8110	2950
YIR012W	18351	14464	9379	112	114	26	7331	5852	3666	403	405	60	2.633	1.397	18239	6928
YIR014W	13082	12720	2350	114	132	219	4507	4424	806	412	426	221	3.167	1.663	12968	4095
YIR015W	13211	12862	3319	112	122	167	4774	4613	1117	416	426	137	3.006	1.588	13099	4358
YIR016W	10816	10487	2883	106	109	25	4042	3850	1020	362	364	56	2.91	1.541	10710	3680
YIR020C	7180	7148	1362	111	120	126	2912	2864	482	381	384	77	2.793	1.482	7069	2531
YIR021W	12723	12252	3593	107	110	58	4807	4623	1518	382	387	75	2.851	1.512	12616	4425
YIR025W	16003	15936	3046	122	123	26	5725	5702	1144	427	431	57	2.998	1.584	15881	5298
YIR028W	11133	10839	2701	103	107	25	4296	4370	988	386	390	62	2.821	1.496	11030	3910
YIR029W	6693	7092	2778	106	146	303	2839	2944	1010	406	418	126	2.707	1.437	6587	2433
YIR031C	14181	14344	3053	104	105	22	5403	5432	1061	436	442	63	2.834	1.503	14077	4967
YIR039C	15226	13367	5874	114	126	218	5592	4983	2062	457	467	155	2.943	1.557	15112	5135
YJL035C	14682	14423	3176	101	104	27	6054	5853	1174	400	400	62	2.579	1.367	14581	5654
YJL149W	5987	5765	1495	115	116	24	2622	2601	637	400	404	60	2.643	1.402	5872	2222
YJL173C	11654	11946	2158	102	103	23	4877	4952	819	433	436	58	2.599	1.378	11552	4444
YJL197W	12395	12025	2128	112	113	25	5127	5018	837	438	438	61	2.62	1.389	12283	4689
YJR115W	45154	44447	5928	116	119	30	8847	8735	1319	387	386	56	5.324	2.412	45038	8460
YKL177W	9866	9805	1973	105	117	172	3951	3875	751	375	383	162	2.73	1.449	9761	3576
YKL187C	11552	11995	2697	107	120	166	4541	4643	1041	369	380	139	2.743	1.456	11445	4172
YKL225W	12913	12569	3812	109	110	24	5415	5299	1500	417	421	59	2.562	1.357	12804	4998
YMR238W	10791	10654	2108	131	136	56	4441	4351	820	451	458	70	2.672	1.418	10660	3990
YMR250W	5721	5875	1209	117	132	229	2617	2635	497	430	444	174	2.562	1.358	5604	2187
YMR277W	4308	4383	3393	108	110	38	2034	2101	1391	394	393	61	2.561	1.357	4200	1640
YMR299C	6539	6602	1752	120	182	500	2942	2990	675	436	465	224	2.561	1.357	6419	2506
YNL338W	13905	12723	6490	107	131	201	2513	2283	934	377	382	63	6.46	2.691	13798	2136
YOR344C	7002	6748	1618	117	131	111	2349	2303	484	433	444	118	3.593	1.845	6885	1916
YOR345C	3411	3355	833	106	108	22	1260	1265	243	366	370	57	3.697	1.886	3305	894
YOR346W	5060	4982	1093	114	118	76	1701	1702	319	390	394	59	3.773	1.916	4946	1311
YOR347C	17714	17082	4409	122	122	26	4974	4725	1198	432	432	57	3.873	1.954	17592	4542
YOR348C	16178	15699	4466	105	106	24	3591	3496	911	386	387	55	5.015	2.326	16073	3205
YOR349W	8681	8268	1855	107	122	221	2336	2276	488	387	398	158	4.399	2.137	8574	1949
YOR350C	2348	2435	535	106	107	21	1218	1258	204	368	367	55	2.638	1.399	2242	850
YOR351C	8578	8448	1515	100	103	29	2797	2755	500	426	429	62	3.576	1.838	8478	2371
YOR352W	6027	5694	2339	104	121	143	1899	1830	617	412	427	118	3.983	1.994	5923	1487

YOR353C	8751	8491	2844	111	114	33	2715	2617	792	399	400	62	3.731	1.899	8640	2316
YOR354C	5368	5331	1073	112	130	177	1878	1862	324	411	418	116	3.583	1.841	5256	1467
YOR355W	4319	4307	1480	106	106	23	1959	1908	554	373	378	58	2.656	1.409	4213	1586
YOR356W	9169	9233	2129	108	111	49	2903	2872	614	385	389	84	3.598	1.847	9061	2518
YOR357C	4421	4369	930	111	112	23	1609	1575	268	414	416	56	3.607	1.851	4310	1195
YOR358W	19977	18873	4045	106	107	22	6345	5970	1239	370	371	54	3.326	1.734	19871	5975
YOR359W	6943	7152	2316	102	114	182	2207	2233	563	426	431	79	3.841	1.942	6841	1781
YOR360C	2629	2663	691	107	112	59	1171	1183	208	369	376	68	3.145	1.653	2522	802
YOR361C	6346	6430	1596	110	112	24	2082	2140	474	424	426	59	3.761	1.911	6236	1658
YOR362C	11621	10657	3624	108	109	23	3434	3140	999	372	373	52	3.76	1.911	11513	3062
YOR363C	8783	8611	1815	124	155	230	2752	2686	485	445	475	231	3.753	1.908	8659	2307
YOR364W	4974	4771	1043	103	105	23	1408	1389	242	366	368	56	4.675	2.225	4871	1042
YOR365C	2232	2257	671	98	101	23	1101	1111	240	386	388	59	2.985	1.578	2134	715
YOR366W	3839	3711	918	100	102	44	1682	1630	330	430	435	91	2.986	1.578	3739	1252
YOR367W	4186	4284	793	104	105	21	1731	1726	267	363	365	56	2.984	1.577	4082	1368
YPL056C	3345	3073	1574	103	106	50	1609	1544	614	374	375	63	2.625	1.392	3242	1235
YPR083W	7378	6262	4174	108	110	28	3018	2700	1536	432	434	66	2.811	1.491	7270	2586
YPR087W	4049	4214	1157	113	114	26	1944	1989	483	441	443	63	2.619	1.389	3936	1503
YPR123C	13848	13058	3769	110	113	30	5098	4829	1346	375	378	59	2.909	1.54	13738	4723
YPR158W	7475	7064	2835	106	114	110	3046	2918	1060	414	421	106	2.8	1.485	7369	2632

Appendix II

Genes significantly affected by the change in the specific gravity of wort.

Experiment	M1	M3	M4	M5	M6	M7	M8
Class	High	High	High	Low	Low	Low	Low
YAL043C-A	1.753	1.109	0.953	0.447	0.579	0.105	0.233
YAL048C	2.021	1.047	0.382	-0.151	-0.103	-0.474	-0.043
YAL049C	0.758	0.602	0.532	0.017	0.116	0.1	-0.015
YAL053W	1.47	0.968	0.432	0.017	0.097	-0.162	-0.03
YAL055W	0.654	0.524	0.491	0.018	0.096	0.061	0.048
YAL056W	1.238	0.967	0.804	0.391	0.415	0.321	0.303
YAL058W	1.845	1.131	0.629	0.131	0.124	-0.284	-0.145
YAL060W	2.067	1.154	0.568	0.167	0.064	-0.317	0.006
YAL061W	1.822	1.146	0.527	-0.003	-0.161	-0.573	-0.128
YAL062W	1.602	0.86	0.632	0.167	0.094	-0.173	0.056
YAL063C	1.561	0.928	0.593	-0.006	-0.037	-0.299	-0.049
YAL064C-A	1.199	0.785	1.041	0.371	0.092	0.147	0.282
YAL064W-B	1.895	0.881	0.706	-0.068	-0.093	-0.506	-0.016
YAL065C	0.833	0.521	0.531	-0.099	-0.025	-0.163	-0.201
YAL066W	0.883	0.784	0.88	-0.006	-0.061	0.29	0.272
YAL067C	1.402	0.875	0.958	0.213	0.091	0.057	0.103
YAL068C	1.254	0.579	0.505	-0.183	0.042	-0.367	-0.236
YAL069W	1.521	0.933	1.198	0.411	0.385	-0.014	0.174
YAR008W	1.417	1.096	0.538	0.114	0.272	-0.077	0.179
YAR018C	1.449	1.13	0.78	0.147	0.127	0.003	0.224
YAR019C	0.569	0.523	0.329	-0.25	0.041	-0.06	-0.113
YAR028W	0.702	0.457	0.457	-0.077	0.172	-0.114	-0.072
YAR029W	1.001	0.723	0.585	-0.067	-0.071	-0.114	0.112
YAR030C	1.102	0.699	0.77	-0.003	0.062	0.032	0.052
YAR033W	1.109	0.72	0.655	0.1	0.084	-0.016	0.049
YAR035W	2.191	1.034	0.508	-0.163	-0.183	-0.77	-0.485
YAR042W	2.175	1.097	0.455	-0.237	-0.356	-0.902	-0.423
YAR044W	1.381	1.209	0.926	0.107	-0.046	-0.019	0.042
YAR050W	1.472	0.709	0.469	-0.039	-0.003	-0.499	-0.23
YAR066W	2.022	0.934	0.253	-0.378	-0.246	-0.879	-0.71
YAR068W	0.971	0.316	0.187	-0.412	-0.211	-0.651	-0.426
YAR070C	1.631	1.053	0.841	0.131	0.247	-0.079	0.247
YAR071W	1.006	0.525	0.565	0.078	0.026	0.012	-0.087
YBL048W	-0.72	-0.657	-0.126	0.064	0.226	0.204	0.304
YBL050W	-0.803	-0.794	-0.435	-0.085	0.188	-0.007	-0.142
YBL053W	-1.021	-0.796	-0.084	0.383	0.449	0.367	0.321
YBL090W	0.873	0.297	0.216	-0.199	-0.172	-0.374	-0.294
YBL098W	1.432	0.72	0.313	-0.19	-0.081	-0.301	-0.203
YBL099W	1.467	0.643	0.501	-0.039	-0.039	-0.248	-0.017
YBL101C	0.901	0.564	0.738	0.081	0.13	-0.036	0.129
YBL102W	1.929	1.166	0.72	0.045	0.182	-0.4	-0.199
YBL103C	1.251	0.828	0.687	0.109	0.052	-0.097	-0.045
YBL106C	1.277	0.403	0.63	-0.145	-0.181	-0.362	-0.16
YBL107C	0.571	0.129	0.516	-0.169	-0.211	-0.158	-0.219
YBL108W	1.456	0.871	1.118	0.35	0.235	-0.021	0.275
YBL109W	3.091	1.586	1.862	0.775	0.692	-0.012	0.623
YBL111C	1.28	0.53	0.62	0.065	0.137	-0.271	-0.15

YBR031W	0.171	-0.093	-0.415	-0.704	-1.027	-0.958	-0.896
YBR048W	-0.087	-0.234	-0.212	-0.648	-0.829	-0.698	-0.673
YBR061C	0.358	0.414	0.057	-0.383	-0.428	-0.398	-0.319
YBR062C	0.442	0.229	0.199	-0.307	-0.344	-0.28	-0.326
YBR069C	0.746	0.313	0.088	-0.402	-0.475	-0.47	-0.284
YBR076W	0.322	0.365	0.185	-0.193	-0.388	-0.27	-0.174
YBR082C	0.622	0.586	0.259	-0.081	-0.174	-0.315	-0.091
YBR092C	0.91	0.602	0.393	-0.019	-0.29	-0.197	-0.101
YBR093C	0.852	0.51	0.309	-0.055	-0.043	-0.216	-0.191
YBR134W	3.965	3.276	3.598	1.6	1.647	1.066	1.327
YBR215W	-1.12	-0.817	-0.523	0.003	0.111	0.069	-0.082
YBR223C	-0.865	-0.402	-0.315	0.078	0.249	0.278	0.102
YBR227C	-0.553	-0.438	-0.292	0.18	0.274	0.132	0.039
YBR233W	-0.259	-0.283	0.063	0.477	0.523	0.344	0.421
YBR245C	-0.444	-0.533	-0.292	0.146	0.249	0.08	0.098
YBR251W	-0.298	-0.329	0.02	0.293	0.597	0.446	0.338
YBR258C	-0.592	-0.543	-0.244	0.179	0.365	0.13	0.109
YBR296C	1	0.63	0.781	0.236	0.292	-0.018	0.033
YBR299W	1.477	1.041	1.083	0.311	0.657	0.116	0.117
YBR301W	0.986	0.411	0.315	-0.236	-0.129	-0.446	-0.194
YBR302C	0.939	0.649	0.857	0.1	0.121	0.085	0.179
YCL024W	-0.736	-0.695	-0.29	-0.056	0.298	0.131	-0.003
YCL049C	1.503	0.805	0.517	0.043	0.115	-0.41	-0.067
YCL050C	1.035	0.57	0.432	-0.064	0.082	-0.303	-0.102
YCL051W	1.648	0.887	0.418	-0.021	0.142	-0.427	-0.203
YCL057W	0.75	0.61	0.813	0.103	0.192	-0.028	-0.045
YCL064C	1.339	0.699	0.697	0.251	0.251	-0.042	0.057
YCL066W	1.603	1.07	0.965	0.546	0.615	0.213	0.457
YCR009C	-0.556	-0.575	-0.288	0.154	0.542	0.046	0.064
YCR032W	2.126	2.141	1.718	1.364	0.929	1.078	1.177
YCR042C	-0.406	-0.221	0.334	0.718	0.966	0.787	0.825
YCR044C	-0.282	-0.213	-0.008	0.424	0.629	0.482	0.363
YCR057C	-0.648	-0.52	-0.151	0.161	0.508	0.278	0.095
YCR090C	1.9	1.18	0.731	0.254	0.382	0.059	0.278
YCR102C	1.328	0.743	0.511	-0.054	-0.043	-0.2	-0.147
YCR103C	1.366	0.959	1.138	0.304	0.137	0.182	0.362
YCR104W	1.047	0.551	0.574	-0.004	0.091	-0.14	0.018
YDL128W	-0.518	-0.456	-0.447	0.054	0.083	0.126	-0.104
YDL139C	-0.374	-0.03	0.086	0.438	0.657	0.605	0.465
YDL150W	-1.356	-0.784	-0.627	-0.116	0.01	0	-0.1
YDL161W	-0.513	-0.406	-0.23	0.182	0.433	0.095	0.186
YDL182W	-0.498	-0.322	-0.222	0.213	0.255	0.278	0.254
YDL186W	-0.78	-0.832	-0.141	0.081	0.373	0.191	0.12
YDL230W	1.165	0.753	0.82	0.22	0.205	0.172	0.337
YDL235C	0.864	0.546	0.539	-0.135	0.032	0.08	0.13
YDL238C	1.275	0.661	0.549	-0.246	-0.15	-0.332	-0.013
YDL240W	1.682	1.042	0.925	0.009	0.144	0.041	0.24
YDL241W	1.189	0.686	0.597	-0.207	-0.058	-0.196	-0.029
YDL242W	0.879	0.446	0.592	-0.259	-0.086	-0.127	-0.061
YDL243C	0.988	0.271	0.253	-0.448	-0.091	-0.573	-0.261
YDL244W	1.044	0.278	0.418	-0.297	-0.05	-0.397	-0.309

YDL245C	1.48	0.909	0.856	-0.071	-0.176	-0.294	0.05
YDL246C	0.926	0.363	0.662	-0.202	-0.052	-0.305	-0.202
YDL247W	0.613	0.109	0.295	-0.504	-0.287	-0.581	-0.537
YDR004W	0.455	0.601	-0.187	-0.478	-0.852	-0.536	-0.519
YDR006C	0.239	-0.02	-0.44	-0.771	-0.989	-0.866	-0.716
YDR008C	0.211	-0.237	-0.58	-0.767	-1.146	-1.121	-1.047
YDR012W	0.14	-0.128	-0.486	-0.763	-0.888	-0.98	-0.899
YDR025W	-0.109	-0.145	-0.267	-0.626	-0.932	-0.753	-0.749
YDR028C	-0.155	-0.168	-0.13	-0.696	-1.092	-0.681	-0.614
YDR032C	0.097	-0.243	-0.794	-0.952	-1.209	-1.283	-1.208
YDR044W	-0.024	0.082	0.055	-0.457	-0.564	-0.46	-0.52
YDR045C	0.093	0.278	0.06	-0.526	-0.624	-0.334	-0.341
YDR048C	0.238	0.241	0.201	-0.262	-0.385	-0.46	-0.226
YDR049W	0.274	0.017	-0.285	-0.704	-1.108	-0.682	-0.817
YDR187C	0.888	1.15	1.099	0.232	-0.009	-0.144	0.289
YDR401W	-0.685	-0.414	-0.339	0.085	0.041	0.25	0.069
YDR406W	-0.434	-0.126	0.026	0.594	0.623	0.593	0.433
YDR415C	-0.842	-0.485	-0.282	0.202	0.089	0.395	0.265
YDR417C	-0.739	-0.839	-0.735	-0.24	-0.205	-0.31	-0.359
YDR424C	-0.693	-0.155	0.031	0.435	0.465	0.607	0.551
YDR438W	-0.448	-0.384	-0.212	0.243	0.26	0.322	0.288
YDR444W	-0.573	-0.507	-0.358	0.183	0.426	0.22	0.164
YDR447C	-0.584	-0.666	-0.454	0.024	0.046	0.079	0.033
YDR448W	-0.546	-0.464	-0.526	-0.02	0.332	0.009	-0.037
YDR451C	-0.421	-0.307	-0.42	0.102	0.215	0.213	0.053
YDR452W	-0.868	-0.442	-0.293	0.197	0.468	0.613	0.243
YDR456W	-0.376	-0.26	-0.138	0.343	0.425	0.303	0.273
YDR457W	-0.498	-0.188	-0.054	0.45	0.575	0.685	0.393
YDR460W	-0.388	-0.241	0.128	0.533	0.686	0.662	0.543
YDR464W	-0.587	-0.454	-0.188	0.23	0.477	0.254	0.216
YDR466W	-0.331	-0.542	-0.203	0.293	0.663	0.386	0.202
YDR468C	-0.216	-0.239	0.026	0.489	0.523	0.536	0.38
YDR469W	-0.548	-0.233	0.157	0.535	0.679	0.788	0.612
YDR473C	-0.813	-0.457	-0.068	0.46	0.607	0.736	0.602
YDR477W	-0.381	-0.609	-0.42	0.124	0.542	0.2	0.133
YDR478W	-0.229	-0.287	-0.01	0.335	0.605	0.443	0.528
YDR479C	-0.35	-0.549	-0.118	0.276	0.638	0.402	0.214
YDR481C	-0.379	-0.069	-0.054	0.391	0.595	0.625	0.561
YDR482C	-0.27	-0.38	-0.293	0.182	0.345	0.14	0.333
YDR484W	-0.156	-0.41	-0.007	0.343	0.537	0.497	0.44
YDR486C	0.119	-0.049	0.103	0.512	0.945	0.586	0.71
YDR492W	0.079	-0.257	0.059	0.45	0.601	0.57	0.518
YDR494W	-0.376	-0.682	-0.341	0.095	0.2	0.512	0.329
YDR517W	1.519	0.754	0.755	0.246	0.206	0.153	0.261
YDR519W	1.469	0.564	-0.029	-0.529	-0.396	-0.837	-0.644
YDR521W	1.697	0.684	0.336	-0.244	-0.316	-0.533	-0.14
YDR525W-A	1.785	1.089	0.734	-0.159	-0.131	-0.349	0.131
YDR527W	1.059	0.365	0.294	-0.444	-0.349	-0.522	-0.235
YDR528W	1.63	0.664	0.773	-0.006	-0.07	-0.253	-0.028
YDR533C	1.364	0.67	0.562	-0.177	-0.149	-0.246	-0.202
YDR534C	0.885	0.087	0.097	-0.488	-0.35	-0.518	-0.619

YDR536W	1.196	0.399	0.567	-0.257	-0.159	-0.409	-0.276
YDR537C	1.861	0.898	0.872	0.073	-0.036	-0.379	0
YDR538W	0.486	0.257	0.433	-0.073	-0.216	-0.356	-0.318
YDR539W	1.558	0.604	0.797	0.031	0.042	-0.2	0.13
YDR540C	2.181	0.698	0.201	-0.576	-0.564	-1.168	-0.857
YDR541C	1.345	0.623	0.652	-0.152	-0.156	-0.332	-0.073
YDR542W	1.168	0.45	0.35	-0.267	-0.034	-0.524	-0.327
YDR544C	2.817	1.436	1.565	0.554	0.335	-0.355	0.341
YDR545W	2.243	1.056	0.85	0.206	0.276	-0.335	0.016
YEL020W-A	-1.022	-0.89	-0.35	0.041	0.295	0.297	0.075
YEL027W	-0.659	-0.636	-0.183	0.255	0.468	0.307	0.258
YEL049W	1.123	0.654	0.552	-0.005	-0.192	-0.418	-0.215
YEL069C	1.683	1.041	0.975	0.091	0.088	-0.262	0.096
YEL070W	1.685	0.786	0.857	0.31	0.307	-0.171	-0.008
YEL073C	1.205	0.683	0.842	0.239	0.209	0.003	0.1
YEL075C	0.929	0.578	0.915	0.044	0.12	0.294	0.021
YEL076C	1.682	0.847	0.938	0.279	0.187	-0.091	0.186
YEL076C-A	1.741	0.782	0.989	0.322	0.166	0.027	0.267
YEL076W-C	1.519	0.955	1.101	0.21	0.388	0.226	0.244
YER002W	-0.835	-0.949	-0.425	0.028	0.181	0.055	0.122
YER030W	-1.183	-0.842	-0.355	0.069	0.172	0.247	0.103
YER112W	-1.014	-0.734	-0.574	-0.266	0.068	-0.163	-0.188
YER116C	-0.895	-0.657	-0.398	0.041	0.059	-0.046	-0.152
YER142C	-0.23	-0.281	-0.033	0.391	0.694	0.591	0.384
YER175C	0.893	0.607	0.592	0.038	0.184	0.016	-0.008
YER178W	1.633	0.785	0.669	0.069	0.024	-0.218	0.03
YER179W	0.88	0.369	0.088	-0.324	-0.278	-0.51	-0.326
YER182W	1.592	0.775	0.812	0.216	0.292	-0.09	0.114
YER183C	1.579	0.854	0.843	0.121	0.217	-0.155	-0.042
YER184C	1.036	0.322	0.356	-0.214	-0.055	-0.369	-0.33
YER185W	1.237	0.765	0.826	0.212	0.321	0.173	0.277
YER186C	1.268	0.565	0.319	-0.207	-0.029	-0.555	-0.342
YER187W	1.405	0.851	1.141	0.42	0.358	0.14	0.322
YER188W	2.114	1.112	1.07	0.221	0.307	-0.106	0.202
YFL040W	1.295	0.616	0.219	-0.213	-0.095	-0.498	-0.282
YFL051C	1.296	0.758	0.797	0.253	0.302	-0.071	0.009
YFL056C	1.032	0.494	0.586	-0.168	-0.022	-0.286	0.071
YFL057C	1.018	0.275	0.515	-0.263	-0.202	-0.269	-0.1
YFL058W	1.022	0.281	0.292	-0.426	-0.062	-0.487	-0.319
YFL059W	1.585	0.649	0.621	-0.244	-0.093	-0.45	-0.217
YFL060C	0.667	0.356	0.599	-0.125	-0.107	-0.008	-0.105
YFL061W	1.733	0.825	0.763	-0.077	0.038	-0.459	-0.053
YFL062W	0.992	0.565	0.763	0.069	0.132	-0.05	-0.006
YFL063W		1.005	1.274	0.515	0.289	0.008	0.354
YFL064C	0.972	0.678	0.962	0.222	0.147	0.375	0.241
YFL065C	1.3	0.756	0.97	0.102	0.205	0.012	0.122
YFR035C	0.88	0.569	0.448	-0.052	0.146	-0.176	-0.238
YFR042W	1.086	0.882	0.988	0.348	0.44	0.394	0.359
YFR043C	1.466	0.768	0.459	-0.126	0.094	-0.199	0.13
YFR048W	1.655	1.136	1.154	0.511	0.468	0.509	0.595
YFR050C	1.266	0.697	0.64	0.102	0.191	-0.088	0.042

YFR051C	2.397	0.89	0.343	-0.362	-0.185	-0.897	-0.688
YFR052W	0.999	0.578	0.46	-0.152	0.189	-0.032	0.036
YFR053C	1.602	0.868	0.595	-0.179	-0.147	-0.501	-0.049
YGL006W	0.835	0.492	0.068	-0.279	-0.677	-0.415	-0.259
YGL010W	0.405	0.148	-0.093	-0.584	-1.014	-0.624	-0.665
YGL026C	0.207	0.451	0.178	-0.181	-0.689	-0.318	-0.352
YGL027C	0.706	0.686	0.271	0.005	-0.513	-0.368	-0.108
YGL030W	0.388	0.03	-0.311	-0.586	-0.897	-0.801	-0.743
YGL052W	1.379	1.132	0.978	0.241	0.282	0.058	0.187
YGL124C	0.56	0.579	0.191	-0.155	-0.331	-0.173	-0.073
YGL126W	1.818	0.91	0.363	0.046	-0.231	-0.599	-0.103
YGL177W	-1.114	-0.833	-0.45	-0.206	-0.007	0.116	-0.089
YGL183C	-1.042	-0.897	-0.483	-0.269	0.096	0.226	-0.022
YGL190C	-0.722	-0.413	-0.001	0.276	0.394	0.554	0.446
YGL203C	-0.595	-0.615	-0.217	0.023	0.315	0.304	0.128
YGL239C	0.494	0.037	-0.034	-0.856	-0.568	-0.638	-0.489
YGL242C	1.177	0.779	0.868	-0.129	0.105	0.011	0.105
YGL245W	0.631	0.34	0.274	-0.476	-0.273	-0.252	-0.278
YGL247W	0.925	0.476	0.592	-0.072	-0.064	0.018	-0.005
YGL249W	0.535	0.346	0.62	-0.353	-0.203	-0.042	-0.085
YGL251C	0.707	0.274	0.625	-0.211	-0.059	-0.038	0.016
YGL252C	1.08	0.392	0.513	-0.226	-0.18	-0.319	-0.194
YGL253W	1.238	0.567	0.407	-0.463	-0.036	-0.467	-0.291
YGL254W	1.551	0.904	1.02	0.014	0.16	-0.068	0.169
YGL255W	1.589	0.791	0.767	-0.037	-0.174	-0.402	-0.241
YGL256W	1.314	0.457	0.335	-0.495	-0.229	-0.681	-0.498
YGL258W	0.939	0.341	0.752	-0.17	-0.128	-0.243	-0.098
YGL259W	0.954	0.341	0.463	-0.293	-0.18	-0.482	-0.272
YGL260W	1.529	0.93	1.058	0.24	0.207	0.03	0.245
YGL261C	1.36	0.633	0.489	-0.166	0.107	-0.233	-0.156
YGL262W	1.645	1.134	1.281	0.567	0.648	0.413	0.614
YGR004W	0.495	0.21	-0.121	-0.349	-0.949	-0.717	-0.535
YGR008C	0.588	0.273	-0.292	-0.495	-1.06	-0.941	-0.793
YGR010W	0.15	0.15	-0.205	-0.567	-0.962	-0.725	-0.68
YGR011W	0.163	0.338	0.229	-0.579	-0.783	-0.572	-0.295
YGR016W	0.369	0.491	0.239	-0.299	-0.632	-0.134	-0.105
YGR017W	0.15	0.065	0.147	-0.418	-0.877	-0.46	-0.538
YGR029W	-0.111	-0.336	-0.523	-0.848	-1.113	-1.081	-0.834
YGR031W	1.118	0.481	0.001	-0.387	-0.802	-0.735	-0.519
YGR032W.2	1.212	0.425	0.42	-0.109	-0.646	-0.378	-0.176
YGR050C	0.569	0.319	0	-0.427	-0.91	-0.589	-0.477
YGR052W	0.659	0.348	0.195	-0.11	-0.609	-0.251	-0.305
YGR073C	0.84	0.625	0.284	-0.163	-0.589	-0.341	-0.331
YGR183C	0.472	0.607	0.51	-0.15	0.051		-0.196
YGR238C	-0.117	0.18	0.214	0.704	0.862	0.704	0.663
YGR244C	-0.145	0.173	0.407	0.836	1.022	0.884	0.784
YGR266W	-0.288	-0.029	0.379	0.663	0.98	0.829	0.693
YGR269W	-0.538	-0.501	0.102	0.728	0.996	0.753	0.679
YGR292W	1.514	0.986	0.956	0.341	0.642	0.156	0.134
YGR294W	1.057	0.426	0.334	-0.234	-0.223	-0.562	-0.338
YGR295C	1.146	0.622	0.662	-0.02	0.325	-0.019	0.004

YGR296W	2.246	1.089	1.014	0.268	0.155	-0.206	0.201
YHL035C	1.253	0.82	1.104	0.514	0.459	0.296	0.335
YHL038C	1.081	0.712	1.005	0.266	0.273	0.275	0.169
YHL040C	1.485	0.622	0.958	0.013	0.158	-0.176	-0.118
YHL041W	0.71	0.46	0.708	-0.201	-0.247	-0.024	-0.051
YHL042W	1.423	0.678	0.85	-0.07	0.21	-0.147	-0.025
YHL043W	1.706	0.74	0.944	0.079	-0.042	-0.271	-0.152
YHL044W	1.266	0.639	1.071	0.271	0.272	0.061	0.149
YHL045W	1.058	0.554	0.812	0.031	0.076	-0.076	-0.03
YHL046C	1.29	0.548	0.56	-0.054	0.045	-0.193	-0.111
YHL049C	1.082	0.689	0.998	0.225	0.17	0.27	0.147
YHL050C	1.741	0.73	0.595	0.012	0.007	-0.524	-0.179
YHR005C	-0.465	-0.692	-0.213	0.388	0.489	0.4	0.235
YHR012W	-0.739	-0.74	-0.429	0.215	0.393	0.267	0.049
YHR014W	-0.5	-0.477	0.001	0.414	0.694	0.518	0.212
YHR015W	-0.471	-0.344	0.12	0.501	0.485	0.699	0.538
YHR017W	-0.643	-0.538	-0.197	0.38	0.509	0.376	0.238
YHR018C	-0.672	-0.601	-0.332	0.121	0.228	0.154	0.104
YHR019C	-0.433	-0.415	-0.158	0.453	0.592	0.321	0.094
YHR020W	-0.335	-0.427	-0.099	0.33	0.421	0.286	0.174
YHR021W-A	-0.486	-0.693	-0.46	0.14	0.187	0.202	0.175
YHR022C	-0.256	-0.436	-0.109	0.45	0.491	0.432	0.325
YHR035W	-0.317	-0.292	0.136	0.516	0.592	0.599	0.411
YHR038W	-0.609	-0.491	-0.348	0.052	0.193	0.033	0.101
YHR040W	-0.45	-0.448	-0.18	0.431	0.491	0.315	0.233
YHR043C	-0.861	-0.463	-0.422	0.136	0.154	0.162	-0.036
YHR127W	-1.266	-0.855	-0.515	-0.268	-0.159	-0.017	-0.092
YHR143W	-0.571	-0.445	-0.137	0.188	0.372	0.228	0.287
YHR156C	-0.817	-0.41	0.036	0.247	0.479	0.516	0.383
YHR211W	1.617	0.882	0.609	-0.027	-0.102	-0.486	-0.011
YHR214W	2.196	0.862	0.484	-0.226	-0.109	-0.69	-0.514
YHR214W-A	1.131	0.452	0.278	-0.236	-0.065	-0.513	-0.25
YHR215W	0.692	0.342	0.456	-0.119	-0.111	-0.24	-0.367
YHR217C	3.316	1.798	1.996	0.847	0.806	0.069	0.577
YHR218W	1.497	0.634	0.766	0.218	0.047	-0.217	0.066
YHR219W	2.076	0.918	0.743	0.067	-0.095	-0.465	0.013
YIL032C	-1.363	-1.074	-0.738	-0.337	0.212	-0.167	-0.346
YIL055C	-0.675	-0.707	-0.281	-0.041	0.288	0.166	0.199
YIL063C	-0.891	-0.723	-0.572	-0.145	0.39	-0.04	0.061
YIL076W	-1.278	-0.771	-0.472	-0.191	0.586	0.133	0.087
YIL079C	-0.722	-0.612	-0.42	-0.026	0.42	0.148	0.078
YIL092W	-0.668	-0.544	-0.495	-0.057	0.23	-0.138	0.028
YIL093C	-0.714	-0.744	-0.373	0.031	0.477	-0.006	0.009
YIL097W	-0.701	-0.508	-0.103	0.023	0.551	0.421	0.314
YIL098C	-1.15	-0.846	-0.378	-0.128	0.301	0.061	0.052
YIL100W	-0.86	-0.776	-0.716	-0.215	0.126	-0.247	-0.222
YIL112W	-0.827	-0.533	-0.194	0.077	0.649	0.408	0.218
YIL118W	-0.702	-0.597	-0.227	0.185	0.542	0.433	0.382
YIL119C	-0.474	-0.178	-0.176	0.238	0.727	0.481	0.919
YIL122W	-0.323	-0.524	-0.352	0.227	0.822	0.239	0.233
YIL126W	-0.657	-0.849	-0.587	-0.055	0.318	-0.044	0.113

YIL127C	-0.747	-0.783	-0.278	0.297	0.728	0.548	0.505
YIL137C	-0.104	-0.34	0.014	0.444	0.838	0.559	0.441
YIL138C	-0.743	-0.939	-0.526	-0.112	0.294	0.207	0.074
YIL144W	-0.106	-0.251	-0.083	0.269	0.657	0.581	0.51
YIL147C	0.255	0.002	0.339	0.684	0.978	0.809	0.85
YIL175W	1.637	0.974	1.277	0.342	0.365	0.139	0.332
YIL176C	1.207	0.571	0.497	-0.188	-0.129	-0.346	-0.186
YIL177C	1.906	0.743	0.747	0.085	-0.027	-0.453	-0.052
YIR027C	1.699	0.661	0.273	-0.231	-0.147	-0.71	-0.417
YIR031C	1.11	0.427	0.384	-0.166	0.026	-0.352	-0.148
YIR034C	1.254	0.381	0.221	-0.416	-0.217	-0.707	-0.516
YIR036C	1.764	0.563	0.295	-0.361	-0.27	-0.947	-0.418
YIR037W	0.833	0.35	0.497	-0.182	-0.207	-0.244	-0.293
YIR039C	1.95	0.977	0.824	-0.021	0.279	-0.498	-0.096
YIR040C	1.153	0.723	0.927	0.16	0.422	0.011	0.144
YJL008C	0.404	0.285	0.15	-0.321	-0.526	-0.301	-0.3
YJL027C	1.302	0.765	-0.176	-0.506	-0.484	-0.816	-0.534
YJL029C	0.155	-0.219	-0.498	-0.818	-0.823	-0.976	-0.845
YJL035C	0.961	0.656	0.17	-0.277	-0.502	-0.62	-0.219
YJL056C	0.895	0.877	0.309	-0.094	-0.348	-0.458	-0.155
YJL059W	0.263	0.261	0.128	-0.281	-0.482	-0.338	-0.384
YJL068C	0.511	0.655	0.258	-0.05	-0.272	-0.325	-0.253
YJL115W	-0.927	-1.061	-0.536	-0.162	-0.076	0.026	-0.153
YJL144W	-0.612	-0.567	-0.171	0.18	0.493	0.247	0.179
YJL165C	-0.894	-0.642	-0.119	0.048	0.513	0.431	0.157
YJL203W	1.022	0.705	0.543	-0.241	-0.077	-0.198	0.22
YJL204C	0.885	0.589	0.542	-0.216	0.016	-0.032	0.009
YJL206C	0.925	0.602	0.644	-0.285	0.06	0.081	0.134
YJL208C	0.56	0.509	0.558	-0.178	0.016	0.037	0.056
YJL209W	1.345	0.963	0.732	-0.141	0.112	-0.052	0.096
YJL210W	0.576	0.109	0.23	-0.407	-0.225	-0.328	-0.291
YJL211C	0.671	0.21	0.209	-0.453	-0.381	-0.488	-0.171
YJL212C	1.419	0.898	1.083	0.203	0.312	0.331	0.434
YJL213W	0.439	0.006	0.134	-0.493	-0.401	-0.477	-0.544
YJL215C	0.635	0.035	0.256	-0.453	-0.311	-0.421	-0.433
YJL216C	0.6	0.724	0.775	-0.22	-0.081	0.183	0.02
YJL218W	1.688	1.013	0.881	-0.012	0.016	-0.092	-0.173
YJL223C	1.033	0.453	0.256	-0.291	-0.146	-0.525	-0.365
YJR018W	1.084	0.712	0.026	-0.391	-0.183	-0.619	-0.486
YJR062C	-1.174	-0.924	-0.546	-0.357	0.012	-0.106	-0.22
YJR115W	3.537	2.821	2.814	0.999	1.083	0.496	0.668
YJR132W	0.646	0.337	0.535	-0.165	-0.153	0.009	0.068
YJR138W	0.825	0.273	0.343	-0.408	-0.279	-0.359	-0.294
YJR139C	0.454	0.148	0.351	-0.506	-0.434	-0.315	-0.248
YJR140C	0.656	0.268	0.329	-0.495	-0.325	-0.423	-0.37
YJR141W	0.347	0.055	0.301	-0.442	-0.581	-0.263	-0.304
YJR142W	0.579	0.348	0.554	-0.36	-0.319	-0.158	-0.181
YJR143C	0.891	0.441	0.663	-0.232	-0.079	-0.235	-0.317
YJR144W	1.11	0.269	0.2	-0.613	-0.517	-0.761	-0.688
YJR146W	0.169	-0.009	0.382	-0.41	-0.365	-0.365	-0.385
YJR147W	0.763	0.218	0.304	-0.342	-0.408	-0.456	-0.402

YJR148W	1.09	0.472	0.157	-0.573	-0.562	-0.56	-0.514
YJR149W	0.365	0.318	0.424	-0.533	-0.264	-0.212	-0.353
YJR150C	0.642	0.017	0.064	-0.767	-0.558	-0.756	-0.699
YJR155W	0.705	0.319	0.355	-0.336	-0.38	-0.289	-0.014
YJR156C	1.09	0.444	0.522	-0.247	-0.076	-0.329	-0.139
YJR157W	1.635	1.004	1.054	-0.078	0.201	-0.092	-0.072
YJR158W	1.359	0.732	0.683	-0.158	-0.009	-0.359	-0.073
YJR159W	1.087	0.499	0.955	-0.054	-0.031	-0.242	-0.038
YJR160C	0.821	0.205	0.425	-0.439	-0.307	-0.346	-0.351
YJR162C	1.146	0.62	1.132	0.419	0.263	0.122	0.239
YKL029C	1.378	1.043	0.44	0.102	0.012	-0.215	0.057
YKL083W	-1.497	-1.21	-0.334	-0.013	0.144	0.375	0.224
YKL097W-A	0.841	0.403	0.1	-0.351	-0.373	-0.775	-0.542
YKL143W	-0.435	-0.206	-0.132	0.211	0.466	0.322	0.272
YKL160W	-0.405	-0.375	0.191	0.581	0.756	0.701	0.352
YKL168C	0.225	0.14	0.548	0.814	0.958	0.885	0.894
YKL172W	-0.569	-0.339	-0.105	0.476	0.703	0.655	0.411
YKL174C	-0.248	-0.145	0.084	0.498	0.766	0.65	0.533
YKL179C	-0.116	-0.057	0.201	0.685	0.914	0.746	0.678
YKL212W	1.291	0.817	0.759	0.175	0.14	0.079	0.075
YKL213C	1.006	0.76	0.739	0.191	0.121	0.142	0.11
YKL215C	1.559	0.894	1.01	0.36	0.192	0.087	0.358
YKL216W	1.217	0.949	0.997	0.14	-0.107	0.091	0.126
YKL217W	1.68	1.004	1.003	0.202	-0.001	-0.112	0.135
YKL218C	1.78	0.981	1.108	0.175	0.006	-0.156	-0.026
YKL219W	1.29	0.716	0.768	-0.018	-0.222	-0.203	0.132
YKL221W	1.331	0.483	0.595	-0.064	-0.073	-0.397	-0.228
YKL222C	1.034	0.572	1.008	0.183	0.12	0.377	0.266
YKL223W	1.368	0.806	1	0.257	0.345	0.084	0.218
YKL224C	1.201	0.518	0.524	0.022	0.04	-0.271	0
YKR020W	-1.592	-1.09	-0.666	-0.34	-0.181	-0.218	-0.271
YKR097W	1.139	0.861	0.8	0.275	0.387	0.054	0.236
YKR099W	1.449	0.781	0.747	0.225	0.391	0.1	0.117
YKR103W	1.525			-0.141	0.054	-0.593	0.028
YLL025W	0.647	0.4	0.237	-0.203	-0.251	-0.338	-0.142
YLL039C	0.993	0.588	0.728	0.209	0.202	-0.102	0.074
YLL049W	1.773	1.339	1.017	0.498	-0.051	0.057	0.271
YLL051C	1.089	0.885	0.901	0.589	0.107	0.406	0.21
YLL052C	1.557	0.906	0.77	0.395	-0.132	-0.201	0.002
YLL053C	1.239	0.913	0.625	0.375	-0.154	-0.014	0
YLL055W	1.609	0.875	0.886	0.35	-0.235	-0.086	-0.141
YLL056C	1.772	1.272	1.157	0.629	0.174	0.253	0.136
YLL057C	1.257	0.831	0.919	0.381	-0.03	0.083	-0.016
YLL058W	1.227	0.971	1.128	0.535	-0.001	0.269	0.281
YLL060C	1.603	0.971	0.826	0.39	-0.109	-0.224	-0.238
YLL061W	1.791	1.193	1.355	0.778	0.309	0.348	0.304
YLL062C	1.458	1.143	1.233	0.555	0.019	0.238	0.349
YLL063C	1.085	0.791	0.908	0.407	0.028	-0.016	-0.02
YLL065W	1.264	0.686	1.207	0.48	0.392	0.188	0.359
YLL066C	1.897	0.853	0.652	0.064	-0.005	-0.469	-0.134
YLL067C	2.323	0.968	0.741	0.137	0.036	-0.483	0.045

YLR036C	1.17	1.033	0.649	0.192	-0.212	-0.093	0.279
YLR061W	0.23	0.215	-0.044	-0.403	-0.399	-0.497	-0.314
YLR109W	2.069	1.271	0.492	0.385	-0.426	-0.357	-0.068
YLR142W	1.92	1.397	0.93	0.763	-0.103	0.135	0.21
YLR146C	2.312	1.833	1.417	1.093	0.446	0.408	0.366
YLR152C	2.244	2.087	1.536	1.244	0.406	0.604	0.557
YLR153C	2.644	2.028	1.443	1.192	0.497	0.39	0.521
YLR154C	1.855	1.169	0.695	0.473	-0.143	-0.22	-0.016
YLR160C	0.685	0.65	0.6	-0.175	-0.144	-0.12	0.359
YLR162W	2.142	1.645	0.898	0.394	0.37	0.296	0.47
YLR164W	1.581	1.248	0.725	0.449	0.064	-0.036	-0.109
YLR165C	1.703	1.662	0.955	0.659	0.17	0.396	0.024
YLR166C	1.873	1.716	0.838	0.591	0.161	0.362	0.12
YLR168C	2.003	1.742	1.168	0.974	0.364	0.565	0.427
YLR170C	1.775	1.572	1.09	0.681	0.235	0.549	0.495
YLR171W	1.561	1.431	0.991	0.787	0.29	0.539	0.412
YLR172C	1.271	1.141	0.608	0.466	-0.054	0.236	0.176
YLR173W	1.626	1.181	0.801	0.58	-0.063	0.14	-0.105
YLR174W	2.306	1.814	1.084	0.831	0.193	0.269	0.319
YLR176C	1.791	1.553	1.09	0.812	0.131	0.679	0.422
YLR177W	1.356	1.455	1.141	0.618	0.06	0.453	0.345
YLR179C	2.262	1.894	1.092	0.844	0.215	0.159	0.115
YLR180W	1.77	1.153	0.887	0.675	0.264	0.127	0.233
YLR186W	1.921	1.289	0.88	0.532	0.124	-0.119	0.022
YLR195C	1.87	1.432	1.16	0.555	-0.107	0.178	0.269
YLR212C	1.794	1.256	0.873	0.726	-0.093	0.244	0.321
YLR213C	1.722	1.064	0.573	0.471	-0.278	-0.164	-0.12
YLR216C	1.533	1.291	0.753	0.483	-0.266	0.098	0.064
YLR219W	1.23	0.689	0.204	0.063	-0.752	-0.524	-0.384
YLR227C	1.432	1.222	0.749	0.531	-0.036	0.223	0.271
YLR230W	1.636	1.386	0.906	0.657	0.06	0.223	0.341
YLR231C	1.919	1.227	0.247	0.098	-0.46	-0.657	-0.431
YLR246W	1.831	1.203	0.551	0.318	-0.1	-0.185	-0.054
YLR280C	0.563	0.17	-0.23	-0.414	-0.707	-0.721	-0.64
YLR372W	-0.721	-0.613	-0.316	0.004	0.046	0.189	0.176
YLR383W	-0.496	-0.584	-0.267	0.233	0.212	0.453	0.319
YLR394W	-0.765	-0.657	-0.433	0.037	-0.215	0.18	0.012
YLR456W	1.212	0.838	0.841	0.189	0.358	0.269	0.372
YLR461W	0.96	0.429	0.381	-0.106	-0.148	-0.289	-0.122
YLR462W	0.923	0.422	0.696	0.022	0.175	0.114	-0.05
YLR463C	1.262	0.74	0.913	0.071	0.101	0.006	0.083
YLR464W	1.524	0.745	0.764	0.107	0.173	-0.128	0
YLR465C	1.602	0.721	0.861	0.192	0.142	-0.114	0.179
YLR466W	2.29	1.061	0.833	0.201	0.15	-0.226	0.213
YLR467W	2.097	0.856	0.901	0.14	-0.07	-0.444	0.024
YML024W	-0.563	-0.633	-0.306	0.059	0.066	0.138	0.011
YML030W	-1.506	-1.231	-1.101	-0.384	-0.388	-0.318	-0.666
YML032C	-0.516	-0.546	-0.332	0.181	0.339	0.154	0.028
YML034W	-0.63	-0.63	-0.184	0.217	0.203	0.204	-0.005
YML037C	-0.444	-0.253	-0.208	0.229	0.251	0.344	0.21
YML038C	-0.538	-0.354	-0.161	0.256	0.534	0.323	0.136

YML047C	-0.545	-0.112	0.191	0.555	0.504	0.686	0.551
YML048W	-0.571	-0.618	-0.225	0.414	0.42	0.375	0.151
YML049C	-0.738	-0.528	0.137	0.864	1.083	0.823	0.654
YML050W	-0.226	-0.285	0.07	0.598	0.686	0.539	0.268
YML054C	-0.379	-0.298	0.093	0.605	0.503	0.522	0.497
YML055W	-0.73	-0.404	-0.314	0.327	0.368	0.313	0.131
YML057W	-0.513	-0.476	-0.01	0.575	0.836	0.506	0.155
YML058W	-0.292	-0.493	-0.358	0.419	0.416	0.235	0.062
YML059C	-0.752	-0.394	0.245	1.003	0.949	0.962	0.694
YML060W	-0.33	-0.361	0.119	0.786	0.784	0.644	0.578
YML061C	-0.527	-0.438	-0.213	0.708	0.466	0.628	0.44
YML062C	-0.816	-0.772	-0.287	0.606	0.56	0.577	0.363
YML064C	-0.49	-0.287	0.358	1.178	1.064	1.052	0.738
YML065W	-0.648	-0.386	0.156	1.006	0.808	0.981	0.617
YML066C	0.065	-0.038	0.433	1.232	1.076	0.84	0.812
YML067C	-0.286	-0.297	0.166	0.832	0.739	0.748	0.66
YML068W	0.189	0.15	0.284	0.996	0.829	0.762	0.712
YML069W	-0.564	-0.589	-0.05	0.853	0.901	0.729	0.558
YML070W	-0.365	-0.56	-0.209	0.704	0.634	0.438	0.268
YML071C	-1.198	-0.737	-0.322	0.574	0.654	0.635	0.388
YML072C	-0.085	-0.304	0.299	1.122	1.065	0.865	0.718
YML073C	-0.542	-0.498	-0.223	0.171	0.302	0.318	0.144
YML075C	0.035	-0.25	0.1	0.923	0.817	0.688	0.639
YML076C	-0.065	-0.018	0.417	1.176	1.087	1.032	0.958
YML077W	0.145	-0.063	0.353	1.013	0.903	0.85	0.864
YML079W	-0.587	-0.49	-0.01	0.873	0.883	0.743	0.564
YML080W	0.365	-0.011	0.293	1.147	1.011	0.869	0.916
YML084W	-0.486	0.078	0.328	0.833	0.618	0.728	0.843
YML093W	-0.049	-0.212	0.25	0.81	0.854	0.796	0.632
YML095C	0.117	-0.153	0.377	1.085	1.013	1.002	0.847
YML121W	1.543	0.846	1.016	0.531	0.37	0.318	0.362
YML130C	1.314	0.762	0.932	0.432	0.385	0.288	0.336
YML132W	0.819	0.425	0.75	0.07	0.137	0.071	0.064
YML133C	1.942	0.797	0.633	-0.004	-0.044	-0.475	-0.131
YMR070W	1.465	0.729	0.607	0.339	-0.027	-0.264	-0.086
YMR227C	-0.805	-0.818	-0.679	-0.009	-0.123	-0.211	-0.135
YMR255W	-0.93	-0.58	-0.417	0.09	0.194	0.576	0.129
YMR257C	-0.136	-0.273	0.035	0.321	0.594	0.574	0.499
YMR260C	-0.581	-0.438	-0.142	0.244	0.408	0.392	0.137
YMR263W	-0.466	-0.474	-0.403	0.217	0.37	0.157	0.066
YMR265C	-0.505	-0.355	-0.005	0.28	0.547	0.36	0.366
YMR266W	-0.589	-0.431	-0.032	0.223	0.494	0.446	0.33
YMR268C	-0.543	-0.283	0.176	0.342	0.596	0.713	0.527
YMR269W	-1.399	-0.837	-0.72	-0.224	0.054	-0.016	0.02
YMR270C	-0.762	-0.426	-0.06	0.269	0.354	0.436	0.31
YMR271C	-0.858	-0.522	-0.121	0.17	0.506	0.439	0.421
YMR272C	-0.427	-0.504	-0.097	0.343	0.552	0.404	0.45
YMR273C	-0.996	-1.055	-0.477	0.032	0.383	0.261	0.007
YMR274C	-0.456	-0.244	-0.141	0.213	0.468	0.344	0.304
YMR275C	-0.651	-0.559	-0.124	0.078	0.454	0.366	0.281
YMR277W	-0.335	-0.158	0.167	0.424	0.598	0.598	0.493

YMR313C	1.848	1.226	0.501	-0.081	0.014	-0.263	0.161
YMR315W	1.639	0.86	0.468	-0.176	0.115	-0.334	-0.126
YMR316C-B	0.866	0.45	0.433	-0.138	-0.139	-0.182	-0.079
YMR316W	0.633	0.368	0.481	-0.15	-0.048	-0.029	-0.068
YMR317W	0.576	0.468	0.368	-0.49	-0.416	-0.481	-0.095
YMR318C	1.654	0.971	0.996	0.295	0.253	0.072	0.209
YMR319C	1.127	0.78	0.723	-0.035	0.075	0.049	0.135
YMR321C	1.03	0.538	0.63	-0.212	-0.341	-0.373	-0.291
YMR322C	1.393	0.696	0.858	-0.059	-0.299	-0.421	-0.256
YMR323W	1.629	0.934	0.955	-0.044	-0.295	-0.604	-0.305
YMR324C	1.034	0.631	1.052	0.135	-0.064	-0.04	0.097
YMR325W	0.694	0.097	0.079	-0.457	-0.339	-0.607	-0.375
YNL052W	0.638	0.614	-0.167	-0.395	-0.513	-0.662	-0.389
YNL057W	0.366	0.261	0.148	-0.15	-0.322	-0.409	-0.489
YNL062C	0.716	0.529	0.058	-0.245	-0.294	-0.289	-0.185
YNL158W	1.626	1.035	0.281	0.033	-0.23	-0.462	-0.116
YNL228W	-1.077	-0.823	-0.768	-0.341	-0.242	-0.251	-0.386
YNL233W	-0.769	-0.188	-0.1	0.321	0.365	0.376	0.399
YNL235C	0.395	0.441	0.166	-0.161	-0.09	-0.313	-0.296
YNL238W	-0.47	-0.421	-0.268	0.242	0.266	0.273	0.12
YNL245C	-1.375	-1.085	-0.841	-0.258	-0.01	-0.068	-0.2
YNL246W	-1.195	-0.909	-0.761	-0.096	-0.05	-0.061	-0.032
YNL250W	-0.949	-0.554	-0.307	0.032	0.293	0.337	0.073
YNL264C	-0.477	-0.475	-0.434	0.058	0.368	0.231	0.156
YNL269W	-0.619	-0.421	-0.421	-0.04	0.259	0.171	0.04
YNL270C	-0.626	-0.442	-0.352	0.089	0.273	0.23	0.152
YNL273W	-0.769	-0.467	-0.153	0.239	0.361	0.503	0.418
YNL274C	-0.405	-0.408	-0.127	0.266	0.427	0.227	0.247
YNL314W	0.694	0.415	0.44	-0.037	0.012	-0.087	0.014
YNL318C	1.238	0.491	0.517	-0.113	0.13	-0.205	-0.114
YNL322C	1.698	0.71	0.484	-0.111	-0.089	-0.515	-0.113
YNL331C	1.119	0.3	0.386	-0.357	-0.258	-0.324	-0.139
YNL332W	0.968	0.345	0.346	-0.339	-0.002	-0.449	-0.261
YNL333W	1.498	0.529	0.327	-0.243	-0.188	-0.527	-0.346
YNL335W	1.927	0.995	0.836	0.115	0.024	-0.345	0.123
YNL336W	1.1	0.504	0.754	0.052	0.254	0.049	0.036
YNL338W	3.335	2.038	3.124	0.863	1.001	0.617	1.048
YNL339C	2.068		0.769	0.113	0.223	-0.366	0.064
YNR007C	-0.086	-0.072	0.163	0.485	0.763	0.633	0.672
YNR008W	-0.635	-0.406	-0.121	0.227	0.568	0.496	0.27
YNR016C	-0.295	-0.307	0.054	0.423	0.57	0.418	0.362
YNR055C	1.432	0.75	0.643	0.171	-0.077	-0.15	0.31
YNR056C	0.821	0.49	0.67	-0.056	0.024	-0.006	0.026
YNR058W	1.404	0.465	0.586	-0.106	-0.173	-0.197	-0.078
YNR059W	0.702	0.482	0.63	-0.146	-0.213	0.158	0.069
YNR060W	1.084	0.569	0.095	-0.588	-0.562	-0.551	-0.772
YNR062C	0.826	0.4	0.477	-0.207	-0.174	-0.222	-0.265
YNR065C	1.137	0.552	0.585	0.035	0.134	-0.071	-0.061
YNR067C	1.11	0.629	1.242	0.245	-0.004	0.201	0.326
YNR070W	0.909	0.469	0.918	-0.095	-0.199	0.229	0.13
YNR071C	1.282	0.532	0.912	-0.138	-0.284	-0.24	-0.051

YNR072W	1.815	1.227	1.115	0.229	0.176	-0.099	0.297
YNR073C	1.411	0.638	0.741	0.148	0.15	-0.219	-0.031
YNR074C	1.425	0.731	0.962	-0.153	-0.291	-0.197	-0.022
YNR075W	0.399	0.124	0.516	-0.398	-0.424	-0.184	-0.201
YNR076W	0.736	0.474	0.354	0.041	-0.116	-0.275	-0.141
YOL093W	-0.923	-0.734	-0.103	0.255	0.642	0.569	0.362
YOL100W.2	-0.526	-0.553	-0.198	-0.007	0.334	0.242	0.147
YOL108C	-1.068	-0.49	-0.518	-0.189	0.129	0.24	0.148
YOL118C	-0.501	-0.479	0.149	0.272	0.539	0.664	0.473
YOL156W	1.049	0.395	0.328	-0.231	-0.1	-0.475	-0.272
YOL158C	1.528	0.704	0.78	-0.051	0.259	-0.324	0.031
YOL159C	0.967	0.46	0.836	-0.138	0.033	-0.036	-0.092
YOL160W	0.751	0.306	0.567	-0.31	-0.036	-0.159	-0.26
YOL165C	0.921	0.478	0.368	-0.25	-0.339	-0.526	-0.048
YOR084W	1.028	0.59	0.271	-0.059	-0.562	-0.563	-0.296
YOR265W	-1.182	-0.753	-0.268	0.01	0.072	0.228	0.079
YOR286W	-0.843	-0.643	-0.263	0.06	0.118	0.269	0.14
YOR290C	-0.776	-0.432	-0.1	0.193	0.374	0.28	0.214
YOR294W	-1.07	-1.164	-0.704	-0.257	-0.143	-0.175	-0.329
YOR295W	-1.208	-0.771	-0.483	-0.101	0.139	0.095	-0.107
YOR297C	-0.652	-0.46	0.108	0.303	0.43	0.516	0.441
YOR298C-A	-0.466	-0.42	-0.433	0.019	0.134	0.092	0.077
YOR304W	-1.03	-0.739	-0.413	0.074	0.219	0.25	0.067
YOR307C	-0.642	-0.676	-0.522	-0.123	0.105	-0.062	-0.078
YOR308C	-0.758	-0.707	-0.372	0.093	0.267	0.2	0.082
YOR313C	-0.668	-0.604	-0.284	0.024	0.318	0.378	0.257
YOR317W	-0.322	-0.338	-0.286	0.142	0.518	0.271	0.118
YOR320C	-0.245	-0.223	0.107	0.404	0.529	0.495	0.438
YOR321W	-0.208	-0.432	0.031	0.468	0.576	0.533	0.45
YOR322C	-0.426	-0.328	-0.092	0.29	0.414	0.309	0.318
YOR327C	-0.592	-0.502	-0.22	0.107	0.222	0.127	0.121
YOR328W	-0.553	-0.66	-0.328	0.114	0.376	0.297	0.02
YOR329C	-0.327	-0.644	-0.109	0.329	0.389	0.186	0.319
YOR330C	-0.671	-0.509	-0.029	0.184	0.361	0.444	0.36
YOR331C	-0.797	-0.914	-0.697	-0.228	-0.026	-0.209	-0.138
YOR336W	-0.39	-0.345	0.071	0.336	0.506	0.465	0.347
YOR340C	-0.606	-0.567	-0.086	0.22	0.428	0.395	0.448
YOR341W	-0.655	-0.491	-0.153	0.382	0.306	0.369	0.32
YOR364W	1.076	0.679	0.809	0.123	0.192	0.074	0.247
YOR372C	1.089	0.82	0.756	0.255	0.176	0.36	0.259
YOR374W	2.303	1.146	0.836	0.294	0.33	-0.357	-0.013
YOR380W	1.633	0.723	0.782	0.175	0.304	-0.096	0.02
YOR387C	1.076	0.437	0.743	-0.174	-0.162	-0.193	-0.117
YOR388C	1.143	0.633	0.828	-0.213	-0.467	-0.479	-0.248
YOR389W	1.025	0.656	0.812	-0.11	-0.442	-0.421	-0.143
YOR390W	1.139	0.693	0.921	-0.033	-0.358	-0.271	-0.129
YOR391C	1.501	0.884	1.06	0.149	-0.278	-0.312	-0.078
YOR392W	1.24	0.866	1.1	0.071	-0.182	-0.189	0.195
YOR393W	1.78	1.087	1.151	0.147	-0.182	-0.374	-0.225
YOR394W	1.299	0.585	0.626	0.046	0.18	-0.189	-0.017
YPL007C	0.418	0.339	0.543	-0.003	-0.404	-0.066	-0.117

YPL008W	0.52	0.232	0.065	-0.331	-0.549	-0.33	-0.388
YPL056C	1.553	2.012	1.978	0.793	0.632	0.325	0.783
YPL217C	-0.717	-0.311	-0.081	0.341	0.457	0.317	0.175
YPL230W	-0.2	-0.156	0.08	0.56	0.824	0.453	0.525
YPL232W	-0.441	-0.513	-0.242	0.144	0.434	0.234	0.154
YPL251W	1.682	1.056	0.711	0.333	0.042	-0.324	0.164
YPL252C	1.638	1.221	0.565	0.183	0.091	-0.336	0.192
YPL258C	0.714	0.378	0.409	-0.073	-0.216	-0.066	-0.046
YPL260W	1.227	0.824	1.074	0.255	0.028	0.244	0.355
YPL261C	0.657	0.449	0.555	-0.142	-0.208	-0.137	0.188
YPL262W	1.364	0.962	0.746	0.024	-0.125	-0.318	0.05
YPL263C	0.603	0.313	0.715	-0.049	-0.339	-0.101	-0.083
YPL264C	1.031	0.68	0.828	0.094	-0.162	-0.103	0.12
YPL265W	0.935	0.81	0.806	0.092	-0.197	0.046	-0.015
YPL267W	0.54	0.315	0.545	-0.171	-0.633	-0.285	-0.294
YPL268W	1.205	1.102	1.067	0.158	-0.02	0.212	0.099
YPL271W	0.876	0.682	0.831	0.194	0.066	0.015	0.132
YPL273W	1.092	0.887	0.981	0.086	-0.008	0.11	0.1
YPL274W	0.96	0.433	0.882	0.068	-0.126	0.073	0.18
YPL275W	0.994	0.487	0.636	-0.206	-0.473	-0.589	-0.426
YPL276W	1.334	0.416	0.305	-0.617	-0.888	-1.074	-0.741
YPL277C	1.032	0.519	0.808	-0.143	-0.523	-0.486	-0.347
YPL278C	0.73	0.388	0.487	-0.472	-0.638	-0.718	-0.606
YPL279C	0.993	0.683	0.908	-0.076	-0.442	-0.268	-0.103
YPL280W	1.284	0.768	0.841	-0.12	-0.385	-0.432	-0.212
YPL281C	1.612	1.006	1.024	-0.031	-0.278	-0.505	-0.194
YPL282C	1.13	0.443	0.518	-0.083	0.045	-0.274	-0.118
YPL283C	2.228	1.109	0.951	0.215	0.196	-0.152	0.273
YPR003C	0.42	0.17	0.01	-0.34	-0.701	-0.476	-0.39
YPR007C	0.241	-0.05	-0.228	-0.661	-0.889	-0.499	-0.606
YPR010C	1.228	0.733	0.113	-0.085	-0.515	-0.54	-0.352
YPR011C	0.688	0.28	0.207	-0.35	-0.678	-0.675	-0.278
YPR014C	0.091	0.004	-0.057	-0.74	-0.95	-0.426	-0.484
YPR091C	-0.973	-0.692	-0.296	-0.007	0.144	0.243	0.075
YPR096C	-0.91	-0.999	-0.576	-0.252	-0.196	-0.152	-0.172
YPR133C	-0.635	-0.514	-0.441	-0.061	0.283	0.079	0.024
YPR133W-A	-0.566	-0.579	-0.124	0.224	0.275	0.332	0.109
YPR136C	-0.484	-0.457	0.005	0.387	0.731	0.543	0.44
YPR147C	-0.322	-0.265	0.044	0.324	0.69	0.618	0.484
YPR148C	-0.589	-0.552	-0.344	0.105	0.457	0.338	0.196
YPR191W	1.43	0.702	0.69	0.104	-0.006	-0.04	-0.103
YPR192W	2.008	0.984	0.651	0.268	0.168	-0.318	-0.1
YPR194C	1.402	0.951	1.088	0.353	0.627	0.269	0.245
YPR196W	1.104	0.884	0.852	0.371	0.352	0.329	0.396
YPR197C	1.155	0.607	0.754	0.121	-0.05	-0.013	0.034
YPR198W	1.518	0.948	0.938	0.168	0.119	0.006	0.256
YPR199C	1.079	0.491	0.78	-0.098	0.041	-0.066	-0.114
YPR200C	0.694	0.526	0.817	0.033	-0.048	0.294	0.083
YPR201W	1.493	0.913	1.119	0.419	0.328	0.274	0.259
YPR203W	1.472	1.01	1.105	0.352	0.357	0.221	0.486