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DEVELOPMENT OF A SCREEN TO IDENTIFY KNOCKOUT STRAINS WITH INCREASED AMPLIFICATION RATES IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Gene amplification is the change from one copy of a gene per genome to two or more copies of a gene per genome. Mutational gene amplification is involved in evolution of new genes, drug resistance, and the progression of malignant tumors. However, it is difficult to study gene amplification because it is a rare event in normal eukaryotic cells. An amplification detection system that can phenotypically identify co-amplification of *ADH4* and *CUP1* genes *in Saccharomyces cerevisae* has been developed in our lab. The recent generation of a collection of gene knockout strains for all 4,600 non-essential yeast genes in combination with the *ADH4*::*CUP1* amplification detection system provides an opportunity to systematically screen for the effect of individual gene knockouts on gene amplification in yeast. We report here on the development of a screening protocol to identify yeast knockout strains that have an increased amplification rate, based on the *ADH4*::*CUP1* amplification detection system. The first four knockout strains we chose to test are, *MRE11* KO, *RAD50* KO, *XRS2* KO, and, *SAE2* KO. These genes were chosen because they are involved in DNA double strand break formation and repair.

Among the four strains tested *XRS2* KO has the largest effect on amplification. *RAD50* KO showed a slight increase in amplification rate, consistent with a previous estimate of the amplification rate of a *RAD50* deletion strain in our lab. Slight or no increase in amplification rates was detected in *MRE11* KO and *SAE2* KO strains but a small increase in amplification rate would not be detected in these experiments. Although the knockout parent strain and the *ADH4*::*CUP1* amplification detection strain have very low amplification rates, we detected an unexpected increase in amplification in crosses

between these strains. Therefore, in future, we propose to introduce the *ADH4::CUP1* system into the knockout strain genetic background to eliminate this problem.

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List of Abbreviations and Symbols

α	Alpha
Δ	Delta represents deletion of a gene when written before the gene name
μg	microgram
ADH	alcohol dehydrogenase
BFB	breakage Fusion Bridge
CHEF	Clamped heterogeneous electric field
CUP	Copper chelator gene
DNA	deoxyribonucleic acid
DBS	double strand breaks
g/l	grams per liter
KO strains	Strains that have deletion in one of the genes
mg	milligram
MMS	methyl methane sulfonate
MRE	meiotic recombination
NBS	Nijmegen breakage syndrome
NBS1	gene involved in Nijmegen breakage syndrome
NHEJ	non homologous end joining
ORF	open reading frame
RNA	ribonucleic acid
RAD	radiation sensitive
UV	ultra-violet radiation
XRS2	X-ray sensitive

Genetic Nomenclature in Yeast

The following nomenclature, specific for yeast, is used throughout this paper. Capital, italicised letters indicate a wild type gene, e.g. *MRE11*. A mutant gene is represented by lowercase, italicized letters, e.g. *mre11*. The protein product of a gene is represented as the gene name followed by a "p", e.g. Mre11p. A deletion of a gene is indicated as *Δmre11*.

INTRODUCTION

SIGNIFICANCE OF PRIMARY GENE AMPLIFICATION

Gene amplifications are changes in the genome that increase the copy number of a part of the genome. Amplifications occur during normal development of some multi cellular organisms. However these changes are not inheritable. Normally the genetic stability in a cell is accurately maintained during processes like DNA replication, recombination and repair. Genetic inheritance from one generation to another is based on exceptional genetic stability over many generations of cells and organisms. However, certain intracellular and extracellular environments play a major role in developing instability in the genome by constantly challenging the cells (Freidberg, 2001). Gene amplification is one result of such genomic instability. Mutational gene amplification is an important process in the evolution of new genes, drug resistance in cancer cells, and in tumor progression. Gene amplification is also important in development of many organisms.

Gene Amplification: An Important Process in Development.

Amplification of DNA plays an important role in development of Drosophila and many diptherian larvae. Most of the larval tissues in these organisms are polytene. Polytenization of the tissues occurs when cell cycle consists of only S phase and gap phase without any mitotic phase (Smith and Weaver, 1991). Chorion gene amplification is another example of amplification as a normal developmental process. Chorion genes are present in two clusters, one cluster on the X chromosome and the other on third chromosome. In the last 16 hrs of oogenesis in Drosophila, chorion genes replicate repeatedly in the ovarian follicle cells. This replicated DNA produces a large amount of

chorion proteins by transcription of the amplified genes (Spralding, 1990). Chorion gene amplification occurs bi-directionally, initiation occurs repeatedly on either side of the origin and the replication fork progresses further in both the directions. By the end of oogenesis each gene cluster is present at its highest copy number. Thus chorion gene amplification is an example of amplification during normal development. In the amplifications involved in development of an organism, the nucleus divides but the cell does not undergo any division. These amplifications therefore remain confined to one generation only and do not get passed on to the daughter cells. As discussed earlier amplifications also occur as mutational events. These mutations are transferred to the daughter cells and can be inherited by offspring. Here, we are going to concentrate on the mutational aspect of gene amplification. Mutational amplifications may have both useful and harmful effects that are discussed in detail below.

Gene Amplification and Evolution

Gene amplification plays an important role in evolution of a genome, since it is the first step in evolution of gene families. When a gene is amplified, one copy of the gene performs the primary function, while the other copies may evolve and aquire new functions. These extra copies are available for either producing increased amounts of their gene products or to become mutated eventually acquiring new functions. In both these cases the amplification results in a change, which may lead to evolution of new genes. Alternatively the extra copies of the gene formed during amplification, become completely inactive causing no change in phenotype. Homeobox genes or hox genes are classic examples of gene families formed by gene amplification. Hom/Hox genes are

involved in the development of the axial axis of an organism. In vertebrates, these genes are tightly linked to each other and organized in four clusters. Each cluster is located on a separate chromosome. Study of hox gene sequences has revealed great similarities, among genes in the same cluster and more amazingly between genes in different clusters. Sequence comparisons of the homeobox genes within the clusters and among different clusters strongly suggest that evolution of the homeobox genes in mouse took place in 2 steps. Individual homeobox genes duplicated to form the ancestral cluster. The ancestral cluster then duplicated several times to create four structurally similar gene clusters (Schughart K. 1989). The most striking feature of hox genes is their organization in a cluster where genomic position correlates with gene expression. This level of organization has been maintained in evolution. The promoters and the enhancers that control hox gene expressions are present in the region upstream of the start point of the cluster. One possible explanation for the evolution of the cluster along with its programmed organization is that enhancer of one gene must be embedded in another gene in such a way that the next gene cannot function unless the gene before it, is expressed (Lewin, 2000). This shows the interdependence of the genes within the cluster, which can be related to the formation of the cluster from one gene. Many other gene families have formed by similar gene amplifications, e.g. globin gene in humans etc. Therefore studying gene amplification is important for understanding evolution of gene families, and will help in understanding the interactions between these related genes.

Gene Amplification and Drug Resistance in Tumor Cells

Gene amplification often results in an increased amount of the gene product of the amplified gene, and thus can lead to increased resistance to certain cytotoxic drugs in cancer cells. Amplification of the gene encoding the dihydrofolate reductase enzyme confers resistance to a chemotherapeutic agent methotrexate. Synthesis of purine nucleotides begins with the reduction of dihydrofolate. The enzyme dihydrofolate reductase catalyses the reaction where dihydrofolate is reduced to trihydrfolate. The metabolic pathway continues further to synthesize nucleotides. But after reduction dihydrofolate is recycled and used again. Thus the dihydrofolate reductase gene is very important for synthesis of nucleic acids. Methotrexate is structurally analogous to the substrate of dihyrofolate enzyme. In normal cells, methotrexate uses reduced folate as a carrier and enters the cell. When methotrexate occupies all the molecules of the dihydrofolate reductase enzyme there is no enzyme left for binding with the substrate remaining in the cell to begin nucleotide synthesis. Therefore normal cells are sensitive to methotrexate. But when the dihydrofolate reductase gene is amplified, the copy number of the gene increases which leads to an increase in the amount of enzyme produced. The increase in the amount of enzyme allows nucleotide synthesis to occur even if some of the dihydrofolate reductase enzyme is occupied by methotrexate. Therefore cells with an amplification of the dihydrofolate reductase gene become resistant to methotrexate. Studies of cells containing different levels of dihydrofolate reductase enzyme in which the levels of the enzyme were proportional to the number of copies of DHFR gene present in the cell showed that resistance to methotrexate increased progressively as the

number of copies of DHFR gene increased in the cells (Schimke, 1984). This is because the DHFR gene amplification does not make the cells totally resistant to methotrexate, but just increases the amount of the enzyme produced so the cells can still synthesize nucleotides even in the presence of a normal dose of methotrexate. Amplifications in normal mammalian cells are often studied by using cells that have been treated with methotrexate and other chemotherapeutic drugs.

Gene Amplification and Cancer

Genomic instability leading to amplification is a frequent event in cancer cells, where amplification of proto-oncogenes plays an important role in tumor progression (Coquelle A. et al., 1997). The degree of amplification of oncogenes is directly proportional to the degree of tumorigenicity in the cell. Gene amplification is frequently observed in human tumors and in cell lines but is rarely seen in normal cells in humans. The lack of amplification in normal mammalian cells may be due to the repair system that effectively rectifies the errors during replication and efficient cell cycle control genes that prevent proliferation of cells with damaged DNA and replication of damaged DNA. Cells are checked for DNA damage at least at three stages of the cell cycle, at the G1/S transition, during the S phase, and at G2/S boundary (Hartwell, 1997). The p53 protein is essential for the checkpoint control in mammalian cell cycle. In the case of damaged DNA or abnormal chromosomes, p53 either arrests the cell cycle in the G1 phase or promotes apoptosis in the cell. Significant association between p53 abnormalities and genetic instability has been detected in cell lines of murine and human origins. Although mutation of the p53 gene is associated with genomic instability, the exact role of p53 in

the increase in the process of gene amplification is not yet known. However, the p53 protein acts as a transcription factor in regulation of several other genes (MDM2, Cyclin G, Bax etc) that are responsible for maintaining genomic integrity and therefore has a direct role in the process of gene amplification (Levine 1997). The genes that are transcriptionally activated by p53 protein include genes that are involved in DNA repair, recombination, replication, etc. It has been shown that mutation in p53 is frequently observed in genetic abnormalities like gene amplification. Therefore it is very important to understand the specific DNA repair, recombination, replication, genes in preventing amplification.

STRUCTURES AND MODELS OF GENE AMPLIFICATION

Structures of Gene Amplification

Structures of gene amplifications have been studied extensively. Gene amplifications are present in a variety of sizes and structures. DNA amplifications vary in size from few hundred base pairs to thousands of kilo bases. Initially DNA amplifications were classified based on their cytological appearances as extended chromosomal region (ECR) and extrachromosomal elements called double minutes (DMs) (Hamlin et al., 1984; Stark et al., 1989). Later, with the advancement of molecular techniques these structures were classified as intrachromosomal amplifications and extrachromosomal amplifications. Intrachromosomal amplifications can be present at the normal location or may be translocated to some other location on the same chromosome or to some other region of the genome. Extrachromosomal amplifications can exist as linear palindromes or circular structures. A frequently observed structure in amplification is a novel joint involving an inverted repeat. Four circular amplifications in which the novel joints involving inverted repeats were found in *Saccharomyces cerevisiae* (Moore et al., 2000). A novel joint can be defined as a point where two DNA segments that are separated from each other become joined together. (FIG 1 shows the structure of novel joints that have been identified in yeast)

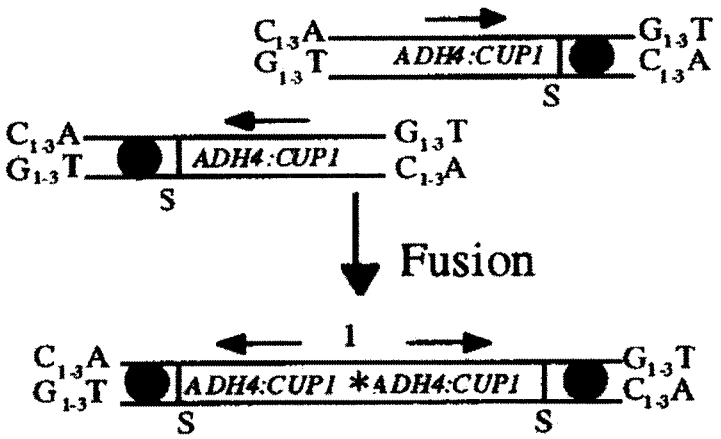

FIGURE 1: Novel Joints Found In Spontaneous Amplifications That Have Been Identified In Yeast.
Fusion of two pieces of a chromosome each with an *ADH4* gene and a *CUP1* gene creates a novel joint in the middle where the two chromosomes are joined together. The star points at the region of the novel joint.

Models of Gene Amplification

Analysis of many different amplification structures has resulted in the proposal of several models of gene amplification. The models of gene amplification propose different mechanisms to explain different amplification structures. However it is also possible that one basic mechanism could result in several different outcomes depending on its structural stability. In most of the models the two initial events are duplication of a specific locus on a chromosome followed by expansion of the duplicated region. Three models of gene amplification are discussed here, the onionskin model, the sister chromatid exchange model and the intrachromosomal recombination model.

Onionskin Model: In this model bi-directional replication is followed by unscheduled DNA replication, which results in a series of partially replicated duplexes, that remain attached together (FIG 2). This structure is very unstable mitotically and can be resolved in three different ways. 1) Multiple recombination events result in intrachromosomally amplified structure. 2) Recombination within a duplex that results in extrachromosomal circular amplifications. 3) Separation of the linear duplex DNA from the partially replicated structure. The chorion gene amplification in development of Drosophila was the inspiration for this model of amplification. The onionskin model of amplification accounts for all types of amplifications and chorion gene in Drosophila development provides evidence for this type of process occurring in the cell. However, there is no physical evidence that shows multiple replications occurring from a single origin of replication in mutational gene amplification.

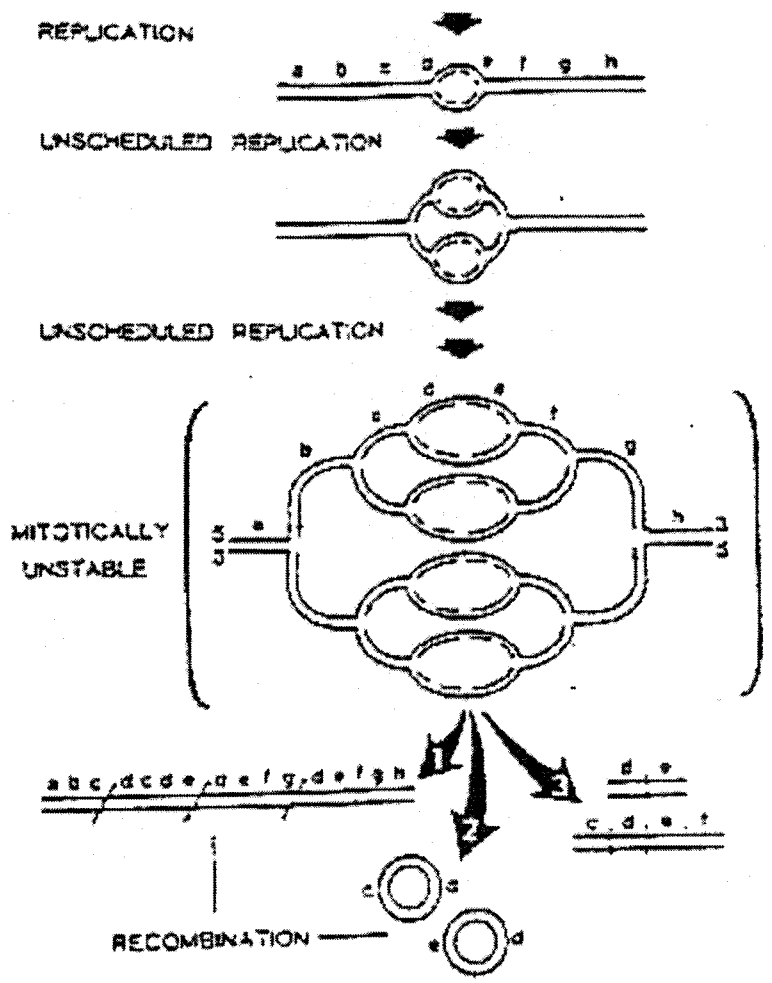

FIGURE 2: Onionskin Model of DNA Amplification.

The figure shows the beginning of unscheduled replication that leads to a structure where multiple replication forks are attached together. Oval bubbles represent the replication forks. The double stranded circles represent circular amplifications formed. This model is from Stark et al., 1989

Unequal Sister Chromatid Exchange: This model suggests that an initial error in recombination between two homologous chromosomes results in deletion of genes in one chromosome and duplication of the genes on the homologous chromosome (Smith et al., 1992). This initial duplication expands rapidly in the following cell cycles due to unequal sister chromatid exchanges (Smith et al., 1992). Although unequal sister chromatid exchange occurs during cell division, this model does not explain the structures like novel joints at inverted repeats or extrachromosomal amplifications that are seen very frequently in gene amplifications.

Intrachromosomal Recombination Model: This model involves 3 steps. The first step is breakage of chromosome near an inverted repeat (Butler et al., 1995). The break may occur between an inverted repeat and a centromere or between an inverted repeat and a telomere. The second step in the intrachromosomal amplification model is recombination between inverted repeats to form DNA hairpins. The last step in this model is bi-directional replication of DNA (FIG 3). If the DNA break is between an inverted repeat and a centromere then it results in an extrachromosomal palindromic structure, which is quite stable. But if the DNA break is in between an inverted repeat and a telomere then a dicentric molecule is formed. This is an unstable amplification structure. The dicentric molecule can then initiate the breakage fusion bridge cycle resulting in other amplification structures.

Breakage Fusion Bridge Cycle: During mitosis the dicentric molecule formed from intrachromosomal recombination model breaks when it gets pulled between opposite

poles. The breakage fusion bridge cycle continues since the broken ends of the sister chromatids keep fusing resulting in dicentric chromosomes. The cycle can be ended only if the broken chromosome ends recircularize to form extrachromosomal circular amplifications or if a telomere attaches to the broken ends of the chromosome to form intrachromosomal amplification. Four spontaneous independent circular amplifications that fit the breakage fusion bridge mechanism of amplification have been identified in our lab. All these amplifications have a novel joint between the amplified DNA and the telomere (Moore et al., 2000). Large palindromic sequences next to inverted repeats that are formed by intrachromosomal recombination mechanism have been identified in *Saccharomyces cerevisiae* (Butler, 1996).

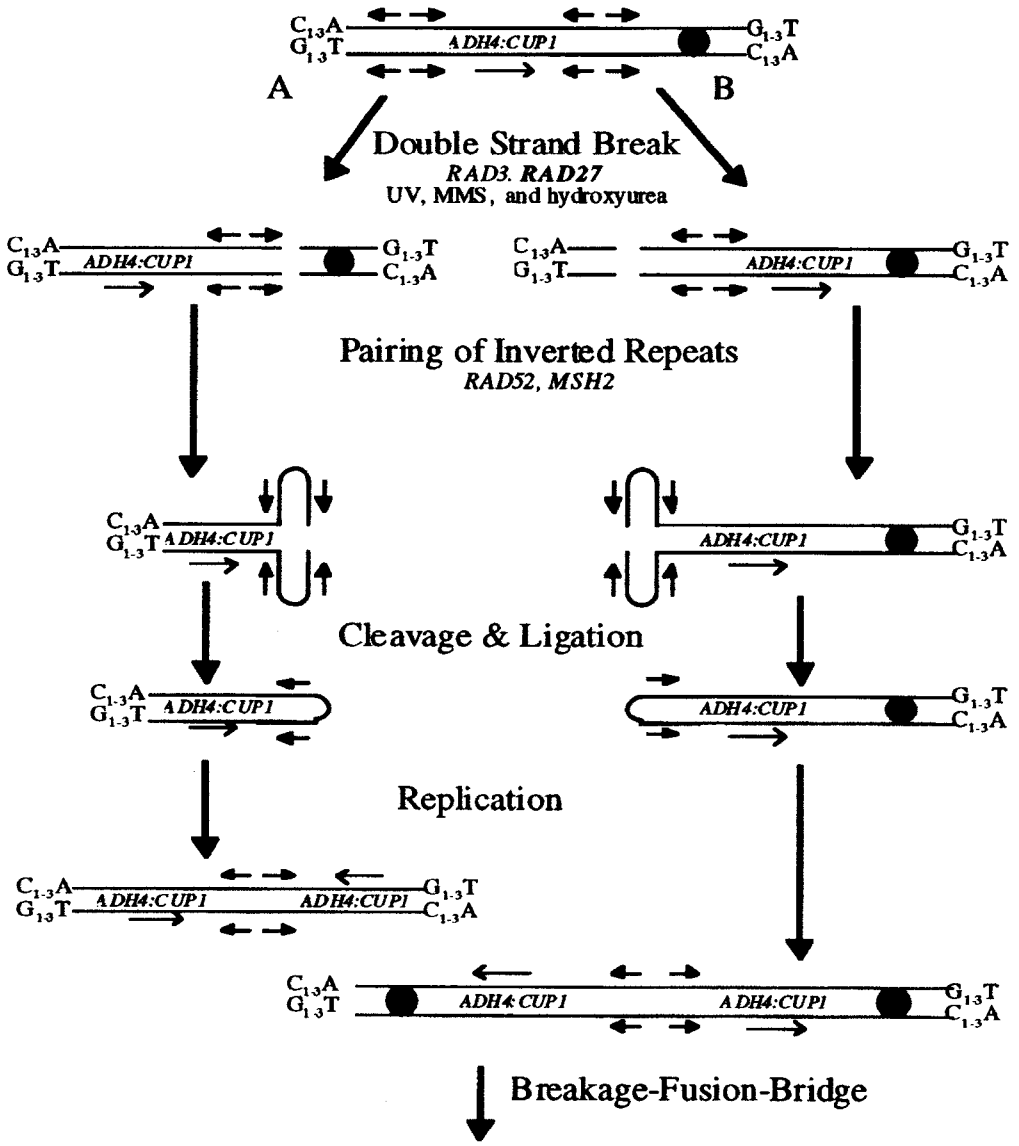

FIGURE 3: Intrachromosomal Recombination Model.

This model of amplification is adapted from Butler et al. (1995) to fit the *ADH4::CUP1* amplification detection system that has been developed in our lab. **A.** Double stranded breaks between an inverted repeat and a centromere results in an extrachromosomal palindrome. **B.** Double stranded breaks between an inverted repeat and a telomere results in a dicentric chromosome that leads to a Breakage Fusion Bridge cycle. Note: Filled circles represent centromeres; open headed arrows indicate direction of transcription of *ADH4* and *CUP1*; close-headed arrows indicate the location of inverted repeats.

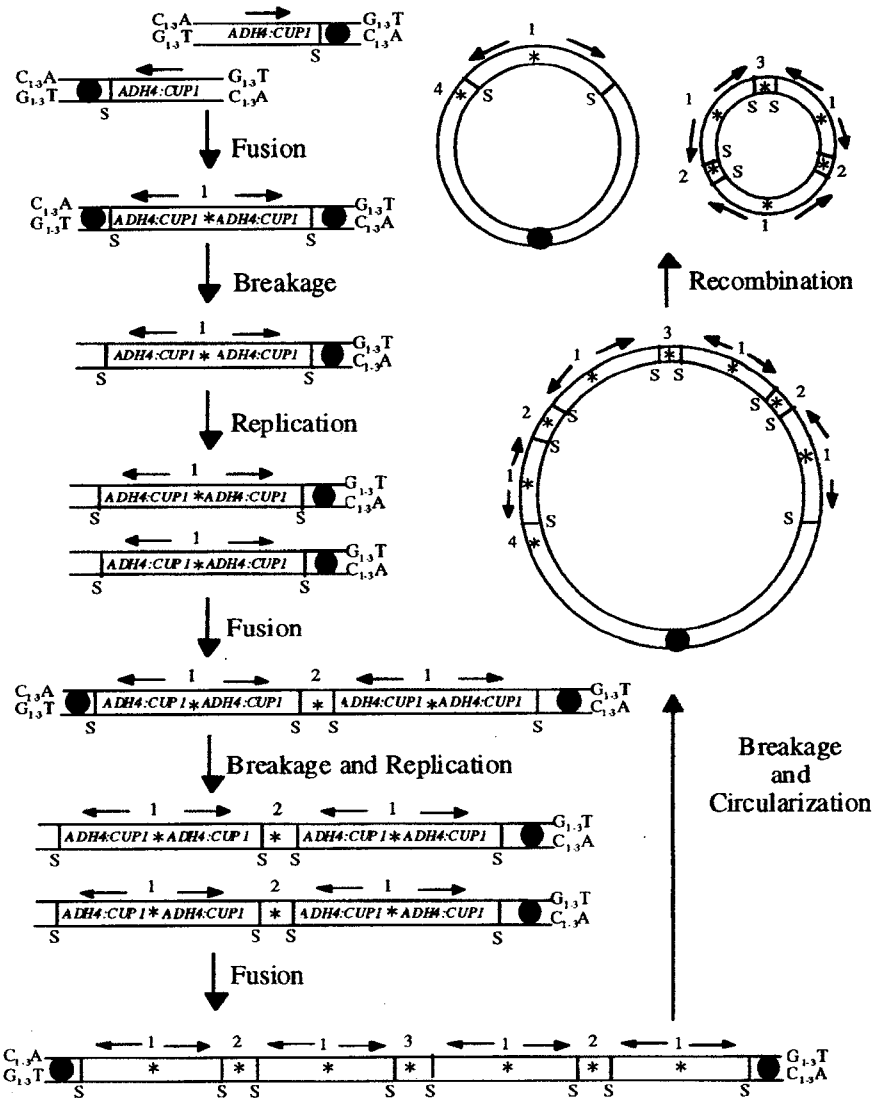

FIGURE 4: Breakage Fusion Bridge Model

This model of amplification is adapted from Butler et al.. (1995) to fit the *ADH4::CUP1* amplification detection system that has been developed in our lab.
Note: Filled circles represent centromeres; open headed arrows indicate direction of transcription of *ADH4* and *CUP1*; close-headed arrows indicate the location of inverted repeats.

MODEL SYSTEM AND MODEL ORGANISM USED IN THE STUDY

Yeast As A Model System To Study Amplifications

Although importance of gene amplification has been very well demonstrated in mammalian systems, it is very difficult to study amplification in normal mammalian cells. The rate of amplification in a normal mammalian cell is $< 10^{-9}$ amplifications/cell/generation. Immortalized cell lines have a much higher rate of amplification ($\approx 1x\ 10^{-5}$ amplifications/cell/generation). Therefore amplifications in mammalian systems are often studied using cultured cell lines. The cultured cell lines are often treated with agents that interfere with DNA synthesis. Alkylating agents like methyl methane-sulfonate, drugs like hydroxyurea, methotrexate (MTX), N-phosphonacetyl-L-aspartate (PALA) can cause double stranded breaks in DNA. Methyl methane-sulfonate blocks replication by breaking the sugar phosphate bonds in the DNA molecule (Friedberg, 1985). Hydroxyurea, MTX, and PALA block replication by depleting the nucleotide pool required for replication (Poupon, 1996). These cultured cell lines have a very unstable genome, making the cells biased toward amplifications or at least a high rate of mutation. Therefore using an unbiased system to study the initial events of amplification in a normal cell is very important. One such system has been developed in our lab, the *ADH4::CUP1* amplification detection system. This system uses *Saccharomyces cerevi*siae (Baker's yeast) as a model organism to select and screen amplifications of a reporter cassette *ADH4::CUP1*. Yeast is an excellent system because it is a single-celled eukaryotic organism. It can be maintained as stable haploid cells. Many DNA repair and recombination systems in yeast are similar to those in higher

eukaryotes. A large number of cells can be grown and studied at a time (as many as 10^8 can be present in 1 ml culture). The generation time in yeast is very fast, approximately 2-4 hrs. All the 16 chromosomes in yeast can be separated on a pulse field gel, this makes it very easy to detect amplifications present along with the normal chromosomes. Molecular techniques can be easily used on yeast like transformation of yeast cells, isolation of genomic and plasmid DNA from yeast cell, addition or deletion of genes in a cell etc.

ADH4::CUP1 Amplification Detection System

ADH4::CUP1 system in yeast uses two genes constructed as a reporter cassette to identify amplifications. *ADH4* encodes an alcohol dehydrogenase isozyme. *CUP1* encodes a low molecular weight copper chelator that is responsible for copper resistance in a cell. Coamplification of the two genes results in antimycin resistance and copper resistance in the cells. The level of copper resistance is directly proportional to the number of copies of the *CUP1* gene present, as the copy number goes increasing the level of copper resistance goes up. Alcohol dehydrogenase (*ADH*) is an enzyme that catalyzes conversion of ethanol to acetaldehyde. There are four isozymes encoded by the *ADH* genes present in yeast, Adh1p, Adh2p, and Adh3p, and Adh4p. *ADH1* and *ADH2* show 90% similarity in their nucleotide sequences and *ADH3* is about 75% identical to *ADH1* and *ADH2*. (Young et al. 1985). *ADH4* does not show any similarity to the other *ADH* genes in yeast but it is similar to an *ADH (zADH2)* found in *Zymomonas mobilis* (Williamson et al., 1987). Adh1p is the classical fermentative isozyme, the cells used in this amplification detection system lack the *ADH1* gene. These cells cannot grow on

medium containing glucose and an antibiotic antimycin A. Antimycin A inhibits respiration by blocking the electron transport chain. The *ADH4::CUP1* yeast strain cannot ferment on glucose medium because it lacks *ADH1*, *ADH2* is repressed by glucose, and, *ADH3* encodes a mitochondrial enzyme therefore it is not functional. *ADH4* is also not expressed under these conditions. Thus the cell can neither respire nor ferment at this point, the only way the cells can survive is by some type of mutational event that will allow the cells to ferment. Amplification of *ADH4* is one such mutation that allows the cells to grow on antimycin A medium (Walton et al.,1986). However, only about one in a hundred antimycin A resistant mutant carries an amplification of *ADH4*. Therefore, a *CUP1* gene was deleted from its normal location and inserted next to *ADH4* so that antimycin resistant, copper resistant mutants that carried co amplifications of *ADH4* and *CUP1* could easily be identified. (Dorsey et al., 1995)This amplification detection system allows detection of as low as 1×10^{-10} amplifications/cell/generation. Some other types of mutations that allow cells lacking *ADH1* to grow on antimycin A in the presence of glucose are 1) Insertion of yeast transposable element (Ty) adjacent to the *ADH2* gene or *ADH4* gene (Williamson et al., 1981, Paquin et al., 1986) 2) mutations in the upstream promoter region of *ADH2* (Russel et al., 1983) 3) mutations in the regulatory regions of *ADH2* and *ADR1* (Denis C, 1984).

Several mutations that increase the rate of amplification in *Saccharomyces cereviseae* have been identified in our lab by using the *ADH4::CUP1* system. DNA double strand break is an important step in amplification. Mutations in two genes (*RAD3* and *RAD27*) that involve increase in the formation of double strand breaks have been identified in our

lab (Peterson et al., 2000; Tseng, 1998) . A high amplification (HAR) strain B9-40 that is sensitive to UV light was isolated. After transforming the genomic library in B9-40Δ*leu2* cells, it was observed that the UV sensitivity in these strains could be complemented by *RAD3* gene. A comparative amplification rate showed that the B9-40 cells with *rad3*-I463K gene show 40 fold higher rate than B9-40 cells with wild type *RAD3* gene. Thus, deletion of *RAD3* a nucleotide excision repair gene, increases gene amplification 40-fold.

Rad27p is involved in processing Okazaki fragments formed during the synthesis of the lagging strand in DNA replication. It is a homolog of mammalian Fen1p that is also involved in processing Okazaki fragments during replication and is involved in base excision repair. Preliminary experiments on *RAD27* show that deletion of *RAD27* gene increases amplification rates 500-fold but more experiments have to be done for a better estimate of the increased amplification rate.

Deletion of *RAD52* has the opposite effect on amplification rate in yeast strains. Yeast strains that have a deletion in the *RAD52* gene show a 100-fold decreased rate of amplification (Peterson et al., 2000). *RAD52* is involved in recombinational repair and DNA double stranded break repair.

In another study it was shown that *MSH2* increases the rate of amplification by 10-fold in *Saccharomyces cereviseae* (Peterson et al., 2000). *MSH2* is a mismatch repair gene that decreases non-homologous recombination. Increase in amplification rate in *Δmsh2* strains and identification of novel joints formed due to recombination between sequences of

limited similarities suggests that non-homologous recombination is an important step in amplification.

All these experiments suggest that DNA replication, repair, and recombination must be important steps in gene amplification. The amplifications in all these genes were studied using the*ADH4::CUP1* amplification detection system. All the amplifications identified so far fit the Intrachomosomal recombination model of amplification very well. Thus it is important to study amplification in other genes that are involved in these processes.

A collection of yeast knockout strains is commercially available from Open Biosystems (Giaever et al., 2002). There are 4600 haploid deletion strains in this collection. All possible gene deletions that allow the cells to survive even after the deletion have been made. Each strain has a deletion in only one gene therefore it is easy to screen the strains independently for detecting amplification. Here we report, a screening system that can be used to identify genes that increase the gene amplification rate in normal eukaryotic cells by using yeast as the model organism. We will be using *ADH4::CUP1* amplification detection system to identify the amplification in these strains. The screening system is tested on four knockout strains, *MRE11* KO, *SAE2* KO, *RAD50* KO, and *XRS2* KO. These genes are involved in DNA double strand break formation as well as repair. As mentioned earlier DNA breaks are important events in amplification. Therefore these four genes were chosen to test the screen.

MATERIALS AND METHODS

Yeast Strains and Plasmids

The yeast strains used in this study are listed in Table 1 and 2. The haploid strain 411B is the standard *ADH1* deletion strain that is used in the *ADH4::CUP1* amplification detection system (Dorsey et al., 1993) The knockout strains used were, *MRE11* KO, *SAE2* KO, *XRS2* KO, *RAD50* KO, and, *ADH1* KO. Mre11, Xrs2, Rad50 work together as a complex referred to as Mre11complex in this paper. A list of other knockout strains studied is listed in Appendix A. The knockout strains were obtained from Open Biosystems. The plasmid p7a was used for making the probe for detecting *ADH4* amplifications in the knockout strains. FIG 5 represents the construction of plasmid p7a with the *ADH4* open reading frame and *CUP1 Nde1/Nde1* fragment.

Media and Growth Conditions

All the yeast strains were initially grown on glucose rich, YP medium (Ciriacy, 1979) containing 10g/l yeast extract, 20g/l peptone, and 10% of 500g/l glucose. Adding 14g/l of agar made all the solid medium. Antimycin A medium was made by using a modified version of YPD medium. (Dorsey et al., 1993).

G418-His$^-$ medium – This medium was used for selecting diploids. *G418-His*$^-$ medium contained minimal medium (1.7 g/l yeast nitrogen base without ammonium sulfate, 5g/l ammonium sulfate, 40mls/l amino acid supplement containing all amino acids except histidine, 14g/l agar). After autoclaving the minimal medium, 50g/l glucose and 200μg/ml G418 was added to the medium (Cheng et al., 2000).

Table 1: The strains used in this study

Strain	Genotype	Source
411B	*MAT a, adh1Δ, HIS3, LEU2, LYS2, URA3*	Peterson et al., (1997)
YKO parent strain	*MAT α, ADH1, his3Δ, leu2Δ, lys2Δ, ura3Δ*	Wach et al., (1994).
SAE2 KO	*MATα, ADH1, his3Δ, leu2Δ, lys2Δ, ura3Δ, Sae2::KanMX*	Winzeler et al., 1999
MRE11 KO	MAT α, *ADH1, his3Δ leu2Δ lys2Δ ura3Δ MRE11::KanMX*	Winzeler et al., 1999
XRS2 KO	*MAT α, ADH1,his3Δ leu2 Δlys2Δ ura3Δ XRS2::KanMX*	Winzeler et al., 1999
RAD50 KO	*MAT α, ADH1, his3Δ leuΔ lys2Δ ura3Δ RAD50::KanMX*	Winzeler et al., 1999
ADH1 KO	*MAT α*, his3Δ leu2Δ lys2Δ ura3Δ *ADH1::KanMX*	Winzeler et al., 1999

Table 2: The knockout strains made in the *ADH1Δ* background

Strain	Genotype	Source
YKO/*ADH1Δ* parent strain	*MAT α, adh1Δ*	This study
SAE2 KO/ *ADH1Δ*	*MATα, adh1Δ, SAE2::KanMX*	This study
MRE11 KO/ *ADH1Δ*	MAT α, *adh1Δ, MRE11::KanMX*	This study
XRS2 KO/ *ADH1Δ*	*MAT α, adh1Δ, XRS2::KanMX*	This study
RAD50 KO/ *ADH1Δ*	*MAT α, adh1Δ, RAD50::KanMX*	This study

Sporulation plates – This medium contained 2.5g/l yeast extract, 15g/l potassium acetate, and 14 g/l agar. After autoclaving 0.5 g/l glucose and 5mls/l of uracil and 5mls/l of leucine was added to the medium. (Kassir et al., 1991)

Allyl alcohol plates – This medium was used for selecting diploids. 10g/l yeast extract, 20g/l peptone, 14g/l agar), After autoclaving the minimal medium, 50g/l glucose and 0.6g/l allyl alcohol was added to the medium. Allyl alcohol is a poison therefore plates are poured in the hood (Ciriacy et al., 1979)

Screen for Selecting Strains Causing High Rate of Amplification

The strains were incubated in 5µl of sterile water and 5µl of sterile glusilase in an eppendorf tube for 2-3 hours. After incubation, 1000µl of sterile water was added to the tubes. Then the tubes were sonicated using a Sonic dismembrator (Fisher Scientific Model # F50). After sonication the tubes were vortexed. Sonication and Vortexing breaks open the asci and releases the spores. The spores were plated on YEPD plates to obtain single colonies. Approximately 20 colonies per strain were transferred to 96 well plates which contained ~100µl of water. These colonies were then stamped from the 96 well plates to G418 plates and antimycin A plates simultaneously.

Chromosomal DNA Preparation

The chromosomal DNA from these strains was isolated using the protocol described by Carle and Olson (1985). The chromosomal DNA isolated from the antimycin A resistant strains was separated on a pulse field gel using a Bio-Rad CHEF (clamped homogenous electrophoretic field) apparatus. For yeast chromosome lengths ranging from 240 –2200

kb, 1% agarose gel made in 1X TBE buffer was used. The buffer overlaying the gel and the buffer used for making the gel were always of the same concentration. The gels were run at 14° C in 6 volts for approximately 21-22 hours. The switch time for all the gels was 60-120 seconds. Chromosomal DNA ran on the gel was transferred to a nitrocellulose membrane using the southern blot analysis procedure as described in Maniatis et al. (1982). The presence of amplifications in the strains was determined by hybridizing the southern blots with P^{32}dATP labeled probe by random priming protocol.

Probe Used for Southern Blot Analysis

The probe used in this study is *ADH4* DNA fragment obtained from plasmid p7a (Eco/Hind fragment). FIG 5 shows plasmid p7a along with the location of the *ADH4* DNA on the plasmid. The probe was labeled with P^{32}dATP at 1 X 10^6 cpm/ml by the random priming protocol (Sambrook et al., 1989)

Amplification Rate

The amplification rates in the knockout strains were calculated by the P_0 method developed by Lea and Coulson (1949). P_0 is a statistical analysis in which number of cultures without mutations are counted instead of the number of mutants per culture to eliminate the growth bias between parental strain and mutant strains. Antimycin A resistant mutants generally grow faster than the parental strain because they can ferment. P_0 method used to determine rate of amplification is described in detail in appendix B.

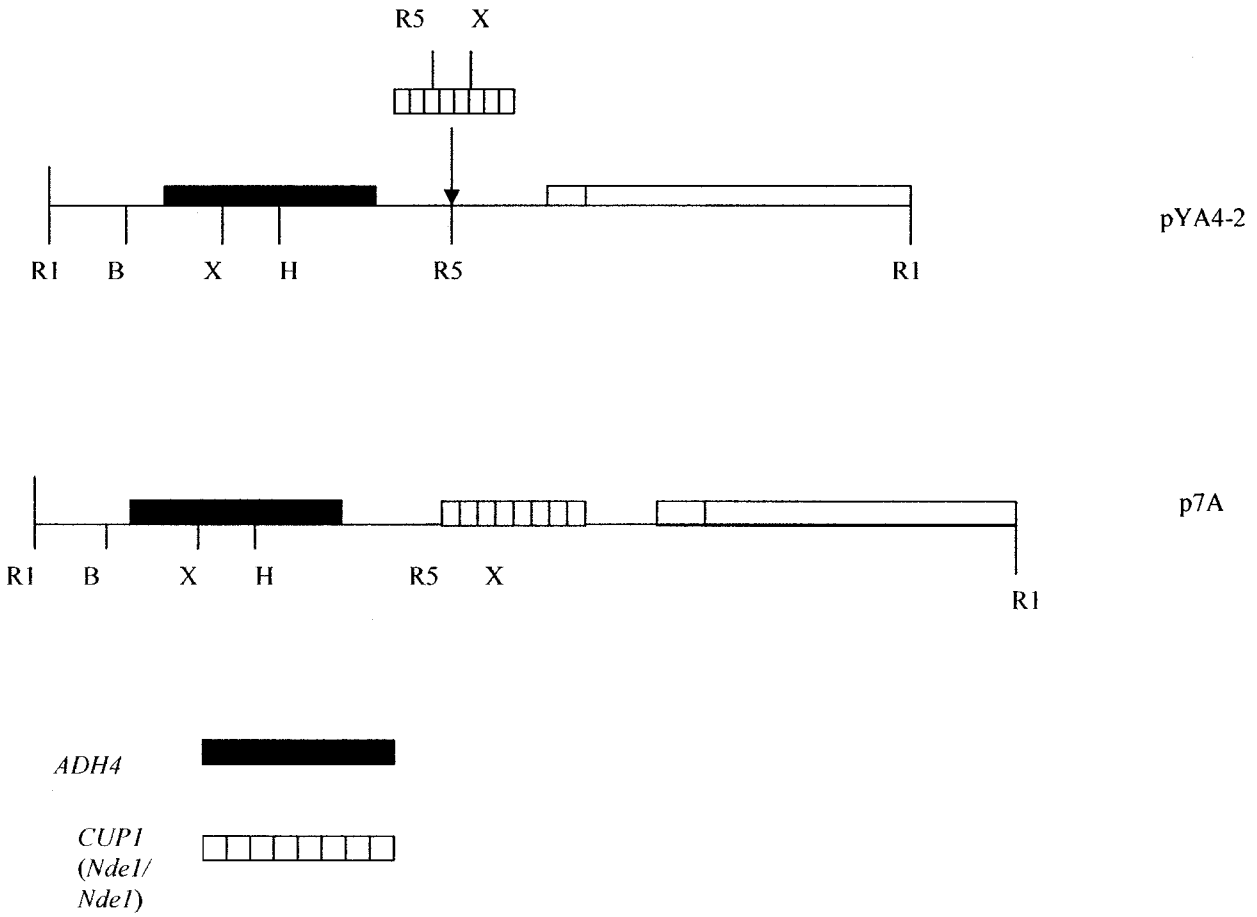

FIGURE 5: Construction of the plasmid p7a
Plasmid p7a was made by inserting the *ADH4* and *CUP1* (*Nde/Nde*) insert into the plasmid pYA4-2

RESULTS

The main goal of this project was to develop a screen to identify genes that alter amplification rates in yeast. The knockout (KO) strain collection constructed under the yeast deletion project (Winzeler et al., 1999 Wach et al., 1994; Giaever et al., 2002) available commercially from Open Biosystems has all the non-essential gene knockouts. There are ~6000 total open reading frames (ORFs) in a yeast cell. However only about 4600 genes can be deleted and the cell still survives, these are the non-essential genes. The ultimate goal of this project is to independently screen each ORF deletion to determine its effect on amplification rates. High amplification rate strains can be identified using a two-step process. First an *ADH1* background is created in the KO strains by mating these strains with 411B (our standard amplification detection strain). The second step is to screen the KO strains using the *ADH4::CUP1* amplification detection system to identify strains with high amplification rates. We chose to test the protocol on four strains carrying mutations in genes that are involved in DNA double strand break repair.

Characteristics of the KO Strains

All the KO strains are haploid. They belong to mating type α (*MATα*); thus they can be mated with any strain that belongs to the opposite mating type, *MATa*. Each strain carries only one gene mutation. In each mutant, a single gene has been deleted precisely from the translational START codon "ATG" through the translational STOP codon "UAA". All the strains carry two markers that important in our screen, the *KanMX* gene that confers resistance to antibiotic G418, and a mutation in histidine biosynthesis gene *HIS3*. The *KanMX* gene has been inserted in place of the knocked out gene in all the

strains. This gene allows the cells to grow in presence of antibiotic G418. The histidine biosynthesis gene is mutated in the strains so they cannot produce the amino acid histidine on their own. Therefore these strains require a histidine supplement in the medium for growth.

Characteristics of Amplification Detection Strain

411B is the standard strain used in the *ADH4::CUP1* amplification detection system. 411B is haploid in nature like the KO strains but belongs to the opposite mating type *MATa*. This strain has a deletion in *ADH1* gene, due to lack of Adh1p the cells cannot ferment and antimycin A represses respiration. Therefore this strain is sensitive to antimycin A. The 411B strain does not have the *KanMX* gene inserted in it. However the *HIS3* gene is intact in this strain therefore the cells can survive without histidine supplement in the medium. One more feature of the 411B strain is that it is resistant to allyl alcohol. Alcohol dehydrogenase converts allyl alcohol to a toxic compound, acrolein. Since 411B strain lacks the *ADH1* gene there is no alcohol dehydrogenase to convert allyl alcohol into acrolein, therefore the cells become resistant to allyl alcohol.

Construction of Knockout Strains in *ADH1* Deletion Background

The KO strains as well as 411B were grown on glucose rich (YEPD) medium for 4-5 days. These strains were streaked as a line on a YEPD plate. Once the growth was sufficiently thick, the cells were replica plated on a YEPD plate. The KO strain and the standard 411B strain were replica plated in a crisscross manner on the YEPD plate as shown in FIG 7a. The strains were allowed to mate overnight on a YEPD plate. The

next day they were transferred on to *G418/his*$^-$ medium. Transferring to *G418/his*$^-$ medium ensures the growth of only diploids on the plate since G418 antibiotic in the medium prevents growth of the haploid 411B strain and absence of histidine in the medium prevents the haploid KO strains from growing on the plate. The diploids were allowed to grow for 4-5 days on *G418/his*$^-$ plates. The diploid strains growing on *G418/his*$^-$ *plates* were then transferred to sporulation plates. Sporulation medium does not contain enough nitrogen for the cells to grow mitotically thus forcing the cells to undergo meiosis. As a result of meiosis, four haploid spores were formed in the asci. The asci were broken open by incubating the cells in the enzyme, glusilase followed by vigorous sonication and vortexing. The spores were then plated on YEPD plates so that single colonies could be obtained. Then, using a replicator the cells were stamped on to G418 plate and an antimycin A plate. The cells were allowed to grow on G418 and antimycin A medium for 5-6 days. After about a week, the plates were checked for G418 resistant, antimycin A sensitive colonies. G418 resistant and antimycin A sensitive colonies were selected because G418 resistance indicates that these cells did not have the knockout gene and antimycin A sensitivity confirms that they had a deletion in *ADH1* gene. At this step the cells did not have any amplifications as indicated by antimycin A sensitivity. Thus an *ADH1* deletion background was created in the knockout strains. The knockout strains with an *ADH1* deletion background are addressed by the name of the strain followed by *ADH1Δ* (see table 2). In the first two attempts, the cells were tested for resistance to allyl alcohol and G418 simultaneously. It was found that the cells do not grow if the medium contains both allyl alcohol and G418, therefore a G418 resistance and antimycin A sensitivity screen was performed.

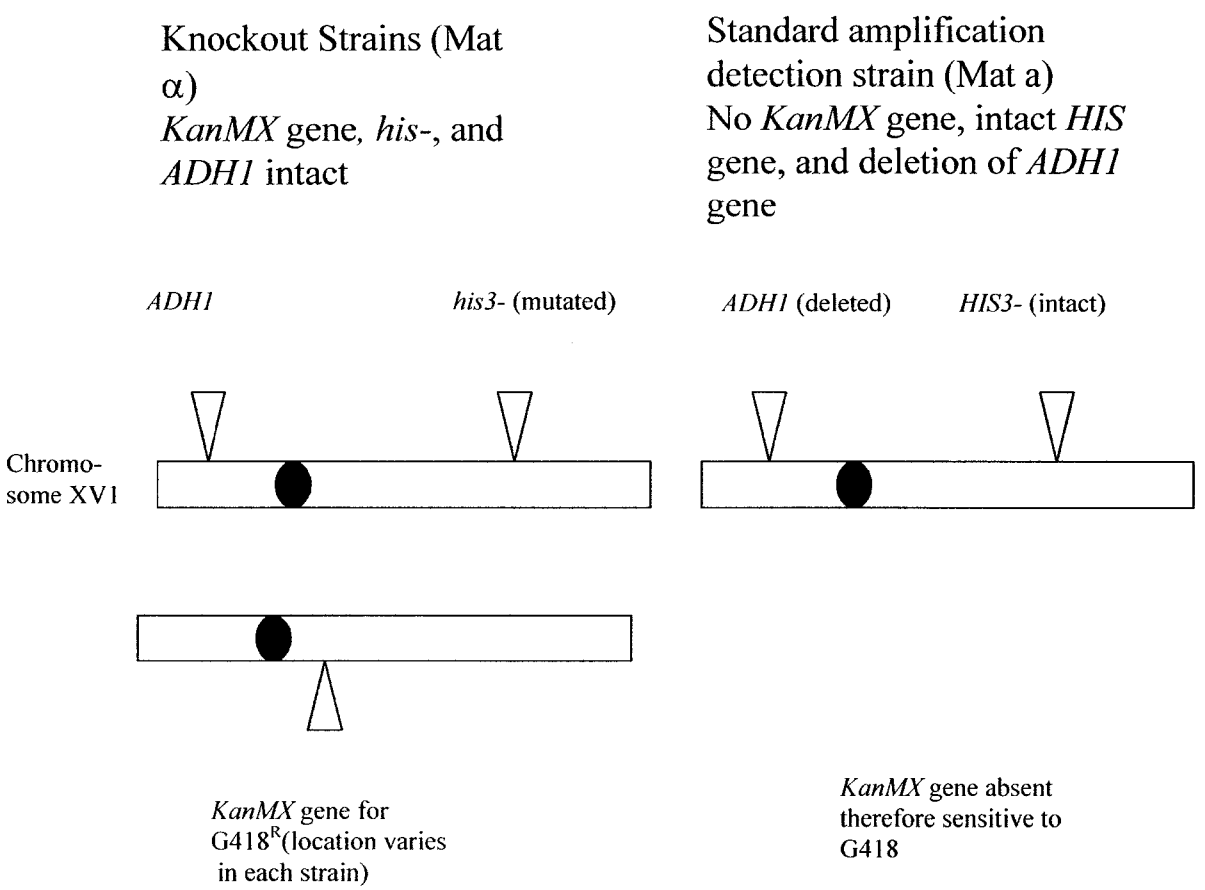

FIGURE 6: Diagrammatic representation of the markers present on the knockout strain and the standard *ADH1* deletion strain.
The approximate locations of *ADH1* and *HIS3* are shown for both the strains (Cherry J. M. et al., 1997). The open rectangle represents chromosome XV. Filled circle within the rectangle represents the centromere for that chromosome. The location on KanMX gene varies in different knockout strains.

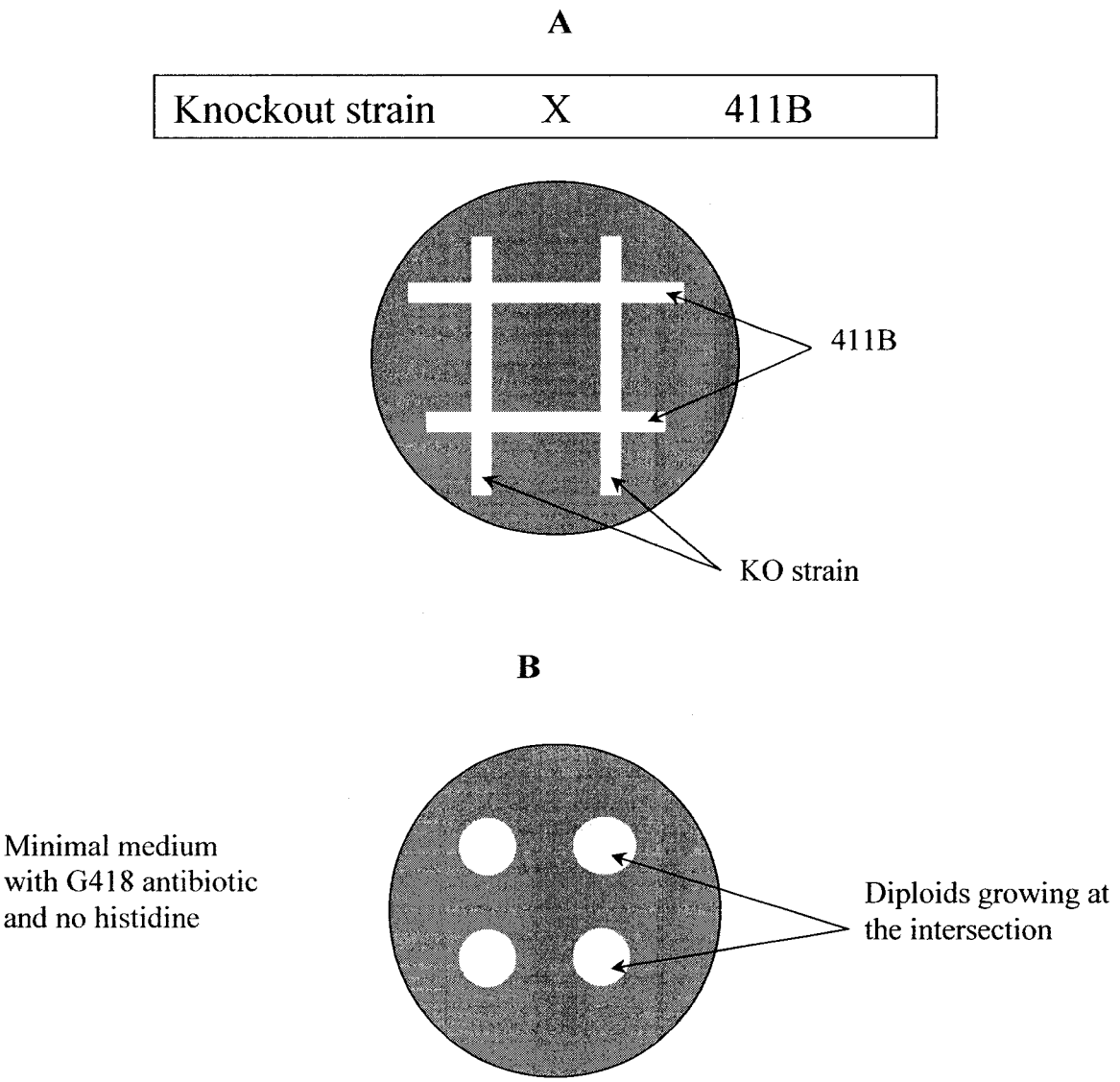

FIGURE 7: Diagrammatic representation of the crossing of the standard *ADH1* deletion strain and knockout strains.
A) The vertical lines represent the standard strain and horizontal lines represent the knockout strain.
B) The growth of the diploids is represented as white circles at the intersection of the two strains.

Estimation of Amplification Rates in Knockout Strains in the *ADH1* Deletion

After the *ADH1* deletion strain was crossed with the knockout strains, the strains were then tested for increased amplification rates. First the knockout strains were plated on YEPD to allow development of amplifications. After isolating G418 resistant/antimycin A sensitive colonies, the number of cells in these colonies was calculated using a hemocytometer. Then appropriate dilutions were made so that approximately 100 cells could be plated on YEPD plates. At least five YEPD plates were required for each strain so that sixty colonies of the same size could be obtained. The cells were allowed to grow on YEPD plates for about 6-7 days and then a single colony was plated on an antimycin A plate. Once again the cells in each colony were counted using a hemocytometer, this was done so that we knew approximately how many cells were plated on each antimycin A plate. By the end of the screen there were 30-60 antimycin A plates for each KO strain. The antimycin A plates that showed growth were removed and chromosomal DNA from one or two colonies from each plate were then subjected to pulse field gel electrophoresis and Southern blot analysis in order to detect the presence of amplifications.

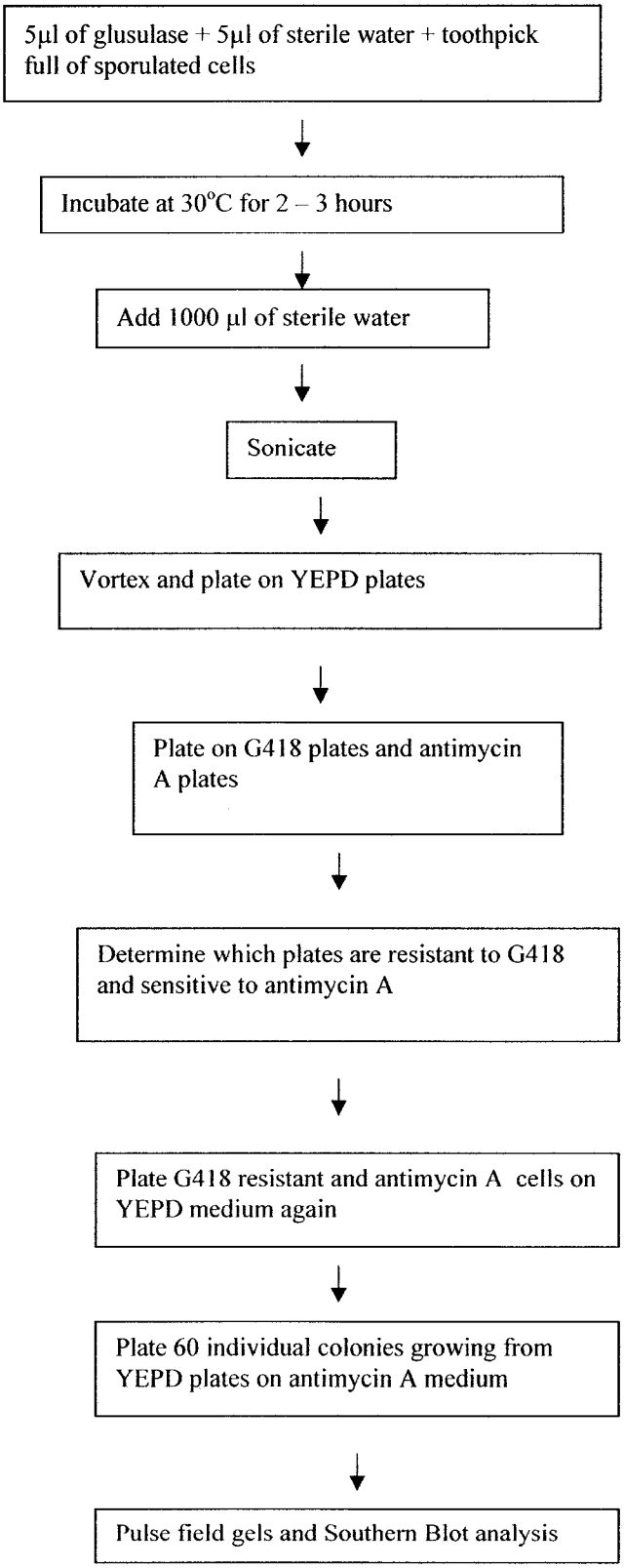

FIGURE 8: Flow chart describing the steps involved in the screening protocol after sporulation of diploids.

Preliminary Amplification Results for *MRE11* KO and *SAE2* KO Strains

The screen was tested using two knockout strains *MRE11* KO and *SAE2* KO; these strains have deletion in their *MRE11* and *SAE2* genes respectively. Only a preliminary analysis was performed on these strains to detect the presence of amplifications. After isolating G418 resistant/antimycin A sensitive colonies from the screening protocol, these colonies were plated on a YEPD plate. Five colonies for both strains were picked from the YEPD plates and plated on antimycin A plates. Out of the five plates, four plates showed growth on antimycin A plates. One colony from each antimycin A plate was then subjected to pulse field gel analysis and Southern blot analysis to determine if these strains increased amplification rates. Out of the four colonies subjected to pulse field gel electrophoresis, three *MRE11* KO/*ADH1Δ* samples show linear amplifications and two *SAE2* KO/*ADH1Δ* samples show linear amplification (FIG 9). These results were quite encouraging; since two or three out of four randomly selected colonies showed amplification therefore these strains appear to have high rates of amplification. The preliminary results also showed that copper screen, which is normally done in the *ADH4::CUP1* amplification detection system is not required here, since the amplifications are frequent enough that they can be identified without the copper screen.

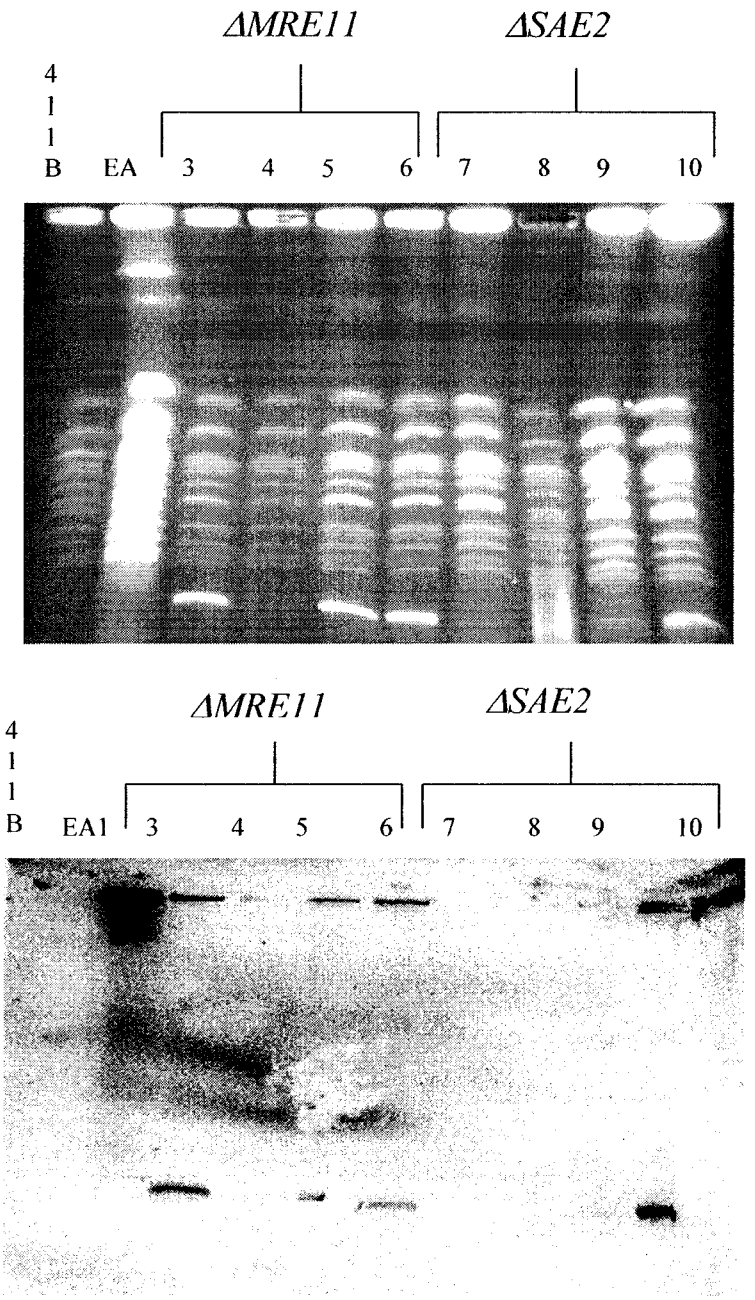

FIGURE 9: Southern Blot Analysis of Chromosomal DNA from *MRE11* KO/*ADH1Δ* and *SAE2* KO/*ADH1Δ* Strains.

A) Chromosomal DNA isolated from *MRE11* KO /*ADH1Δ* and *SAE2* KO/*ADH1Δ*strains. Lane 1: Wild type strain without amplifications therefore there is no hybridization seen in this lane. Lane 2: Spontaneous circular amplification, therefore the probe hybridizes near the top of the gel where circular molecules are normally seen. Lanes 3-6: *MRE11* KO /*ADH1Δ* strains show three linear amplifications. Lanes 7-10 *SAE2* KO/*ADH1Δ* strains show 2 linear amplifications. All the linear amplifications are seen as dark bands at the bottom of the gel.

B) Southern Blot analysis of the gel probed with *ADH4* and *CUP1*DNA sequences.

Rate of Amplification of *MRE11*KO/ *ADH1Δ* and *SAE2* KO/ *ADH1Δ*:

Antimycin A resistant colonies of the *MRE11KO/ ADH1Δ* strain were analyzed by pulse field gel and Southern blot analysis to detect amplifications. Chromosomal DNA from these cells was isolated and separated on pulse field gel. The gel was blotted and probed with the *ADH4* probe (FIG 10). A single linear amplification was detected in the nine *MRE11*KO/ *ADH1Δ* samples that were analyzed. The amplification rate of the *MRE11* knockout strain is estimated to be 4 +/- 8 X 10^{-9} (See Table 3). Normally, only one out of a hundred spontaneous antimycin A resistant mutations are due to amplification, but one out of nine antimycin A resistant mutants showed amplification in the *MRE11* knock out strain. This suggests that although the rate of amplification is low amplifications occur frequently compared to other types of mutations in the *MRE11* knock out strain. The size of the colonies that we could plate on antimycin A plate were very small (~10^6 cells/colony). If larger colonies were plated we would have detected more amplifications. No amplifications were detected in the *SAE2* KO/ *ADH1Δ* strain (FIG 11). Even though there were many more antimycin A resistant mutants in SAE2 KO/ *ADH1Δ* strain than *MRE11*KO/ *ADH1Δ* strain. Only 10^6 cells per colony were plated while screening SAE2 KO/ *ADH1Δ* strain. Thus the small sample size could be the reason why no amplifications were detected in this strain.

Table 3: Amplification rates of knockout strains and the parent strain

	Name of the Strain	Number of cells per colony	Medium number of antimycin A mutants / plate	Number of amplifications /Number of antimycin A resistant mutants	Number of independent amplifications /total number of colonies tested	Rate of amplification by P_0 method
1	YKO/ *ADH1Δ*	$1.3x10^{7}$	18 (0-123)*	13/41	9/60	$1.3+/-0.8x10^{-8}$
2	*MRE11*KO/ *ADH1Δ*	$4x10^{6}$	2 (0 – 65)*	1/5	1/5	$4+/- 8x10^{-9}$
3	*SAE2* KO/ *ADH1Δ*	$5x10^{6}$	5.5(0 – 53)*	0/24	0/24	$< 3 +/-6.7x10^{-9}$***
4	*RAD50* KO/ *ADH1Δ*	$1.2x10^{7}$	16.5 (0 – 189)*	2/38	2/38	$4+/-6.0x10^{-9}$
5	*XRS2* KO/ *ADH1Δ*	$2.8x10^{7}$	19(1 – 140)*	11/49	11/27 **	$1.1+/-0.53 x10^{-8}$***
6	*ADH1* KO	$3.7x10^{7}$	20 (1 – 112)*	0/29	0/29	$< 0.9+/-0.2x10^{-10}$

* Range of the number of Antimycin A mutants in each strain.
** Only 27 out of the 49 antimycin A resistant mutants grew well enough for chromosomal DNA isolation.
*** Note: There were no amplifications detected in these strains. The amplification rates were recorded in the table by calculating the amplification rate of a single amplification and then indicating that the amplifications in these strains were less than the calculated rate of amplification.

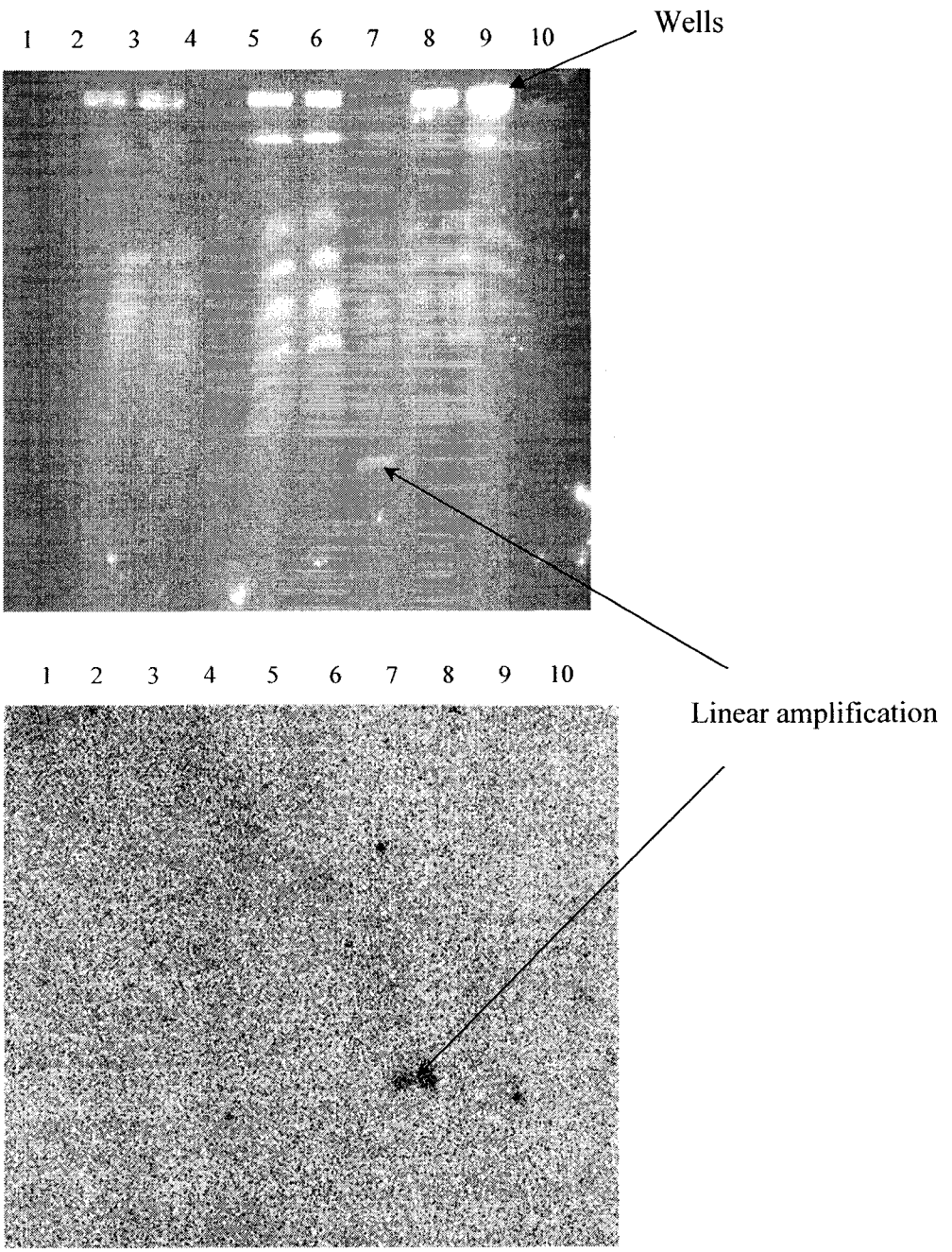

FIGURE 10: Southern Blot Analysis of Chromosomal DNA from *MRE11*KO/ *ADH1Δ*.

A) Chromosomal DNA separated on pulse field gel. The locations of the linear amplification are indicated in the figure.
B) Southern blot analysis of the above gel probed with *ADH4* and *CUP1* DNA sequences.

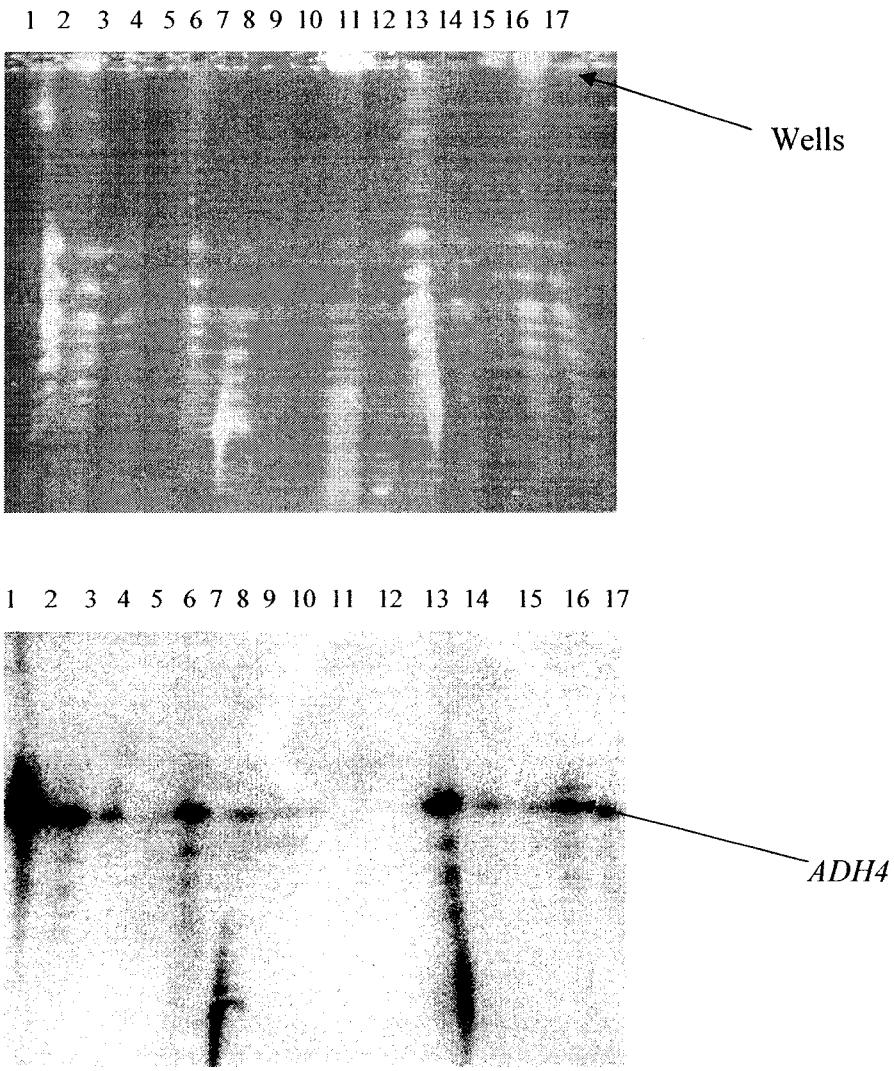

FIGURE 11: Southern Blot Analysis of Chromosomal DNA from *SAE2* KO/ *ADH1Δ*.

A) Chromosomal DNA separated on pulse field gel. Lanes 1-16 contain chromosomal DNA from *SAE2* KO/ *ADH1Δ*. Lane17 represents chromosomal DNA from the normal strain without amplification. The band the runs across the center of the gels in almost all the lanes represents the normal location of ADH4 gene on chromosome 7.
B) Southern Blot analysis of the above gel probed with *ADH4* and *CUP1* DNA sequences.

Rate of Amplification of *RAD50* KO/ *ADH1Δ* and *ΔXRS2* KO/*ADH1Δ*:

The *RAD50* KO/ *ADH1Δ* strain showed two linear amplifications, therefore the estimated amplification rate in this strain was rate was 4 +/- 6 X 10^{-9} amplifications/cells/generation (see Table 3). In the Southern blot analysis of *RAD50* KO/ *ADH1Δ* two linear amplifications can be seen at the bottom of the gel (FIG12). Above the linear amplifications, hybridization to the *CUP1* gene located at its normal location on chromosome VIII can be seen. The probe used contains DNA from *CUP1* and *ADH4* gene (FIG 5). The *CUP1* band is not visible in the *MRE11*KO/ *ADH1Δ* and SAE2 KO/ *ADH1Δ*. All the strains are crossed with 411B, our standard amplification detection strain, this strain has the *CUP1* gene deleted from its normal location. All the knockout strains have *CUP1* gene at its normal location on chromosome VIII. Therefore half of the spores formed by crossing the knockout strains with 411B have *CUP1* at its normal location and half the spores do not. The *ADH4* gene located on chromosome VII does not show hybridization in this figure. Chromosome VII being fairly large gets degraded faster than the smaller chromosomes and thus does not hybridize to the probe.

The *XRS2*KO/*ADH1Δ* strain showed the most changes in this study including amplifications and other genomic changes that could be observed even on the ethidium bromide stained gel (FIG 13). The major genomic rearrangements in *XRS2*KO/*ADH1Δ* have not been seen in any other strains that have been studied earlier in the lab e.g. *Δrad27* mutant. There were 11 circular amplifications detected by Southern blot analysis on this strain. The amplification rate was calculated to be 1.1 X 10^{-8} amplifications/cells/generation. Along with the rate being high another interesting observation was that in every lane where a circular amplification was present there was

no band present at the normal location of *ADH4* on chromosome VII. This indicates that when circular amplifications are formed in this haploid strain the one copy of chromosome VII appears to get circularized. Thus chromosome VII is not at its normal location.

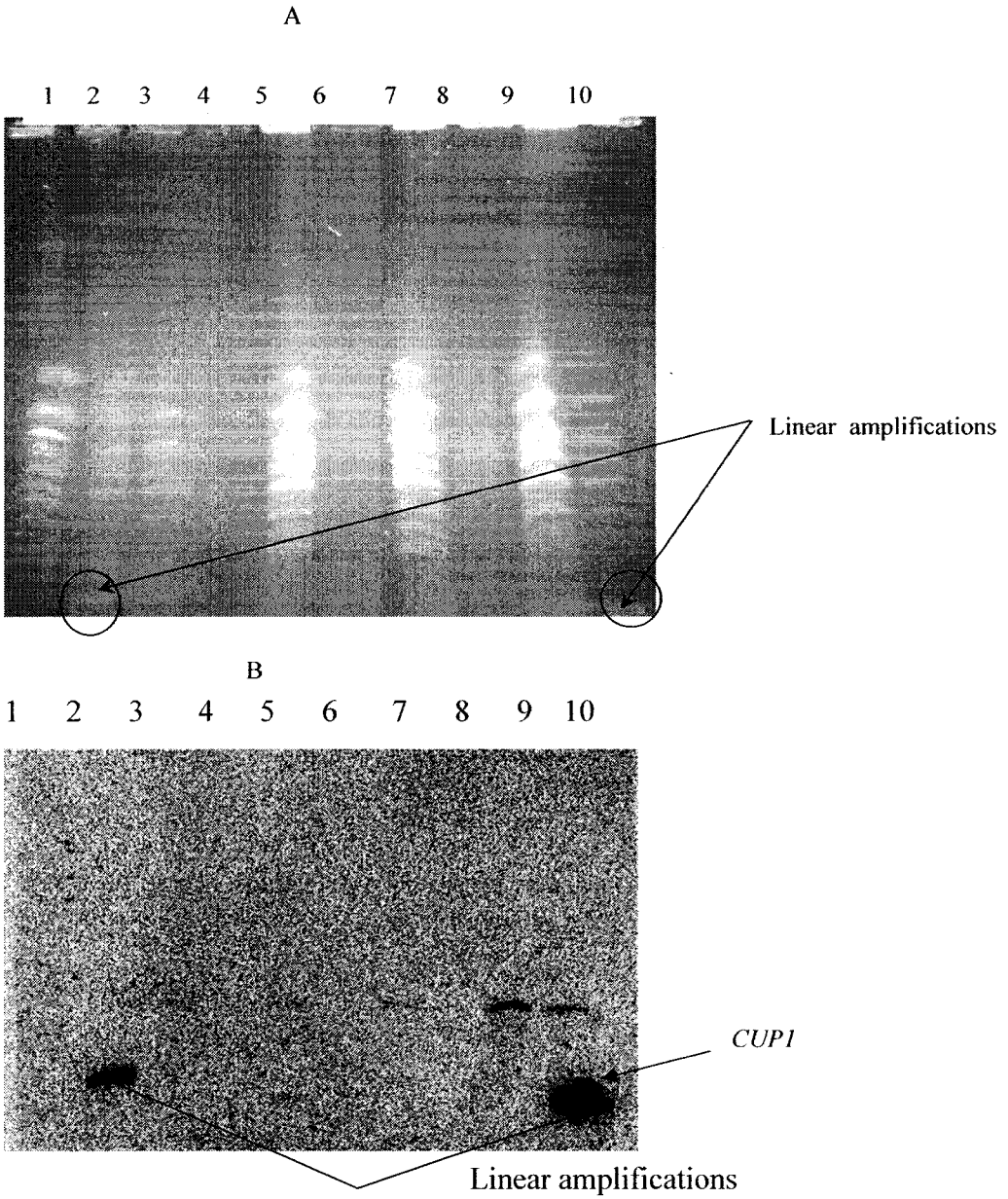

FIGURE 12: Southern Blot Analysis of Chromosomal DNA Isolated From *RAD50* KO/ *ADH1Δ*.
A) Chromosomal DNA separated on pulse field gel. Lane 1 contains chromosomal DNA from wild type strain. Lanes 2-10 contain chromosomal DNA from *RAD50* KO/ *ADH1Δ*.
B) Southern Blot analysis of the above gel probed with *ADH4* and *CUP1* DNA sequences.

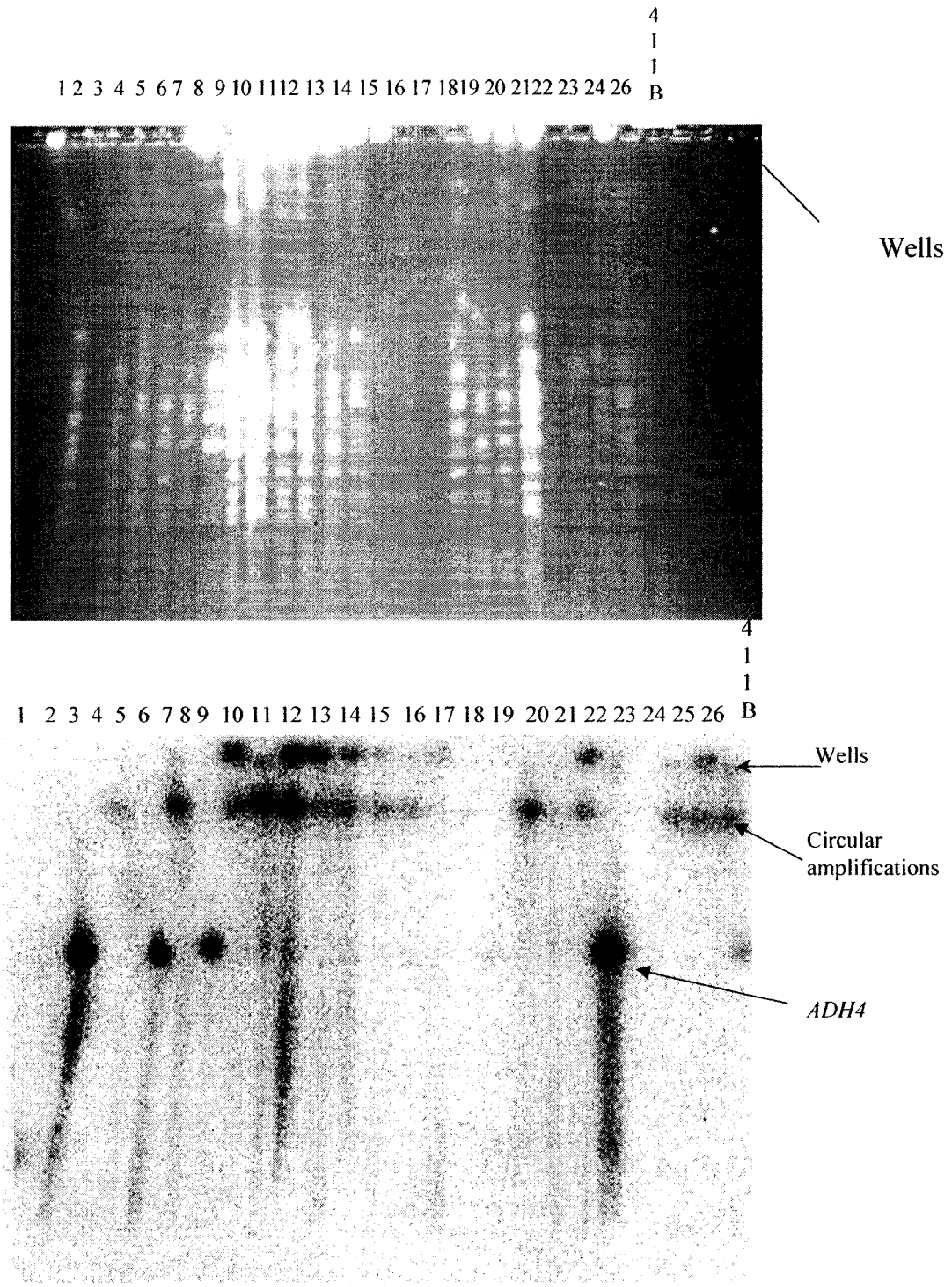

Fig 13: Southern blot analysis of chromosomal DNA isolated from *ΔXRS2* KO/ *ADH1Δ*.
A) Chromosomal DNA separated on pulse field gel. Lane 1 contains chromosomal DNA from wild type strain. Lanes 2-10 contain chromosomal DNA from *ΔXRS2* KO/ *ADH1Δ* strain.
B) Southern blot analysis of the above gel probed with *ADH4* and *CUP1* DNA sequences.

Rate of Amplification in the YKO/*ADH1Δ* Parent Strain:

All the knockout strains were made from a parent strain 4742. This strain is referred to as YKO strain throughout this paper. YKO strain was used as a control to determine amplification rate in the other knockout strains since it is a wild type strain. The amplification rate in the YKO strain was expected to be very low. The amplification rate of a wild type strain (411B) without any amplifications was determined to be $1+/-1x10^{-10}$ (Peterson et al.). The YKO/*ADH1Δ* strain was constructed similar to the other knockout strains. Out of 60 antimycin A plates, 41 plates showed growth. One or two colonies were picked from these plates depending on how many colonies were present. Chromosomal DNA was isolated from the YKO strain and it was then separated on pulse field gels. The pulse field gels were then analyzed on Southern blots that were probed with *ADH4* DNA sequence probe. Intense hybridization of the *ADH4* probe towards the bottom of the gel indicates linear amplifications in these strains. Nine linear amplifications were found in these samples (FIG 14). The estimated amplification rate of the YKO parent strain is very high ~1.3 +/- 0.8 X 10^{-8} (see Table 3)when compared to the previously estimated spontaneous amplification rate in the wild type strain(411B) in our lab. Two reasonable explanations can be given for the high rate of amplification in the YKO parent strain. 1.YKO strain itself has a high amplification rate in the background. 2. Crossing the YKO strain to 411B(amplification detection strain) resulted in a high amplification rate. To check the background amplification rate of the knockout strains we decided to screen the *ADH4* knockout strain. This particular strain was chosen

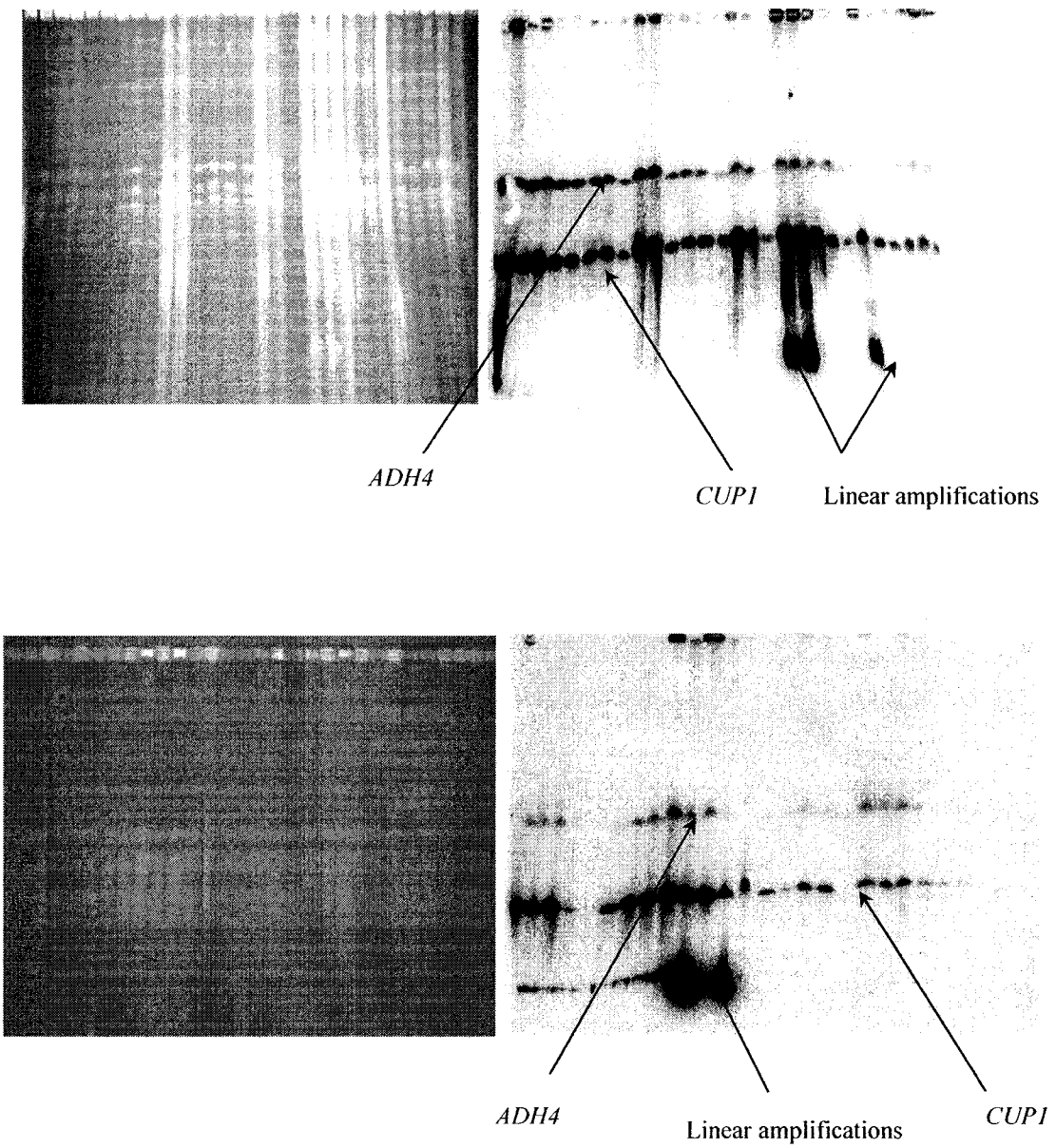

FIGURE 14: Southern blot analysis of chromosomal DNA isolated from YKO/*ADH1Δ*.
A) Chromosomal DNA separated on pulse field gel.
B) Southern Blot analysis of the above gel probed with *ADH4* and *CUP1* DNA sequences.

since this strain already had an *ADH1* deletion in it and therefore could be used in our *ADH4*::*CUP1* amplification detection system.

Amplification Rate of *ADH1* KO Strain:

The amplification rate in this strain was done similar to the other KO strains in that antimycin A mutants were isolated and subjected to pulse field gel and Southern blot analysis. However this strain was not mated with 411B like the other strains, since it already had a deletion in *ADH1* gene. There were no amplifications detected in this strain(FIG 14). This indicates that the KO strain genetic background does not result in a high amplification rate. Therefore the cross between the knockout strain and the standard amplification detection strain(411B) must be causing the high rates of amplification in the YKO/*ADH1Δ* strain.

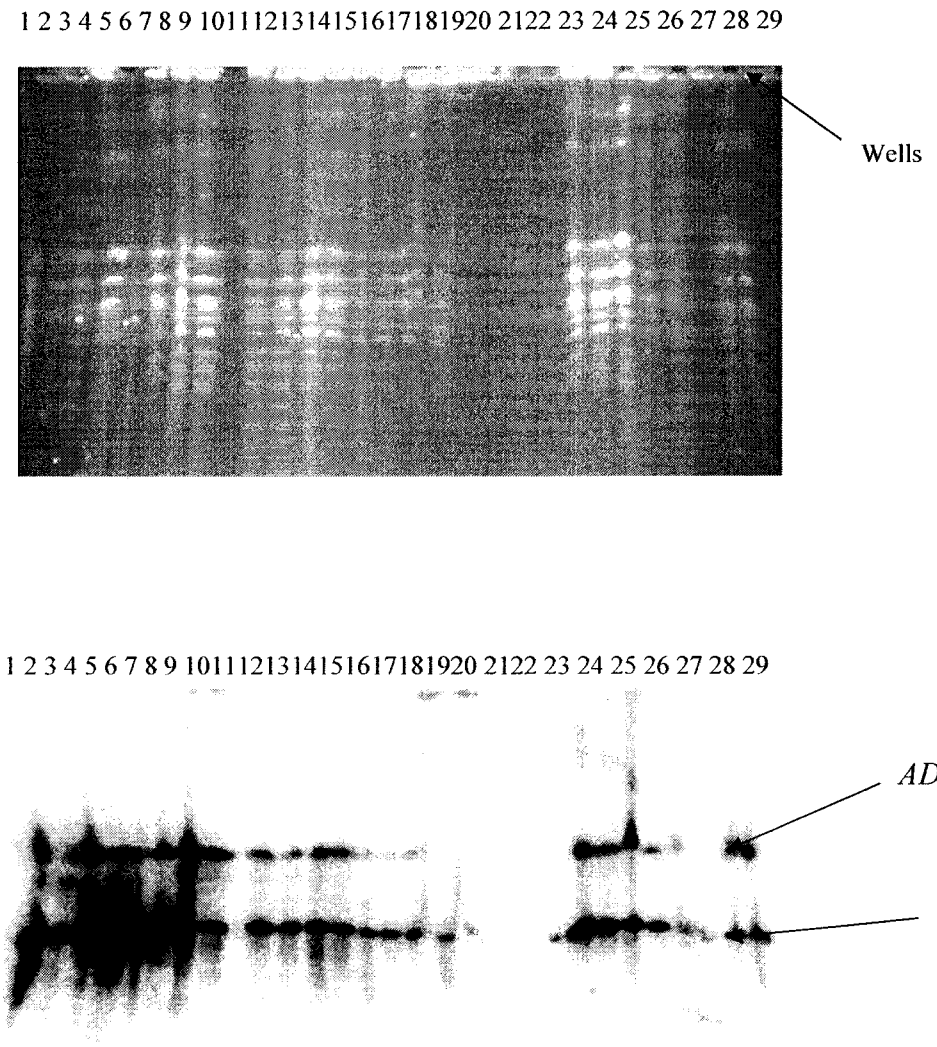

FIGURE 15: Southern Blot Analysis of Chromosomal DNA from *ΔADH1* Strain.

A) Chromosomal DNA from *ΔADH1* strain separated on pulse field gel.
B) Chromosomal DNA isolated from *ΔADH1* strain was analyzed by Southern blotting probed with *ADH4* and *CUP1* sequences. The normal locations of *ADH4* and *CUP1* genes are indicated in the figure.

DISCUSSION

Development of the Protocol for Screening the Knockout Strains

In this study a screening protocol was developed for identifying the yeast knock out strains that increase amplification rates in yeast. Identifying the knockout strains with increased amplification rates will provide us an understanding of the contribution of specific genes in controlling amplification rates in yeast. Once the genes involved in increasing amplification rates are known, we will be able to predict the effect of specific mutations in gene amplification. All the amplifications identified in our lab so far suggest that DNA double strand breaks, recombination and replication are all important in gene amplification. These amplifications fit very well with the intrachromosomal recombination model of gene amplification. The first step in this model is a double strand break near an inverted repeat followed by recombination between the inverted repeats and lastly replication of DNA hairpin. Based on this model we developed a list of 60 genes that are predicted to be responsible for increasing amplification rates in yeast because of their function in the yeast genome. The list of these genes can be found in appendix 1. The genes include DNA repair genes, genes involved in recombination, replication, formation of double strand breaks, and telomere replication genes, telomere regulator genes. Since 60 genes can be predicted to be responsible in increasing amplifications rates just based on their function, it is important to develop an efficient way to screen all the knockout strains carrying mutations that cause increased amplification rates.

As an initial test we have chosen to screen four of the knockout strains (*MRE11*KO, *SAE2*KO, *RAD50* KO, and *XRS2* KO) from the list. The first step in the screen involved

crossing the knockout strain to our amplification detection strain in order to create an *ADH1* deletion background in the knockout strains. This step was pretty straightforward; different markers present in the knockout strains and the standard *ADH1* deletion strain were used in this step for making diploid strains. The markers include the *KanMX* gene (confers resistance to G418 antibiotic) that was inserted in place of the deleted gene in all the knockout strains and the mutation in *HIS3* gene present in all the knockout strains. To make diploid of the knockout strain and the standard strains, the cells were first mated on the YEPD medium and then transferred to G418/*His*$^-$ medium. It was found that the cells have to be grown on G418/*His*$^-$ for at least 4 days for proper growth of the diploid cells. While mating knockout strains and the standard *ADH1* deletion strain replica plating the strains in a crisscross fashion causes less contamination than if the knockout strains were replica plated onto a lawn of the standard strain. Crisscross mating is possible if we are working with one or two strains. However if more than two strains are to be screened at a time, then the standard *ADH1* deletion strain should be replica plated on a plate using a replicator and a 96 well plate. The knock out strains should then be replica plated on top of the standard strain.

In the second step of the screen the diploid strains were allowed to sporulate in order to obtain haploid spores. The four types of spores that would be formed were:

a) G418 resistant and antimycin A sensitive spores

b) G418 resistant and antimycin A resistant spores

c) G418 sensitive and antimycin A resistant spores

d) G418 sensitive and antimycin A sensitive spores.

Out of the four types of spores formed we were looking for the G418 resistant and antimycin A sensitive spores. During sporulation of the diploid strains, it was observed that most of these strains take more than a week to sporulate. Therefore it is necessary to make thick sporulation plates so that the plates can survive long incubations. Thin plates dry out to quickly. After sporulation, the spores were broken open and selected for *G418* resistance and antimycin sensitivity. These particular spores were selected because *G418* resistance in these spores indicates that the spore did not carry the knockout gene and antimycin A sensitivity indicated that these spores had *ADH1* gene deleted. It was observed that during this selection antimycin A resistant spores grew faster than the antimycin A sensitive spores. The spores grew very slowly on G418 medium, therefore G418 resistance was checked after letting the cells grow for a week at least. The cell isolated at this point had not developed any amplification therefore they were antimycin A sensitive. If the cells had amplifications, they would be able to grow on antimycin A plates. These cells were allowed to grow on glucose rich medium to give them a chance to develop amplifications. The presence of amplifications was determined by growing the cells on antimycin A medium and then analyzing antimycin A resistant cells. Four mutant strains, *MRE11*, *SAE2, RAD50* and *XRS2* knockout strains were studied here. All these genes are involved in processing double strand breaks, which is predicted to be an important event in amplification.

Role of DNA Double Strand Break in Amplification

Double-strand breaks are often seen in chromosomal DNA during the normal cell cycle, upon exposure to ionizing radiation or as intermediates during DNA rearrangements.

These double strand breaks are repaired very efficiently either by homologous recombination or non-homologous end joining (NHEJ) (Paull et al., 1998). DNA double strand breaks (DSBs) are potentially lethal if they are not repaired accurately. In homologous recombination, a sister chromatid, homologous chromosomes or other homologous sequences are used as template for synthesis of DNA to restore the gap between the broken ends of the chromosome. Homologous recombination is thought to be an accurate repair pathway for DNA double strand breaks. In *Saccharomyces cerevisiae* homologous recombination is the preferred pathway of DSB repair there aren't many repetitive DNA sequences in the genome (Moreau et al., 2001). Nonhomologous recombination (NHEJ) is thought to be an error prone repair pathway since it is sometimes accompanied by DNA sequence deletions (Furuse et al., 1998). NHEJ between sequences that have very less sequence similarity or are completely different is potentially mutagenic. However in situations where the survival of the cells depends upon joining of nonhomologous sequences, this becomes the preferred pathway. NHEJ is also predominant in mammalian cells. According to Rattray et al. (2001), the mammalian genome consists of highly repetitive DNA sequences and therefore there is less chance that NHEJ may occur in the open reading frame of a gene in these genomes. In *Saccharomyces cerevisiae*, however the genome is mostly composed of unique sequences therefore NHEJ is not the preferred pathway of DSB repair . DNA double strand breaks are often associated with inverted repeats or large palindromic sequences that can form a hairpin or a cruciform (Lobachev et al., 2002). Gross chromosomal rearrangements like large inverted repeats are often observed in genetically unstable cells.

In *Saccharomyces cerevisiae*, the Mre11 protein is involved in double strand DNA double strand break (DSB) formation as well as DSB repairs. The first step in intrachromosomal recombination is formation of DNA double-strand breaks followed by formation of inverted repeats that creates novel joints in the DNA strand. Since *MRE11* plays an important role in DSB formation and repair it is important to understand whether mutation in *MRE11* causes increase in the rate of gene amplification.

MRE11: Core Gene of the Mre11 Complex

Mre11 protein is involved in DSB repair and DSB formation during meiosis. The Mre11 protein has two domains, the N-terminal and the C-terminal, that function independently. The N terminal domain of the Mre11 protein is responsible for DSB repair. This terminal shows similarity with the E.coli SbcD nuclease subunit, therefore probably is a nuclease (Haber, 1998). The C terminal is required during meiosis for formation of DSBs near the sites that are hotspots for recombination or chromatin modification (Furuse et al., 1998). Due to the importance of Mre11p in formation and repair of DSBs, the *MRE11* knockout strain seemed to be a potential strain that might be involved in increasing the gene amplification rate in yeast. By using our screening protocol it was found that the rate of amplification of *MRE11* KO/ *ADH1Δ* strain is 4 +/- 8 X 10^{-9} amplifications per cell. In normal *Saccharomyces cerevisiae* cells amplification is very rare ~ 10^{-10} amplifications/cell/generation. We identified one amplification among 5 independent antimycin A resistant mutants in only 10^6 cells. Considering that normally one in hundred antimycin A mutants show amplification, it can be concluded that amplifications

are frequent among antimycin A resistant mutants in the *MRE11* knockout strain. Thus deletion *MRE11* appears to affect the amplification rate in yeast.

Mre11p, however does not work by itself, it is a core unit of a complex called Mre11 complex. Mre11 complex consists of the Mre11 protein and two other proteins, encoded by the genes *RAD50* and *XRS2*. All the three genes of the complex were first identified in *Saccharomyces cerevisiae* mutants that were hypersensitive to DNA damage. It was also observed that the yeast strains that have mutations in any of the three genes in the complex show similar phenotypes like hypersensitivity to DNA damage, cells lacking meiotic division, unusual telomere shortening etc. These results suggested that Mre11p, Rad50p and Xrs2p might be working as a complex. (D'amours et al., 2002). Like Mre11p, Rad50p is a homolog of E.coli SbcC nuclease subunit, suggesting that Rad50p also has a nuclease activity (Lobachev et al., 2002). The *SbcCD* complex is required for hairpin loops formed at the DNA double strand breaks. *MRE11* and *RAD50* are similar to the bacterial *SbcD* and *SbcC* respectively; they also have a comparable role in maintaining inverted repeat stability. Like the SbcCD complex Mre11p and Rad50p also have endonuclease and exonuclease activities that can process hairpin structures formed by inverted repeats. It has been observed that not just Mre11p but Rad50p and Xrs2p are also involved in DSB formation and DSB repair by non-homologous end joining and homologous recombination. Since our results show that the *MRE11* knockout strain increases the rate of amplification, we decided to screen *RAD50* and *XRS2* knockout strains for increased amplification rates.

***RAD50*: Its Role in Double Strand Break Formation and Repair.**

Rad50p is a ~ 150kda protein consisting of amino and caboxy termini that have nucleotide binding motifs. The carboxy and amino termini are separated by a heptad repeat regions that form two coiled structures with a globular domain in the middle. The two termini in Rad50p contain ATP binding motifs that are crucial for Rad50p function. In the presence of Mre11p the two ATP binding motifs come very close together when ATP is bound to these motifs. Based on the structure of Rad50p, it has been established that Rad50p binds to DNA and holds it together. Since Rad50p binds to the two strands of DNA, several ways have been described in which Rad50p might perform DSB repair. 1. Holding the two DNA strands together facilitates the search for homologous sequence during recombination, which is essential for DSB repair. 2. The two strands brought together by Rad50p stimulate DNA ligase activity. 3. The broken DNA strand and its sister chromatid are joined together by Rad50p to avoid total separation of the broken DNA section from the rest of the chromosome. Also because during homologous recombination sister chromatids are used as templates more often than the homologous chromosomes. Yeast cells with a deletion in the *RAD50* gene show a high rate of interchromosomal recombination. By using our screen we found that *RAD50* knockout strains have an increased amplification rate of $4 +/- 6.0 \times 10^{-9}$ amplifications/cell/generation. The rate of amplification in *RAD50* knockout strain determined in this study is not statistically different than the rate determined in an earlier study done by Whitsken S. in our lab ($1.23 +/- 0.9 \times 10^{-9}$amplifications/cell/generation). In her study she had used a *RAD50* deletion strain and detected circular amplifications

and extrachromosomal amplifications along with linear amplifications. However we could only detect linear amplifications here. Whitsken S. (1998) isolated a high amplification rate strain B20-28 that is sensitive to MMS. It was observed that *RAD50* could complement the MMS sensitivity in this strain. In another study, B20-28 strain showed an amplification rate of $9+/-0.3x10^{-8}$ amplifications/cell/generation (Kordich 1996). This rate is higher than the amplification rate determined in this study and the amplification rate done by Whitsken S. These observations suggest that point mutations in *RAD50* might increase amplification rates more than deletion of *RAD50*. Alternatively, B20-28 could contain additional mutations that affect amplification.

XRS2: Could be the Major Contributor in Development of Amplification

Another member of the Mre11 complex, Xrs2p is a homolog of vertebrate Nbs1p. Mutations in *NBS1* gene cause Nijmegen breakage syndrome (NBS) which is a genetic disorder that causes genomic instability, defects in triggering cell cycle delays and hypersensitivity to DNA double strand breaks (D'Armours et al., 2002). Since *XRS2* is homologous to *NBS1*, it also functionally analogous to *NBS1* gene. Xrs2p is involved in formation and repair of double strand breaks, meiotic recombination, spore formation etc. Mutations in *XRS2* and *RAD50* genes show similar mitotic and meiotic phenotypes suggesting that *XRS2* is also involved in homologous recombination like *RAD50*. Patients with NBS show many phenotypic changes including; developmental defects like microcephaly, compromised immune system, and a high rate of cancer. The cells carrying *NBS1* mutations show genomic instability, hypersensitivity to double strand break inducing agents, delays in cell cycle checkpoint in case of damaged DNA. In our

study we found that strains with deletion in *XRS2* do show many genomic rearrangements that are so prominent that they can be observed even on an ethidium bromide stained gel before the southern blot analysis. These observations regarding *XRS2* and our results from the *XRS2* knockout strain amplification rate make it very important to pursue studying the effect of *XRS2* mutations on amplification in further detail.

All the knockout strains from the *MRE11* complex show at least a slight increase in their amplification rates suggesting that these genes are important in maintaining genomic stability.

*SAE2*KO/*ADH1Δ*: Does This Strain Increase Amplification?

Like Mre11p, Sae2p is also involved in causing DNA double-strand breaks. The separation of function mutations of *MRE11* and *RAD50* result in phenotypes that are similar to *sae2* null mutants (Rattray et al., 2001). However Mre11 complex plays variety of roles in DNA metabolic processes, telomere maintenance, nuclease activity etc. Thus it has been proposed that Mre11 complex must be involved in regulating Sae2p activities. The genes in Mre11 complex and *SAE2* are also found to be essential in recombinational repair of DNA double strand breaks near inverted repeats that form hairpin like structures. (Lobachev et al., 2002). In our study we observed that *SAE2*KO/*ADH1Δ* strain does not increase amplification rates. However this result was obtained from a small sample size of 10^6 cells per plate. Therefore it is necessary to repeat this experiment using larger colonies containing more cells.

Comparison between the amplification rates of the knockout strains and the YKO parent strain

The amplification rates of the knockout strains, when compared to the amplification rate of the YKO parent strain, seemed to show no increase at all. In fact the parent strain had the highest rate of amplification. The high rate of amplification in the YKO parent strain was quite surprising because we were expecting almost no amplifications in this strain. The two possible reasons for such a high rate of amplification in the parent strain are that, this strain had a high amplification rate in the background already or it developed amplifications when it was crossed with the amplification detection strain 411B. Therefore we used the *ADH1* knockout strain to determine the amplification rates in the knockout strain background. The *ADH1* knockout strain has a deletion in the *ADH1* gene therefore doesn't need to be crossed with the amplification detection strain (411B) before using it in the *ADH4::CUP1* system. We detected no amplifications in the *ADH1* KO strain, and the rate of amplification in the *ADH1* KO strain was similar to the amplification rate of our standard amplification detection strain 411B that was done earlier in the lab.

Thus it was established the background of the knockout strains does not increase the rate of amplification. Therefore we came to the conclusion that crossing the amplification detection strain (411B) with the knockout strains must be increasing the amplification rates. In order to solve this problem we could use the standard *ADH1* deletion strain, instead of 411B for making diploids in the first step of the screen. This way the differences in the genetic background of the two strains could be eliminated. However,

the *ADH1* knockout strain cannot be used without making some changes. One change is that the *KanMX* gene, which is present in place of the *ADH1* gene, should be replaced with some other gene. The presence of *KanMX* gene would make it almost impossible to select diploids after mating. The *URA3* gene seems to be a good candidate for replacing *KanMX* gene in *ADH1* knockout strain. The *ADH1* knockout strains have a mutation in *URA3* gene so this could be used as a selectable marker. In addition if a normal copy of *URA3* were inserted in this strain it would also decrease the number of antimycin A mutants formed. The product of the *URA3* gene is important in the biosynthesis of thymine. Thus when the *URA3* gene is mutated, it affects production of thymine in the cells. Resulting in point mutations that lead to antimycin A resistance in the cell. Also, if we delete *CUP1* gene from its normal location and place it next to *ADH4* in the *ADH1* knockout strain, then this strain can be used in the amplification detection system. After making these changes we would be able to compare the amplification rates of the knockout out strains with the parent strain. In the future, it would all be helpful to determine *CUP1* gene amplification along with *ADH4* gene amplification in the knockout strains.

Modifications needed in the protocol in order to screen many mutants at a time

Once the problem of creating an *ADH1* deletion background in the knockout strains is overcome, then it would be possible to obtain an accurate rate of amplification for these strains. Along with the knockout strains of *MRE11* complex, all the genes present in yeast strains that alter amplification rates also need to be identified. The screen as it is currently designed can successfully screen two knockout strains at a time. However

screening more strains (at least 48 strains on a plate) at a time could speed up the process of identifying all the genes responsible for increased amplification rates in yeast. This can be achieved by growing and mating the strains using 96 well plates. One problem observed by using 96 well plates is that after sporulation, it becomes very difficult to isolate G418 resistant and antimycin A sensitive colonies. This is because the cells are stamped on a plate using a replicator device. This could be avoided by streaking the strains individually on a plate but this causes long delays in the screening process. Another way to speed up the screening process is by using colony hybridization protocol that is based on amount of DNA bound to the filters. If this procedure is successful in identifying high amplification rate strains than as many as 48 strains can be screened at a time on one plate. Thus the next step in improving the screening protocol would be to overcome the problem of isolating many G418 resistant antimycin A sensitive colonies on a single plate and using colony hybridization procedure for identifying strains with increased amplification rates.

Conclusions

From this study we have preliminary data indicating that the genes in the *MRE11* complex may affect amplification rates. Among the genes, in the *MRE11* complex, we found that *XRS2* probably has the largest effect. It was very interesting to see that some of the genomic rearrangements taking place in this strain are visible even before Southern blot analysis was performed. No amplifications could be detected in *SAE2* KO/*ADH1Δ* strain, here, but amplifications might be more detectable if a large sample size was used.

The screen described in this protocol can be used to identify the strains that increase amplification. However the background problem in the cross of amplification detection strain and the knockout strain must be solved in order to accurately estimate amplification rates of the knockout strains. The similarity between the amplification rate found in this study and the earlier study on *ΔRAD50* strain is very encouraging. Lastly, the screen must be scaled up in order to be able to screen many mutants efficiently.

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Appendix A

#	Gene	ORF name	Function	Mat alpha	Mat a
1	RAD52	YML032C	Recombination protein	103-C-4	3-D-6
2	MLH1/PMS2	YMR167W	Mismatch repair in mitosis and meiosis	104-A-12	4-C-7
3	SGS1	YMR190C	Maintains genome stability	104-C-8	4-E-2
4	RAD14	YMR201C	Involved in nucleotide excision repair	104-D-4	4-E-12
5	RAD58/MRE11/XRS4/NGS1	YMR224C	double strand DNA break formation as well as processing	104-E-6	4-G-2
6	YKU70	YMR284W	DNA binding protein	104-H-11	5-C-3
7	RAD17	YOR368W	DNA damage checkpoint control	107-C-4	7-F-7
8	SAE1/CBC2/MUD13/CBP20	YPL178W	pre-mRNA splicing factor	108-H-4	9-D-2
9	MLH3	YPL164C	Mismatch repair	109-A-2	9-E-2
10	RAD55	YDR076W	X-ray damage repair, full sporulation and viable spores.	110-E-3	11-A-7
11	MSH6/PMS3	YDR097C	Mismatch repair in mitosis and meiosis	110-F-5	11-B-11
12	XRS2	YDR369C	MRE11/RAD50/XRS2	111-C-5	11-H-4
13	RAD30/DBH1	YDR419W	DNA replication proof reading	111-F-3	12-C-5
14	RAD23	YEL037C	Ubiquitin like protein. Damaged DNA binding	112-A-7	12-F-9
15	RRM3/RTT104	YHR031C	Involved in rDNA replication and Ty1 transposition	114-A-9	15-A-1
16	RAD27/ERC11/RTH1	YKL113C	Flap endonuclease, required for Okazaki fragment processing	117-C-4	18-D-5
17	RAD9	YDR217C	Regulation of cell cycle	121-B-5	23-G-1

#	Gene	ORF name	Function	Mat alpha	Mat a
18	SIR4	YDR227W	SIR4 binding to Rap1 initiates sequential association of Sir and other proteins thus spreading heterochromatin protein along the chromosomes	121-G-7	23-G-10
19	EST2	YLR318W	Telomerase catalytic subunit.	122-D-11	23-B-7
20	RAD1	YPL022W	UV endonuclease	123-G-12	25-C-10
21	MSH3	YCR092C	damaged DNA binding	124-G-7	26-C-4
22	RAD6/ UBC2	YGL058W	Involved in DNA repair and sporulation, Ubiquitin-conjugating enzyme	126-F-12	28-B-10
23	MSH2/PMS5	YOL090W	Repair single-base and insertion-deletion mispairs	131-B-1	32-H-9
24	RAD51	YER095W	Processing ds breaks, synaptonemal complex formation, meiotic gene conversion and reciprocal recombination	131-F-3	28-B-10
25	RAD5/ REV2,SNM2	YLR032W	DNA helicase, involved in DNA repair	131-H-6	32-H-9
26	MLH2	YLR035C	Mismatch DNA repair	131-H-8	33-D-11
27	RAD50	YNL250W	Involved in joining ds breaks and recombination	132-B-12	33-G-1
28	RAD10	YML095C	Degrades ssDNA (endonuclease)	132-D-6	33-G-3
29	YKU80	YMR106C	DNA end binding complex	132-G-12	34-A-8
30	RADH/ RADH1 /HPR5, SRS2	YJL092W	Required for proper timing of commitment to meiotic recombination and transition from meiosis to mitosis	133-H-8	34-B-12

#	Gene	ORF name	Function	Mat alpha	Mat a
31	RAD61	YDR014W	DNA repair, exact Function unknown	137-A-11	34-F-3
32	RAD57	YDR004W	X-ray damage repair, meiotic recombination, wt levels of sporulation and viable spores	137-A-4	35-G-2
33	RAD28	YDR030C	Transcription coupled DNA repair	137-B-12	38-H-4
34	RAD18	YCR066W	DNA repair	137-G-9	38-G-8
35	RAD26	YJR035W	Transcription coupled DNA repair	138-C-12	39-A-5
36	RAD7	YJR052W	NER factor 4 complex	138-D-12	39-E-11
37	MSH5	YDL154W	DNA repair, meiotic intrachromosomal recombination	139-A-3	40-A-5
38	RAD54/RDH4/TID1	YBR073W	ds break repair,heteroduplex formation, meiotic recombination	140-E-11	40-F-2
30	PMS1	YNL082W	Mismatch repair in mitosis and meiosis	142-G-6	42-E-4
40	SIR3	YLR442C	Histone Binding, Silencing regulator of HML,HMR and telomere	145-D-6	43-G-4
42	RAD16/PSO5	YBR114W	Nucleotide excision repair, DNA damage recognition	145-H-6	43-G-4
43	RAD59	YDL059C	Involved in mitotic recombination	146-G-4	46-D-8
44	SAE2	YGL175C	DNA double strand break formation and repair	148-D-3	46-H-7
45	RAD4	YER162C	Nucleotide excision repair, DNA damage recognition and binding	148-H-7	47-G-3
46	RAD24/RS1	YER173W	Cell cycle exonuclease (checkpoint protein)	149-A-2	49-D-1

#	Gene	ORF name	Function	Mat alpha	Mat a
47	DMC1/ISC1	YER179W	Involved in meiotic recombination, repair of ds breaks	149-A-8	49-H-4
48	MSH4	YFL003C	DNA repair required for spore viability	150-A-1	49-H-11
49	RAD2	YGR258C	Incision step of NER of DNA	150-B-3	50-A-5
50	PIF1/TST1	YML061C	Repair and recombination of mt DNA, chromosomal telomere formation and elongation	170-A-5	50-G-2
51	MSH1	YHR120W	Mitochondrial DNA repair	170-D-4	50-H-1
	TEL genes				
52	PXR1	YGR280C	Telomerase Regulator/RNA binding protein	239-G-1	15-E-6
53	TLC1/TER1		Template for synthesis of g-rich strand of Telomere		
54	CDC13	YDL220C	Regulator of telomere replication, recruits telomere to telomerase, required for G2/M transition etc	247-H-5	
55	TEL2	YGR099W	Telomere binding protein	223-A-5	
56	EST1	YLR223C	Telomere elongation protein	220-D-5	
	RAD genes				
57	RAD3/REM1	YER171W	NER factor 3 complex	263-C-5	
58	RAD53/LSD1, MEC2,SPK1	YPL153C	Required for wild type spore viability, dispensable for sporulation.	211-A-7	
59	RAD56	No ORF	Cell protection against oxidative damage		
61	SSL2/LOM3/RAD25	YIL143C	DNA helicase homolog	252-C-5	

Appendix B

The P_0 method used to estimate amplifications rates in the knockout strains

The P_0 method is a statistical analysis that was developed to describe Poisson distribution of the mutants in bacterial populations. The P_0 method was developed by Luria and Delbruck (1943). This method relates to the logarithmic growth rate of mutants and the formation of mutants in a given population, and it requires the mutants and the parental population must have equal growth rates. However, the experimental populations and the parental populations do not always have equal growth rates. To eliminate the bias between the differences in the growth rates of the experimental populations and the parental populations, Lea and Coulson (1949) described a modified version of the P_0 method described earlier. We used this modified version of the P_0 method to calculate the frequency of *ADH4::CUP1* amplifications in the knockout strains. The 95% confidence intervals were also calculated to eliminate the difference in the growth rate of the cells with increased *ADH4* activity that ferment and grow fast and the parental strain that do not ferment.

P_0 is calculated as follows:

$$P_0 = e^{-m}$$

... Where, P_0 represents the fraction of cultures with no amplifications. Lea and Coulson (1949) describe that the sensitivity of the analysis is maximized when the P_0 value is at its optimum (between 0.15 and 0.8)

Example

ADH4::CUP1 amplification was detected in 9 out of a total of 60 antimycin A resistant mutants, where average number of cells per culture was 1.3×10^7. Therefore there were 51 cultures without amplifications.

$P_0 = 51/60$
Thus, $e^{-m} = 0.85$

The natural log of both the sides gives,

$$\ln(51/60) = \ln(e^{-m})$$

$$-0.1625 = -m$$

Therefore, m = 0.1625amplifictions per culture

To determine number of amplifications per cell, the above number is divided by the average number of cells per culture.

$$\frac{0.1625 \text{amplifications per culture}}{1.3\text{x}10^{7} \text{ cells per culture}} = 1.3\text{x}10^{-8} \text{ amplifications per cell}$$

To determine the 95% confidence interval levels, the standard error value was calculated as follows:

$$\sigma_m = [(e^{-m} - 1)/N]1/2$$

...where N represents the total number of cultures.

Therefore the standard error in this example is

$$\sigma_m = [(1.1764 - 1)/60]^{1/2}$$

$$\sigma_m = 0.0542$$

95% confidence interval is than calculated by dividing the standard error value by the average number of cells per culture and multiplying by 2.

Therefore 95% confidence interval in this example is :

$$\frac{0.0542}{1.3 \text{ x } 10^{7}} \text{ X } 2 = 0.8\text{x}10^{-8}$$