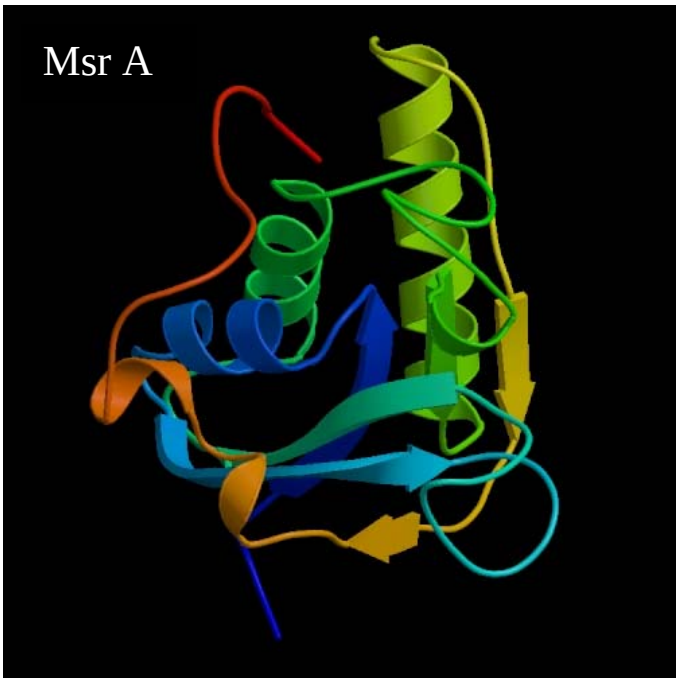
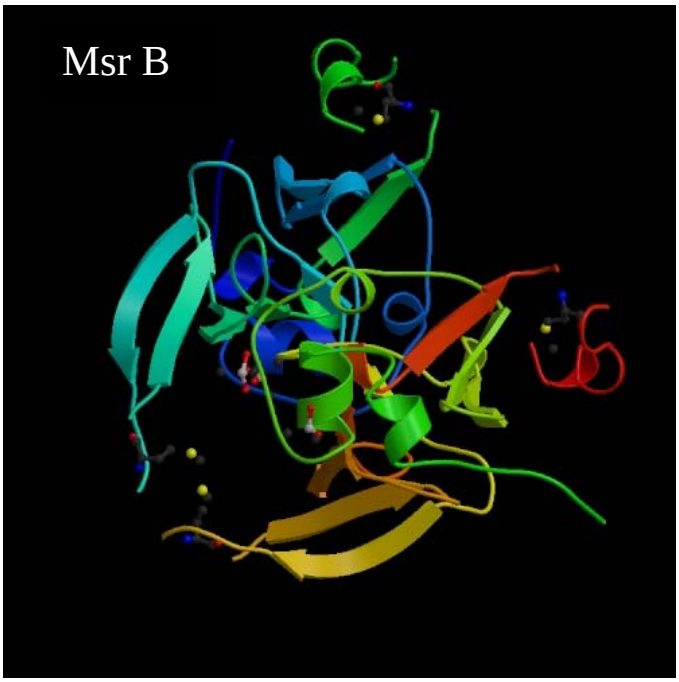
Role of Schizosaccharomyces pombe Methionine Sulfoxide Reductase (msr) Genes in Oxidative Stress Resistance

Author: Heather Elaine DeFoer

Role of *Schizosaccharomyces pombe* Methionine Sulfoxide Reductase (*msr*) Genes in Oxidative Stress Resistance





Heather DeFoer

Advisor: Dr. Clare O'Connor

Biology Department

April 29, 2005

Table of Contents

Abstract

As organisms get older, the proteins in their cells also age, and as this happens, the amino acids that make up these proteins may become chemically modified and begin to lose their integrity. One example of an age-related modification occurs when the amino acid residue methionine is oxidized by a reactive oxygen species to methionine sulfoxide. Methionine sulfoxide reductase is an enzyme that repairs this damage to the protein by catalyzing a reaction that reduces methionine sulfoxide back to methionine. The fission yeast *Schizosachharomyces pombe* was used as the experimental model to study methionine sulfoxide reductase *in vivo*, taking advantage of the variety of tools available with which to study the organism. In *S. pombe* there are two genes encoding methionine reductase activities, *msrA* and *msrB*. The first goal of this project was to construct yeast strains in which the endogenous *msrA* (Δ *msrA*) *msrB* (Δ *msrB*) genes had been inactivated. This was accomplished via homologous recombination reactions in which the *msr* genes were replaced with a selectable marker for biosynthesis of uracil (*ura4*$^{+}$). After the construction and verification of the two knockout strains, the sensitivities of the strains to reactive oxygen species were tested. Both strains showed reduced resistance to oxidative stress. Future experiments will include more detailed analyses of the abilities of the strains to survive oxidative stress. Finally, the two knockout strains of yeast will be mated with one another in order to produce a double *msr* knockout (Δ *msrA*, Δ *msrB*), in order to examine the effects of a complete lack of methionine sulfoxide reductase activity on the organism.

Chapter 1: Introduction

Proteins are formed from amino acids that are joined together via peptide bonds, leading to the formation of a long polypeptide chain that is then folded into the specific quaternary structure that allows for the appropriate functionality within the cell. However, as proteins age, they are susceptible to chemical damage, due to the fact that the amino acids themselves are inherently reactive. This damage decreases the integrity of the amino acids, and can cause changes in the structural formation of the individual side chains of the residues. Any change in the structure of an amino acid causes a change in the primary structure of the protein, and can also have a negative impact on the ability of the polypeptide to fold into the appropriate tertiary configuration necessary for functional activity in the cell. Some amino acids are more susceptible to cellular damage, and methionine is one of these residues. On the average, proteins contain approximately 2% methionine residues, which usually play important roles in protein function. Thus, when they are damaged, it is likely that there will be significant impact on the structure of the protein and its functionality (Ruan et al., 2002). Fortunately, however, some kinds of protein damage are reversible, due to the activities of protein repair mechanisms inside the cell. One example of this kind of reversible damage involves methionine residues, which are oxidized by certain reactive oxygen species (ROS), such as hydrogen peroxide, to methionine sulfoxide, which has an oxygen atom double bonded to the sulfur group in the side chain (see Figure 1). Two kinds of protective mechanisms in the cell contribute to their resistance to ROS. One subset involves enzymes that reduce the levels of the ROS in the cell, while a second subset (the one of primary interest here) involves enzymes that repair the damage to nucleic acid, lipids or proteins caused by the ROS (Grimaud et al., 2001). Protein repair enzymes in the cell, called methionine sulfoxide reductases, reduce the methionine sulfoxide back to the correct methionine residue,

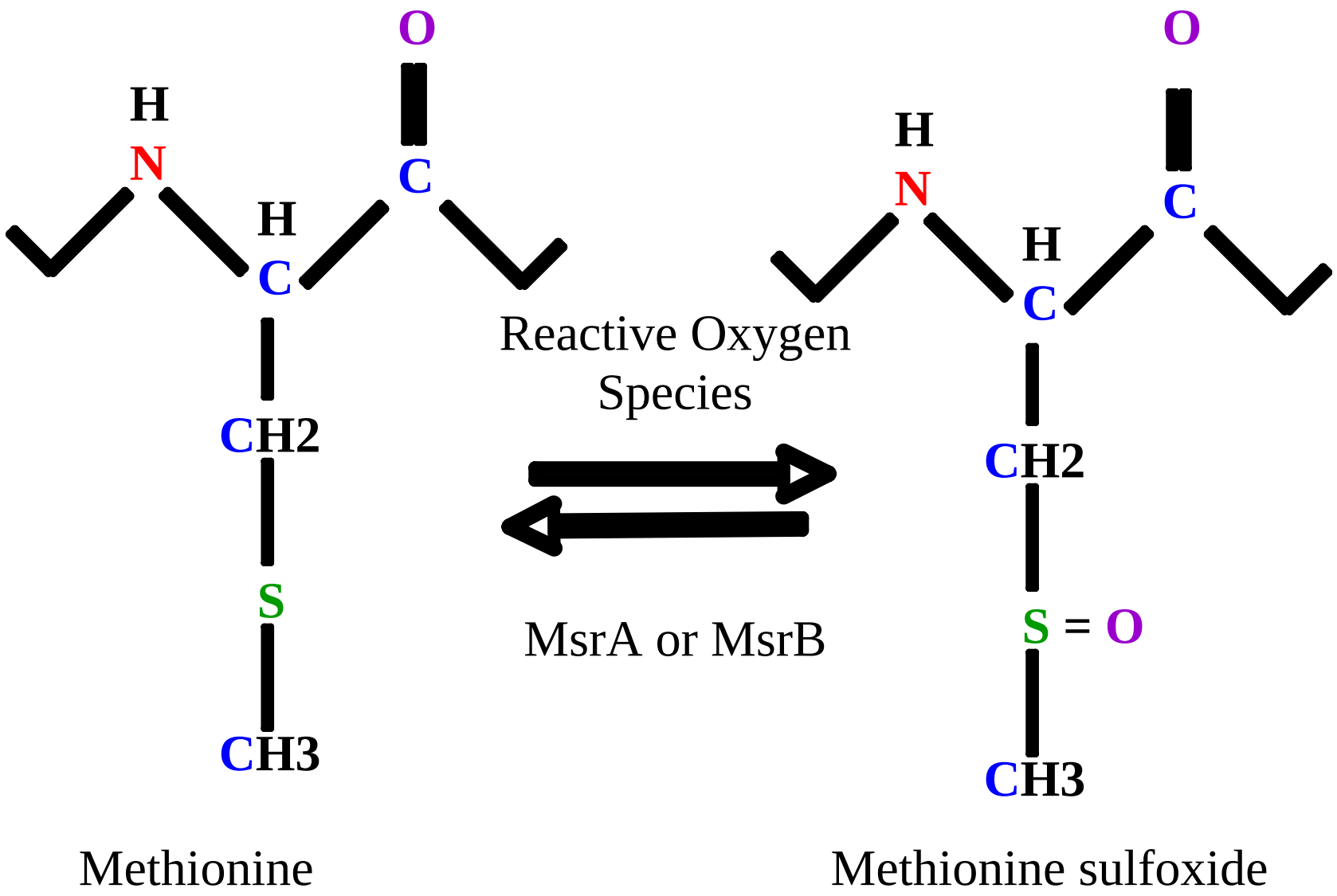


Figure 1: Reactive oxygen species oxididize the amino acid residue methionine into methionine sulfoxide through the addition of an oxygen atom to the sulfur atom in the side chain of methionine. Methionine sulfoxide reductases convert oxidized methionine back to normal methionine through a reduction reaction.

thereby reversing the damage to the protein, and ensuring that the appropriate structure and function are maintained. The Msr proteins are responsible for the reduction of not only methionine sulfoxide residues within a protein, but also for free methionine sulfoxide residues present in the cell (Grimaud et al., 2001).

Methionine residues in proteins have been found to be especially susceptible to oxidation by reactive oxygen species (ROS), and the result is one of two stereoisomers, either a methionine-S-sulfoxide (Met-S-SO) or a methionine-R-sulfoxide (Met-R-SO). These ROS are found to be the by-products of normal aerobic metabolism, and it is believed that these species are responsible for the aging of organisms. The product of the *msrA* gene, the antioxidant enzyme MsrA, has been found to reduce the Met-S-SO stereoisomer, while the product of the *msrB* gene, the antioxidant enzyme MsrB, has been found to reduce the Met-R-SO stereoisomer (Koc et al., 2004). Despite the fact that the two proteins share a similar function, they do not share any recent origin, based on the lack of similarity in their respective amino acid sequences. ClustalW analysis of protein homologies, a program found at the European Bioinformatics Institute website, was performed in order to compare the protein sequences of MsrA and MsrB. From the results shown in Figure 2, it is obvious that there is little sequence homology between the two proteins, given that the homology score is only 3%. The two *msr* sequences appear to have evolved independently of one another. The identification of *msrB* was, in fact, a serendipitous discovery, given its lack of similarity to *msrA*. In *Neisseria gonnorhea* and certain other human pathogens, the MsrA coding sequence was fused to another region of approximately 150 amino acids, and this domain was named MsrB. Further studies indicated that the proteins did not interact, but that both domains had methionine reductase activity. The

SeqA Name	Len(aa)	SeqB Name	Len(aa)	Score
1 S.pombe_spac29E6.05c_	187	2 S.pombe_spbc216.04c_	138	3

```
CLUSTAL W (1.82) multiple sequence alignment


S.pombe_spac29E6.05c_       MQIAIIAAGCFWGVQEVYLRKFIPAAAILKTSVGYTGGITADPTYKEVCT 50
S.pombe_spbc216.04c_        MGFPLEKSEDEW-KKELGPEKYR---IMRQKGTEHPGAGRFTHQFPKQGV 46
                            * :.:  :   *  :*:  .*:     : :... :.*.      : :  .

S.pombe_spac29E6.05c_       NTTNHAEALKIEFDEKLTSYDKIIEFFFAMHDPTTSNQQGNDIGTQYRSA 100
S.pombe_spbc216.04c_        FVCAACKELLYKASTKFESHCGWPAFFDNLPG-KVKRIEDNSYGMHRVEA 95
                             .   .: *  : . *: *:     **  : . .... :.*. * :  .*

S.pombe_spac29E6.05c_       IFTTNPEQATIAKRVMNEVQAKHYPNKKIVTQILPAGKWWDAEDYHQLYL 150
S.pombe_spbc216.04c_        VCAN------CGGHLGHIFKGEGYSNPTDERHCINS----ASLEFHNEAT 135
                            : :.       . :: : .:.: *.* .   : : :     : ::*:

S.pombe_spac29E6.05c_       EKNPDGYRCSSRMNIKCQVNQKVLLTHSIRFFALECF 187
S.pombe_spbc216.04c_        NDN---------------------------------- 138
                            :.*
```

Red → residues AVFPMILW (small, hydrophobic side chains)
Green → residues STYHCNGQ (hydroxyl, amine, basic side chains)
Blue → residues DE (acidic side chains)
Magenta → residues RHK (basic side chains)

* → residues in the column are identical in the two protein sequences
: → conserved substitutions (based on the colors shown above) observed between the two sequences
. → semi-conserved substitutions observed between the two sequences

Highlighted → indicates the score of sequence homology between the two proteins, and the conserved residues

Figure 2: ClustalW Protein Analysis for Sequence Homology Results for MsrA (SPAC29E6.05c) and MsrB (SPBC216.04c)

catalytic activity of the MsrA protein was calculated to be 1000 times greater than that of the MsrB protein, proving that the second protein has a lower efficiency when reducing free oxidized methionine residues (Grimaud et al., 2001).

The structure of the MsrA protein has been determined via X-ray crystallography. Its structure involves a β-sheet, with α-helices around it, and a catalytic cysteine residue located at the amino terminus of the α-helical dipole. The structure of the MsrB protein has not yet been elucidated, although it is believed to be β-rich. This information leads to the suggestion that the two proteins, which exhibit stereo-specific reduction activity when presented with racemic mixtures of methionine sulfoxide residues, evolved independently of one another (Kumar et al., 2002).

In the yeast, *Saccharomyces cerevisiae*, both *msrA* and *msrB* genes were identified based on homology analyses. Following the cloning and overexpression of the recombinant enzymes, the MsrA protein was found to be significantly more active with the Met-S-SO form of the methionine sulfoxide, while the MsrB protein was demonstrably less efficient in reducing this stereoisomer. It was also shown that MsrA is responsible for the reduction of free methionine sulfoxide residues, and that MsrB does not participate in this process when MsrA is present in the cell. This indicates that MsrA is the major and dominant form of the protein, while MsrB assists in a minor capacity (Koc et al., 2004). Considerable evidence has implicated ROS damage as a major factor in the aging process. In this scenario, one could predict that the deletion of *msrA* gene from the genome of the yeast would result in a decrease in the viability of the organism, while an overexpression of the gene would increase the viability of the organism. On the other hand, a deletion or overexpression of the *msrB* gene would be expected to have little to no effect on the viability of the yeast. These predictions were verified, when it was demonstrated

that the Δ*msrA* mutant had a 26% reduction in viability, and overexpression of *msrA* increased the viability by 25%. The deletion and overexpression of *msrB* did not significantly affect the viability of the yeast, but a Δ*msrA*Δ*msrB* double mutant had a reduction in viability greater than that for the Δ*msrA* mutant. This demonstrates that both MsrA and MsrB are important to cell viability, but that MsrA protein plays a more significant role (Koc et al., 2004). Thus, although the two proteins are not similar in sequence (as demonstrated in the ClustalW results in Figure 2), this is not a problem based on the fact that the two proteins function on different stereoisomers of the methionine sulfoxide residue, and this difference in function is represented in the relative dominance of the MsrA protein in the organism. These experiments were carried out under aerobic growth conditions for the yeast, which demonstrates the presence of ROS during this kind of metabolic activity. When similar experiments were performed on yeast under anaerobic conditions, the deletion and overexpression of the *msr* genes did not affect the viability of the organism, consistent with the absence of ROS under anaerobic conditions, and it follows that cells growing via anaerobic metabolism and characterized by a lack of ROS would be expected to live longer (Koc et al., 2004).

The presence of *msr* genes has also been demonstrated in bacteria, and studies have demonstrated that a deletion of the *msrA* gene reduced cell viability in a manner similar to that seen in yeast. This is based on an increased sensitivity of mutant cells to oxidative stress conditions, which results in a decrease in survival and an overall lower rate of survival for the cells (Moskovitz et al., 2001).

Methionine sulfoxide reductase is a widely distributed enzyme. In addition to bacteria and yeast, the enzyme is also found in insects and mammals. In an experiment involving the fruit fly, *Drosophila melanogaster*, a line of flies was constructed that overexpressed bovine *msrA*

(*bmsrA*) genes. For lines in which the *bmsrA* gene was overexpressed throughout the body, a significant lifespan increase of 37% in females and 44% in males was found. When the overexpression of the MsrA protein was localized to the nervous system, lifespan was increased even more dramatically in both the males (from 45 days to 80 days) and females (from 58 days to 95 days), representing approximately 70% in both cases. When these overexpressors were then subjected to oxidative stress conditions, only 10% of the male flies died, as compared with 70% in the control group (non-overexpressors). It appears that the overexpression of the protein in the transgenic organism delayed the onset of certain inevitable mortality factors that normally dictate the longevity of the organism, including decline in physical activity and reproductive stamina. Thus, not only is the lifespan of the organism increased in this case, but so is the quality of that life (Ruan et al., 2002).

The methionine sulfoxide reductase genes are found to be relatively ubiquitous, with homologs found in many different organisms, from bacteria and yeast to mammals, including humans. It has been shown that MsrA in particular is found in many types of mammalian cells, including pigment cells in the eye, brain neurons, macrophages, liver, kidney, and cerebellum in the brain.

An experiment performed with mice, high levels of the MsrA protein were detected in the liver and kidney tissues of wild type mice. It was found that the deletion of the *msrA* gene revealed the presence of a second form, the *msrB* gene (as had been previously demonstrated in yeast). The activity of this second form of the MsrB protein was found to be variable, with a value in kidney and liver tissues equivalent to only 35% of the wild type value, but a value in brain and lung tissues equivalent to 75% of the wild type levels (Moskovitz et al., 2001). This confirms the data from the yeast study, showing that the *msrB* gene is only a minor contributor to

the reduction of methionine sulfoxide residues under normal circumstances, while the *msrA* gene is the primary protein repair enzyme. It was determined that the Δ*msrA* mutant mice had a lifespan reduction of approximately 40% when compared with the wild type strain (Moskovitz et al., 2001). This also corroborates early results with yeast indicating that organisms without the functional protein repair enzyme have a shorter lifespan.

In addition to differences in lifespan, there was a notable phenotypic difference between the Δ*msrA* mutant mice and the wild type strain in the walking behavior of the animal. Mice with the *msrA* deletion began walking on their tiptoes approximately six months after birth, which was interpreted as symptom of ataxia, the inability to coordinate muscular movements. Motor system dysfunction, like that caused by ataxia, can be linked to cerebellar-based anomalies, and these abnormalities can be connected to a decrease in resistance to oxidative stress. This heightened sensitivity to the effects of oxidative stress in mutants could be brought on by a loss of functional Msr protein, given that the MsrA protein has been found at high levels in the brain, especially in the neurons and the cerebellum. If the protein is not present, then there would be an increase in the number of oxidized methionine residues in proteins in these areas of the brain, which could prove to be detrimental to neuronal function (Moskovitz et al., 2001).

Additional earlier studies have also demonstrated that overexpression of the MsrA protein in both yeast and human T lymphocyte cells extends the lifespan of the cells by protecting them from the effects of oxidative stress (Moskovitz et al., 1998). It has also been shown that there is a decrease in the activity of the methionine sulfoxide reductase in patients with Alzheimer's disease, which further implicates the importance of the Msr proteins in maintaining proper neuronal function (Gabbita et al., 1999).

In this project, my goal was to extend these studies to the fission yeast, *Schizosaccharomyces pombe*, which can be used as a model organism for studying protein repair. *S. pombe* was selected for these experiments based on the wide array of tools available for its study, as well as its rapid generation time of the yeast (approximately 2.5 hours). The *S. pombe* genome contains two genes predicted to code for the methionine sulfoxide reductase enzyme, labeled *msrA* and *msrB*. *S. pombe* is also an excellent model system for studying the effects of oxidation, given that the yeast grows in colonies that can be easily observed, and its growth media can be easily manipulated in order to place oxidative stress upon the yeast.

The first step in the experimental procedure was to construct two *msr* gene knockouts. These deletions were generated based on the principle of homologous recombination. Primers were designed, corresponding to flanking regions on either side of each gene, one set for *msrA* and another set for *msrB*. These primers were used to clone the segments of the genome on either side of the respective *msr* gene. These flanking segments were then inserted into a cloning vector, on either side of a *ura4*$^+$ selectable marker. After this vector construct had been constructed, the segment containing the *msr* flanking regions and the *ura4*$^+$was amplified via polymerase chain reaction (PCR). This segment, the final product of the cloning procedure, was then transformed into a wild type yeast strain *S. pombe*. During the transformation procedure, homologous recombination should occur, causing the replacement of the wild type *msrA* or *msrB* gene with the *ura4*$^+$ selectable marker, effectively deleting the *msr* gene and replacing it with another, larger, gene. The resultant transformants from the plating of the transformation reaction could then be screened via growth on uracil-deficient media, and any colonies that grow will have had to incorporate the *ura4*$^+$gene, possibly in place of the *msr* gene. Possible mutants were

verified by PCR, and these mutants, once verified, were subjected to various oxidative stresses in order to determine the effect of the deletion on the viability of the yeast.

Chapter 2: Materials and Methods

Materials

All reagent grade chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Casamino acids and yeast extract were purchased from Difco (Sparks, MD). Cosmid c29E6 and cosmid c216 were purchased from the Sanger Center. Culture plates, tubes, and pipet tips were purchased from Fisher (Pittsburg, PA). Oligonucleotides were purchased from MWG Biotech (High Point, NC). PCR reagents were purchased from Perkin-Elmer (Branchburg, NJ). Primm Columns were purchased from Primm Labs (Cambridge, MA). Qiagen Spin Miniprep and Midiprep Kits, and the Qiagen Min Elute Gel Extraction Kit were purchased from Qiagen (Valencia, CA). Restriction enzymes and buffers were purchased from New England Biolabs (Beverly, MA) and from Promega (Madison, WI). Salmon sperm DNA was purchased from Pharmacia, Molecular Biology Division (Peapack, NJ). SeaKem GTG agarose for electrophoresis gels was purchased from Cambrex Bio Science Rockland, Inc. (Rockland, ME). SYBR Green was purchased from Molecular Probes (Eugene, OR). The TOPO TA Cloning Kit was purchased from Invitrogen Corporation (Carlsbad, CA).

Growth Media

Escherichia coli and other bacteria cultures were grown in both liquid and solid media. The media was prepared as indicated below, and was then sterilized for thirty minutes in the autoclave. The composition of the media and notes on media preparation are given below.

Luria Broth liquid media: 25 g/L of Luria Broth

Luria Broth solid media with ampicillin: 25 g/L Luria Broth, 20 g/L agar (for 2% agar plates). After sterilization and cooling, ampicillin (from a stock solution with a concentration of 50 mg/mL) was added to achieve a final concentration of 50 μg/mL. The plates were stored at 4°C.

Yeast cultures were grown in a variety of sterilized liquid and solid media. The media was prepared as indicated below, and was then sterilized for thirty minutes in the autoclave. The various types of media were prepared as follows:

YEA liquid media: 5 g/L yeast extract, 2 g/L casamino acids, 30 g/L D-glucose

YES liquid media: 5 g/L yeast extract, 30 g/L D-glucose, 225 mg/L adenine, 225 mg/L histidine, 225 mg/L leucine, 75 mg/L uracil

YES solid media: YES liquid media with 20 g/L agar (for 2% agar plates). The plates were stored at 4°C.

PM complete liquid media: 3.0 g/L phthallic acid, 3.4 g/L sodium phosphate ($Na_2HPO_4 \bullet 7H_2O$), 5.0 g/L ammonium chloride (NH_4Cl), 30.0 g/L D-glucose, 20.0 mL/L 50X PM salts, 75 mg/L L-histidine, 75 mg/L uracil, 75 mg/L adenine, 150 mg/L leucine. This solution was brought to pH 5.6 with 1 M potassium phosphate (KOH), and then autoclaved. After the liquid media had been sterilized and cooled, 1.0 mL 1000X PM vitamins and 0.1 mL PM minerals were added.

PM complete solid media: 3.0 g/L phthallic acid, 3.4 g/L sodium phosphate ($Na_2HPO_4 \bullet 7H_2O$), 5.0 g/L ammonium chloride (NH_4Cl), 30.0 g/L D-glucose, 20.0 mL/L 50X PM salts, 75 mg/L L-histidine, 75 mg/L uracil, 75 mg/L adenine, 150 mg/L leucine, 20 g/L agar (for 2% agar plates) This solution was brought to pH 5.6 with 1 M potassium phosphate (KOH), and then autoclaved. After the liquid media had been sterilized and cooled, 1.0 mL 1000X PM vitamins and 0.1 mL PM minerals were added before the plates were poured. The plates were stored at 4°C.

PM –uracil liquid media: 3.0 g/L phthallic acid, 3.4 g/L sodium phosphate ($Na_2HPO_4 \bullet 7H_2O$), 5.0 g/L ammonium chloride chloride (NH_4Cl), 30.0 g/L D-glucose, 20.0 mL/L 50X PM salts, 75 mg/L L-histidine, 75 mg/L adenine, 150 mg/L leucine. This solution was brought to pH 5.6 with

1 M potassium hydroxide (KOH), and then autoclaved. After the liquid media had been sterilized and cooled, 1.0 mL 1000X PM vitamins and 0.1 mL PM minerals were added.

PM –uracil solid media: PM –uracil liquid media with 20 g/L agar (for 2% agar plates) This solution was brought to pH 5.6 with 1 M potassium phosphate (KOH), and then autoclaved. After the liquid media had been sterilized and cooled, 1.0 mL 1000X PM vitamins and 0.1 mL PM minerals were added before the plates were poured. The plates were stored at 4°C.

50X PM salts: 53.5 g/L magnesium chloride ($MgCl_2$), 0.75 g/L calcium chloride ($CaCl_2$), 50 g/L potassium chloride (KCl), 2.0 g/L sodium sulfate (Na_2SO_4). The solution was filter sterilized and stored at 4°C.

1000X PM vitamins: 1.0 g/L pantothenic acid, 10.0 g/L nicotinic acid, 10.0 g/L inositol, 10.0 mg/L biotin. The solution was filter sterilized and stored 4°C.

10,000X PM minerals: 5.0 g/L boric acid, 4.0 g/L manganese sulfate ($MnSO_4 \bullet H_2O$), 4.0 g/L zinc sulfate ($ZnSO_4$), 2.0 g/L ferrous chloride ($FeCl_2$), 1.6 g/L molybdic acid, 1.0 g/L potassium iodide (KI), 0.4 g/L copper sulfate (Cu_2SO_4), 1.0 g/L citric acid. The solution was filter sterilized and stored at 4°C.

Cosmids

The cosmids used in experimental procedure (c29E6 for *msrA* and c216 for *msrB*) were obtained from the Sanger Center. The cosmids were streaked on Luria Broth (LB) plates with kanamycin added to achieve a final concentration 50 μg/mL. The plates were allowed to grow at 37°C overnight. Once colonies were observed on the plates, 3 mL liquid starter LB cultures (with kanamycin added to a final concentration of 50 μg/mL) were inoculated with colonies and these cultures were grown overnight at 37°C with shaking. The 3 mL starter cultures were then added to 100 mL LB media (with kanamycin added to final concentration of 50 μg/mL), and these

larger cultures were incubated overnight at 37°C with shaking. Cosmids were isolated from the cultures using the Qiagen Midi Prep, and the isolated cosmid DNA was run on a 1% agarose gel to estimate the concentration and purity of the DNA.

Yeast Culture

Small aliquots of yeast strains were taken from frozen stocks stored at –80°C, streaked on solid YES media, and incubated at 30°C for three days. Once colonies were observed on the plates, several of the colonies were selected and used to inoculate 2 mL cultures of YEL media. The cultures were grown at 30°C overnight with shaking. The cells in each culture were examined under a microscope and were counted using a hemacytometer in order to determine the concentration of cells per mL of media, which allows for a determination of the doubling time of the particular yeast strain. The culture can then be diluted to allow the cultures to grow to the desired concentration.

Molecular Cloning

Molecular cloning was performed based on standard procedures (Sanbrook et al., 2001).

Restriction Enzyme Digest

In a restriction digest, the enzyme concentration should be adjusted to a 5-10 U/μg of DNA. Once the concentration of the DNA to be digested is determined, the individual concentrations of the enzymes (as recorded on the company label for the enzyme) are used to determine the appropriate amount of enzyme to be added. Restriction enzymes are typically supplied at a concentration of 10,000 U/mL, or 10 U/μl. In addition, a 10X buffer is also added to the restriction enzyme digest, to a final 1X concentration. The correct buffer was determined using either the New England Biolabs or Promega catalog, which records the ideal buffer for each enzyme, as well as the appropriate buffers for digesting DNA simultaneously with two

enzymes. The restriction digest reaction was prepared and then put in a 37°C water bath from between two to four hours, depending on the amount of DNA being digested. For vector digests, there was additional enzyme added to digest more DNA, and the length of the digest was typically increased to four hours. There were four enzymes used for restriction digests, and they are recorded below with their appropriate concentration and their purpose in the procedure

Enzyme	**Concentration**	**Purpose**
EcoRI	12 U/μl	Linker on right side of right arm for *msrA* and *msrB*
KpnI	10 U/μl	Linker on left side of left arm for *msrA* and *msrB*
NotI	10 U/μl	Linker on left side of right arm for *msrA* and *msrB*
XhoI	20 U/μl	Linker on right side of left arm for *msrA* and *msrB*

Table 1: Restriction Enzymes, Concentrations, and Purposes

Phenol-Chloroform Extraction on Vector DNA

One hundred μl of buffer-equilibrated phenol was added to the pAP5 purified DNA suspended in TE. After the phenol was added, and the mixture was vortexed, the DNA with phenol was centrifuged and the aqueous layer was removed. A pipet was used to remove the aqueous layer, and equal volume of chloroform was added to this aqueous layer, and the mixture was then vortexed and spun in a microcentrifuge. The aqueous layer was then removed and one-tenth volume of 3 M sodium acetate (pH 5.3) and two and one-half volumes of 95% ethanol were added. The purified and phenol-chloroform extracted plasmid pAP5 vector DNA was ethanol purified and resuspended in 50 μl of TE.

DNA Gel Electrophoresis

Gels containing 1% agarose were prepared by mixing 0.30 grams of GTC Seakem Agarose with either 30 mL 1X TAE buffer [80 mL of 50X stock diluted to a final volume of 4 L in dH_2O; stock solution of 50X per liter: 242 g of Tris base, 57.1 mL of glacial acetic acid, and 100 mL of 0.5 M EDTA (pH 8.0)] or SYBR Green reagent. The agarose solution was then

placed in a microwave for between one to one and a half minutes, until the solution began to boil. The gel mixture was then poured into the gel box, with a comb in place to create the necessary wells. The gel was then covered with 1X TAE or 0.5 TBE buffer [400 mL of 5X stock diluted to a final volume of 4 L in dH_2O; stock solution of 5X per liter: 54 g of Tris base, 27.5 g of boric acid, and 20 mL of 0.5 M EDTA (pH 8.0)], and then run for one hour at between 80 and 100 volts. Bands were purified from the gel if necessary (in which case, a wide-toothed comb was used to create the wells in the gel), and a razor blade was used to cut the large bands from the gel. The section of the gel were put into a Primm Column and broken up with the side of a toothpick. The column was spun for 3 minutes in a microcentrifuge. One-tenth volume of 3 M sodium acetate (pH 5.3) and two and one-half volumes of 95% ethanol were added to the resultant flow through from spinning the column to precipitate the DNA. The purified DNA was then resuspended in 50 μl of TE.

Ligation and Transformation

Ligation reactions were prepared using a 1:10 molar excess of the vector to the insert DNA. Two 10 μl reaction were prepared in ligase buffer, one with T4 DNA ligase, and one without. The ligation reaction was performed overnight at 16°C. Then, the Invitrogen TOPO cloning kit was used to transform competent *E.coli* cells with the ligation product. Two μl of the ligation reaction was added to one vial of One Shot Chemically Competent *E.coli* cells from the kit. The cells were incubated on ice for 30 minutes, and then the cells were heat shocked in a 42°C water bath. The tubes were immediately placed on ice, and then 250 μl of room temperature SOC medium was added to each tube. The tubes were capped and then shaken horizontally at 37°C for one hour. The cells were then plated on LB/Amp plates, 200 μl on one plate and 50 μl on the other plate. The plates were then incubated at 37°C overnight.

PCR Screening from E.coli colonies

Aliquots containing 12.5 μl aliquots of PCR Master Mix were separated into individual PCR tubes. One colony was selected from the appropriate transformation plate with a toothpick, streaked on a new patch plate, and then the toothpick was dipped in the Master Mix aliquoted in the PCR tube. Several colonies were selected, streaked on the patch plate, and mixed in an individual PCR tube with Master Mix, The tubes were then placed in the thermocycler for 5 minutes at 98°C. A primer mix was prepared, consisting of nuclease-free water and 1.6 μM of each primer. Aliquots (12.5 μl) were added to each PCR tube, and a normal PCR reaction followed. The thermal cycles were as follows: 2 minutes at 94°C, 40 cycles of {30 seconds at 94°C, 30 seconds at 55°C, 3 minutes at 72°C}, 5 minutes at 72°C, and hold at 25°C.

DNA Sequencing

Dideoxy nucleotide chain termination method of DNA sequencing was performed at the Boston College sequencing facility (Chestnut Hill, MA), using the Beckman Coulter CEQ 2000 System. A stop solution of 1.5M sodium acetate (NaOAC) and 50 mM ethylenediaminetetraacetic acid (EDTA) was prepared fresh for each sequencing experiment. In addition, 95% and 70% ethanol were prepared and stored at –20°C. The molar concentration of the template DNA (the DNA to be sequenced) was then determined in order to ensure that no more than 1.5 μg of template was used in any reaction. The template DNA was diluted with water to give the proper concentration of 50-100 fmol. The plasmid template DNA, once mixed with water, was heated for either 1 minute at 96°C or 5 minutes at 86°C. After this preheating, the template was allowed to cool to room temperature before the sequencing reaction was prepared. The sequencing reaction was then prepared: 4 μl of DTCS Quick Start Master Mix, 1 μl of primer, and 5 μl of the template DNA, giving a total reaction volume of 20 μl. The PCR

program for the sequencing reaction was set up with the thermal cycles as follow: 33 cycles of {20 seconds at 96°C, 20 seconds at 50°C, 4 minutes at 60°C}, and hold at 4°C, using a ramp speed of at least 3°C/second on the thermocycler. After the PCR cycles were completed, the PCR tubes were spun down in a microcentrifuge to recover condensed reaction volumes. Next, 0.5 μl of glycogen (from the sequencing kit) was added to the PCR reactions, and the 4 μl of the stop solution (NaOAc and EDTA solution) was then added. The solution was then transferred to 1.5 mL Eppendorf tubes for ethanol purification, and 60 μl of 95% ethanol (stored at –20°C) was added to each sample. The tube was briefly vortexed, and then spun in a microcentrifuge at 4°C for 15 minutes. The ethanol was then aspirated away from the pellet, and 200 μl of 70% ethanol (stored at -20°C) was added to each sample. The tubes were then spun in a microcentrifuge at 4°C for 4 minutes. The ethanol was aspirated away from the pellet, then another 200 μl of 70% ethanol was added to the pellet, and the tube was spun in a microcentrifuge at 4°C for 4 minutes. After this ethanol purification, the pellet was air dried for 30 to 40 minutes. The pellet was then resuspended in 33 μl of sample loading solution (SLS), which was found in the sequencing kit. The sample was then stored at -20°C until the sequencing via capillary electrophoresis was performed by the Boston College technician.

Glycerol Frozen Stock Preparation

Glycerol frozen stocks of transformed bacteria were prepared by mixing 850 μl of an overnight culture with 150 μl of sterile glycerol and immediately transferring the cells to a -80°C freezer. Stocks were made for both pKP4 and pHD4 plasmids containing the left and right arms, with the *ura4*$^{+}$ gene between them. Colonies were taken from PCR patch plates and grown in 5 mL of LB (Luria Broth) media, with the addition of 5 μL of ampicillin (stock concentration of

50 mg/ μL). The cultures were grown overnight at 37°C with shaking, and frozen cultures were made with 850 μL of the sample culture and 150 μL of glycerol, which was then vortexed and stored in a -80°C freezer.

Transformation of Yeast Cells

Yeast cultures were collected via centrifugation at 3000Xg for five minutes, and the pelleted cells were then washed with 20 mL of sterile distilled water and resuspended in 1 mL sterile water. The cells were then transferred to an Eppendorf tube and were pelleted by centrifugation for thirty seconds at 3000Xg. The cells were washed with 1 mL of lithium acetate/TE and then resuspended in 100 μL in lithium acetate/TE [prepared by mixing 10X lithium acetate and 10X TE and diluting the filter sterilized 10X stocks: 10X lithium acetate – 1 M lithium acetate, adjusted to pH 7.5 with acetic acid, 10X TE—0.1 M Tris-HCl, 0.01 M EDTA, pH 7.5]. The cells were mixed with 2 μL of salmon sperm carrier DNA (stock concentration of 10 mg/mL) and 10 μL containing approximately 1 μg of the transforming DNA (either pKP4 and pHD4). The cells were then incubated for ten minutes at room temperature, after which 260 μL of 40% PLATE buffer [8 g polyethylene glycol (PEG), 2 mL 10X lithium acetate (as prepared above), 2 mL TE (as prepared above), 9.75 mL sterile water] was added. The cells were gently mixed and incubated for thirty minutes at 30°C. After thirty minutes, 43 μL of DMSO (dimethyl sulfoxide) was added, and the cells were heat shocked for precisely five minutes at 42°C in a water bath. The cells were transferred to 10 mL of YEL liquid media and grown at 30°C with shaking for forty-eight hours. After the cultures had grown to a density greater than approximately 10^6 cells/mL, 200 μL of undiluted cells, as well as 10^{-1} and 10^{-2} dilutions of the cells were plated on PM –ura plates (pombe media without uracil); one set of plates each for pKP4 and pHD4 transformations. The cultures were plated using sterile glass beads and they

were incubated for three days at 30°C. Single colonies were then selected and plated on a new PM –ura patch plate, one each for pKP4 and pHD4. The plates were grown overnight at 30°C, then colonies were selected from each plate (for both pKP4 and pHD4). These colonies were grown on separate PM –ura plates until a there was a lawn of colonies. A sterile pipet tip was used to collect cells and put them in a mixture of 85% sterile water and 15% glycerol, in order to make frozen stocks. These stocks were then stored in a -80°C freezer.

Zymolase Colony DNA Isolation

The zymolase solution was prepared by mixing 2.5 mg/mL zymolase enzyme (lyticase), 1.2 M sorbitol, and 0.1M sodium phosphate, pH 7.4. A yeast colony or a yeast cell pellet from an overnight liquid culture was selected using a sterile pipet tip (the use of wooden toothpicks or dowels was avoided in this procedure to prevent any detrimental effects to the release of DNA from the cells or to the PCR reaction itself). The cells were rinsed off of the pipet tip using 10 μl of the zymolase solution. The cells were pipetted up and down with the zymolase in order to rinse the pipet tip and spheroplast the cells. The cells were then incubated from 15 minutes at 37°C. Immediately after the addition of the zymolase, and then at five-minute intervals during the incubation process, 2 μl of cells were mixed on a glass slide with 10 μl of dH_2O. These slides were examined under a microscope, and after the fifteen-minute incubation period, the presence of spheroidal cells (spheroplasts) was apparent, indicating the deterioration of the yeast cell wall as a result of the zymolase enzyme. Two μl of the spheroplasted cells were used in a 25 μl PCR reaction mix containing 12.5 μl PCR Master Mix, 2 μl primer 1 (10 μM concentration, or 1 μl of a 20 μM concentration), 2 μl primer 2 (10 μM concentration, or 1 μl of a 20 μM concentration), 2 μl spheroplasted yeast cells (DNA), and 6.5 μl nuclease-free water. A PCR reaction was then carried out, with the thermal cycles set as follows: 2 minutes at 94°C, 40

cycles of {30 seconds at 94°C, 30 seconds at 55°C, 3 minutes at 72°C}, 5 minutes at 72°C, and hold at 25°C.

Peroxide Sensitivity Experiment

Two mL of YEA media was inoculated with a single colony from a streak plate of the desired strain of yeast. The cells were placed in a rotating wheel in a 30°C incubator overnight. The cells were then counted under a microscope using a hemacytometer, and this number was used to determine the volume of cells need for a 2 mL culture of cells with an initial concentration of 2×10^6 cells/mL. A 2 mL culture of YEA was then inoculated with this calculated volume of cells from the overnight culture, and the new cultures were placed in a rotating wheel in the 30°C incubator for 2 hours. While the cells were growing, 30% H_2O_2 (10 M) stock solution was diluted in YEA media in microcentrifuge tubes to make a 1 M solution and then this 1 M solution was diluted in YEA to make a 0.1 M solution. After 2 hours, the cultures were removed from the incubator, and 100 μL of the 0.1 M H_2O_2 solution was added to each culture, to give a final concentration of 5 mM H_2O_2 per culture. One spot plate was prepared for each culture being tested, with four different cell concentrations plated at each of four time points (including time zero). At each time point, 100 μL was removed from each culture, and three serial 1:10 dilutions were made from this 100 μL aliquot, to give 1:1, 1:10, 1:100, and 1:1000 dilutions. Five μL was pipetted onto the plate for each dilution, and the plates were allowed to grow on the bench until the spot plates had been completed with spots for each of the four time points. The cultures were placed back in the rotating wheel in the 30°C incubator, until the next time point at 1 hour, at which point 100 μL was removed from each culture, the dilutions were prepared, and the 5 μL spots were added to the spot plates. Dilutions and spots on the plates were also made at 2 hours and 4 hours after addition of the H_2O_2 solution.

The plates were then grown in a 30°C incubator for three days, until the spots were visible and could be counted. After three days, the plates were removed from incubator, photographed, sealed with parafilm, and placed in the refrigerator.

Chapter 3: Results

I. Construction of targeting vectors for homologous recombination

Cloning of homologous flanking DNA sequences

MsrA: The cloning strategy used for working with the *msrA* gene is shown in Figure 3. Cosmid c29E6 (Figure 4) was used as a template in PCR reactions designed to amplify flanking sequences for the *msrA* gene. The cosmid was obtained from the Sanger Centre, and the primers were obtained from the MWG Biotech Company. The downstream flanking sequence, or "left arm," corresponded to nucleotides 7455 to 7673 of cosmid c29E6. The primers used to generate this fragment were SP16 and SP17 (shown in Figure 6), generating an amplification product 219 base pairs (bp) in length. The SP16 primer has an added linker containing a *KpnI* restriction enzyme recognition site, and the SP17 primer has an added linker containing a *XhoI* recognition site. The upstream flanking sequence, or "right arm," corresponded to nucleotides 8383 to 8620 of cosmid c29E6. The primers used to generate this fragment were SP18 and SP19 (shown in Figure 6), producing an amplification product 238 bp in length. The SP17 primer has an added linker containing an *EcoRI* restriction enzyme recognition site, and the SP18 primer has an added linker containing a *NotI* recognition site.

The PCR product of the left arm (Lane B of Figure 8) was then cloned into the pCR 2.1-TOPO vector using the TOPO TA cloning kit, thereby creating the plasmid pKP1 (Figure 3). Plasmid pKP1 was then digested with the *KpnI* and *XhoI* restriction enzymes to release the left arm of *msrA*, which was then isolated from a 1% agarose gel using the MinElute Gel Extraction Protocol. The pAP5 plasmid, containing the *ura4*$^{+}$ gene, was also digested with the *KpnI* and *XhoI* restriction enzymes. An overnight ligation reaction of the left arm and the pAP5 plasmid was performed using the T4 DNA ligase, and the ligation products were then transformed into TOP-1 cells. PCR screening was used to identify *E.coli* colonies carrying the correctly

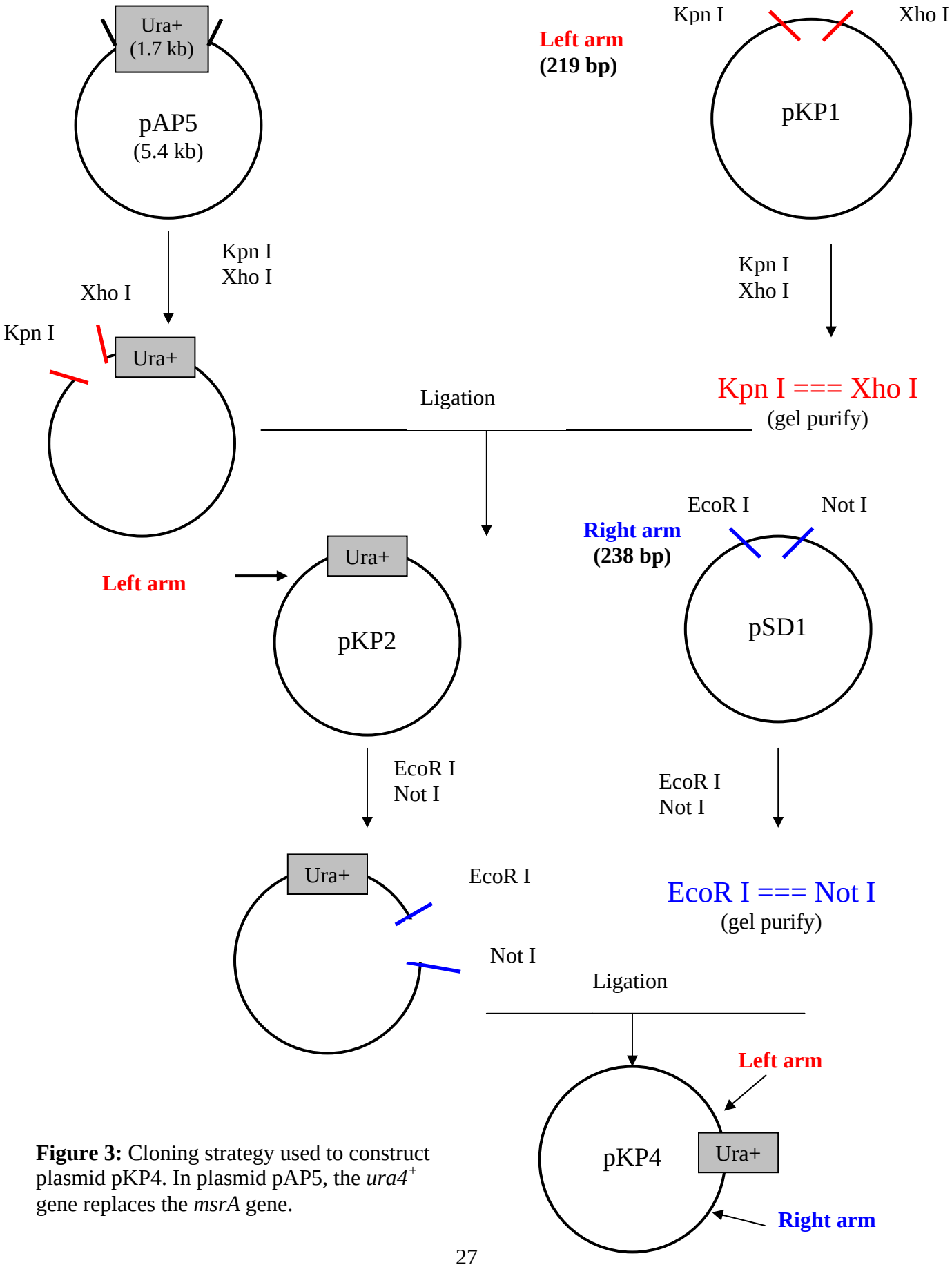


Figure 3: Cloning strategy used to construct plasmid pKP4. In plasmid pAP5, the *ura4*$^{+}$ gene replaces the *msrA* gene.

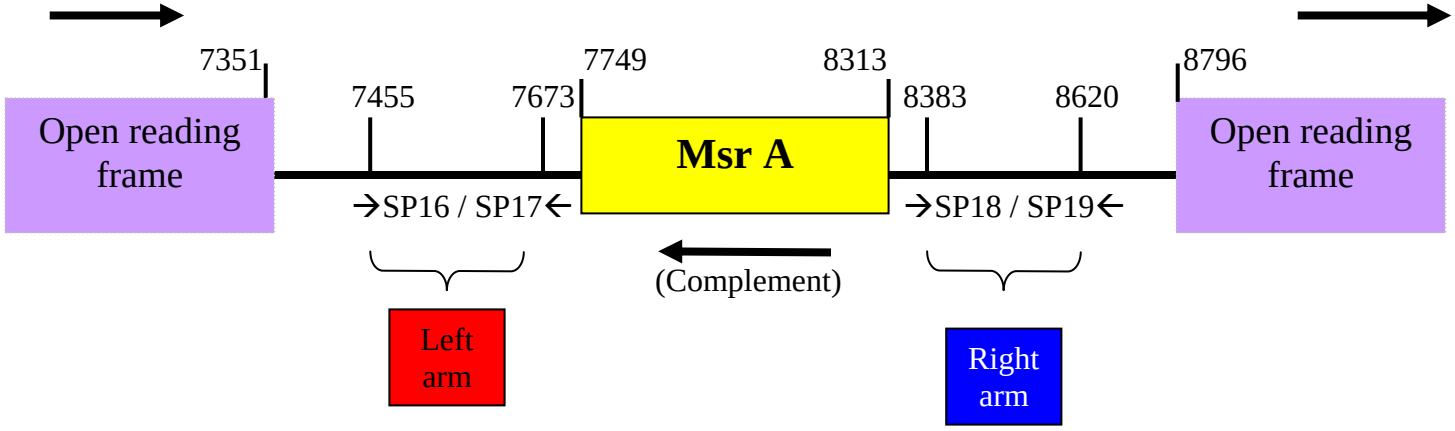


Figure 4 : Map of Cosmid c29E6

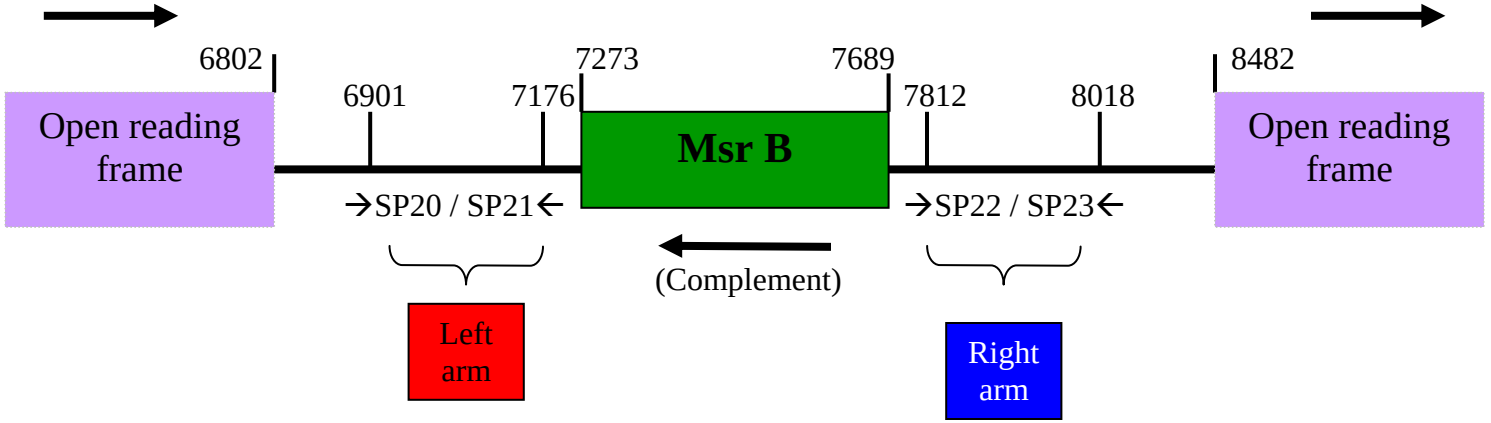


Figure 5 : Map of Cosmid c216

Figure 6 : Cosmid c29E6 primers

SP16 → Forward left arm: nucleotides 7455 to 7480

GGT ACC-5'-GAT CTA CTT CCA TAC ATC CAA AAC C-3'
KpnI linker

SP17 → Reverse left arm: nucleotides 7673 to 7650

CTC GAG-5'-TAC ACC ATG TCT GGT TCT GAC CAG-3'
XhoI linker

SP18 → Forward right arm: nucleotides 8383 to 8406

GAA TTC-5'-CGT GGA ACA GTG CTT CAT GTT TC-3'
EcoRI linker

SP19 → Reverse right arm: nucleotide 8620 to 8596

GCG GCC G-5'-CAA TCG GGA ATT AGC GTG ACT ATC G-3'
NotI linker

Figure 7 : Cosmid c216 primers

SP20 → Forward left arm: nucleotides 6901 to 6920

CGT ACC-5'-GAC CTA TGA AGC GTA CGC AA-3'
KpnI linker

SP21 → Reverse left arm: nucleotides 7176 to 7156

CTC GAG-5'-CAA CGA ACA AGT GCA AAA CC-3'
XhoI linker

SP22 → Forward right arm: nucleotides 7812 to 7834

GAA TTC-5'-CGT GGA ACA GTG CTT CAT GTT TC-3'
EcoRI linker

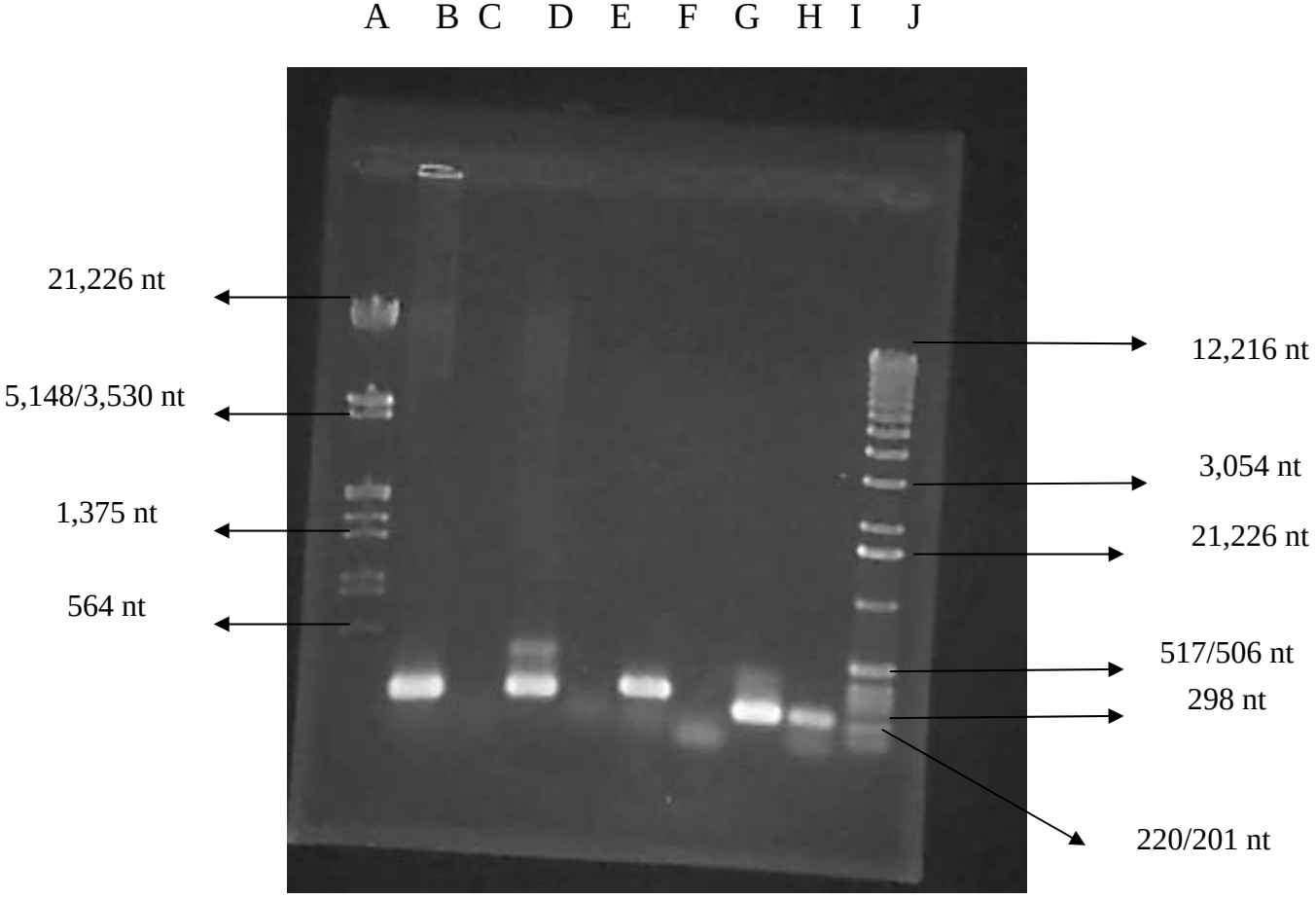
SP23 → Reverse right arm: nucleotides 8018 to 7996

GCG GCC GC-5'-TAG CGT GTA GGA TCA AGA GTA AC-3'
NotI linker

constructed pKP2 plasmid, using the SP16 and SP17 primers. Small aliquots of the PCR product were run on a 1% agarose gel to verify that the left arm had indeed been inserted into the pAP5 plasmid. The gel results, as seen in Figure 9A, verified the correct size of the PCR product (219 bp), and the pAP5 plasmid with the pKP1 left arm insert was labeled pKP2. The sequence of this plasmid product was then verified using the dideoxy nucleotide chain termination method of DNA sequencing, and a BLASTN search on the resultant sequence showed high homology to both the *S. pombe ura4*$^{+}$ gene and the cosmid SPAC29E6, as shown in Figure 9B.

The PCR product of the right arm (238 bp) was amplified using primers SP18 and SP19, and cloned into the pCR 2.1-TOPO vector, thereby generating the plasmid pSD1. Correct construction of the clones was verified using PCR, as shown in Figure 10A. This plasmid product was then submitted for dideoxy nucleotide chain termination method of DNA sequencing, and a BLASTN search on the beginning part of resultant sequence produced good matches with the *S. pombe* cosmid SPAC29E6, as shown in Figure 10B. Plasmid pSD1 was then digested with the *NotI* and *EcoRI* restriction enzymes, in order to release the right arm of *msrA*. The correct size of the arm was verified using a 1% agarose gel, and the right arm was then purified from the gel using the MinElute Gel Extraction Protocol. The pKP2 plasmid, containing the *ura4*$^{+}$ gene and the pKP1 left arm was then digested with the *NotI* and *EcoRI* restriction enzymes. An overnight ligation reaction was performed between pKP2 and the right arm, and the ligation products were used to transform TOP-2 cells (Figure 3). Next, PCR screening from the *E.coli* colonies was used to identify successful transformants among ten colonies from the transformation plates, using the SP16 and SP19 primers, as shown in Figure 11. The pKP2 plasmid (with the *ura4*$^{+}$ gene and the pKP1) is 5.0 kb in size, and the *ura4*$^{+}$ gene itself is 1.7 kb, while right arm is 238 bp, giving a total size of the right and left flanking sequences with the

Figure 8

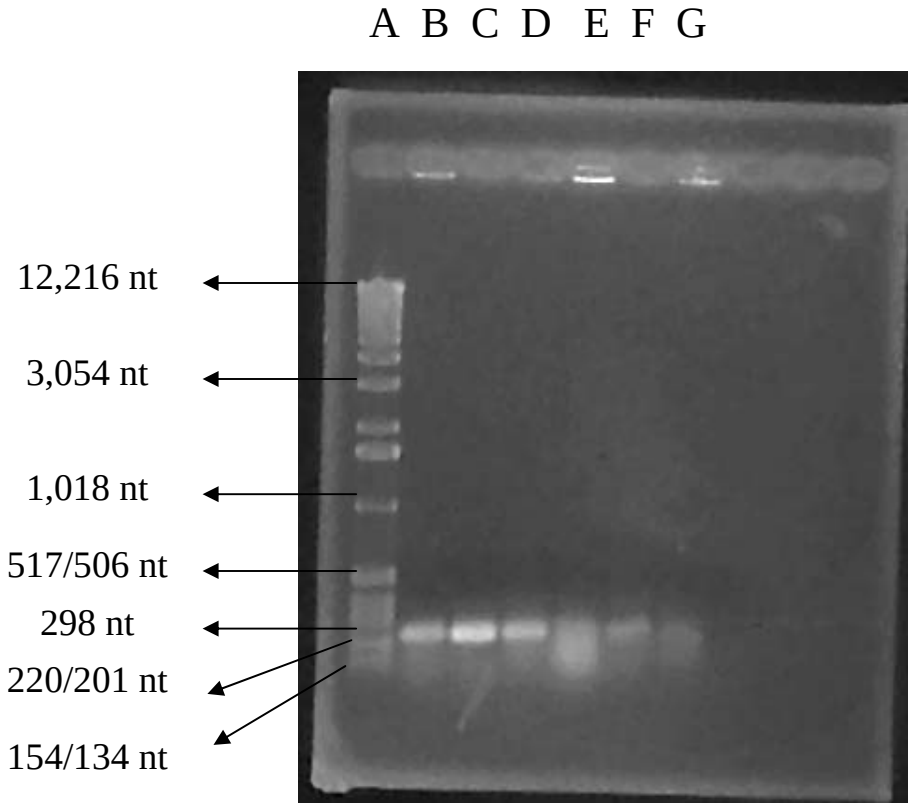


Amplification of *msrA* and *msrB* flanking sequences
A 1% agarose gel was used to separate the amplification products of a PCR reaction. Left and right arms of *msrA* and *msrB* were amplified in separate reactions. The PCR products were then transformed into the pCR 2.1-TOPO vector and then sequenced to confirm that the flanking regions had been successfully cloned.

Lane A: DNA Standard Marker III
Lane B: Left arm of *msrA*
Lane C: PCR control reaction for *msrA* left arm (no primers added to the DNA)
Lane D: Right arm of *msrA*
Lane E: PCR control reaction for *msrA* right arm (no primers added to the DNA)
Lane F: Left arm of *msrB*
Lane G: PCR control reaction for *msrB* left (no primers added to the DNA)
Lane H: Right arm of *msrB*
Lane I: PCR control reaction for *msrB* right arm (no primers added to the DNA)
Lane J: DNA Standard Marker X

Figure 9

A.



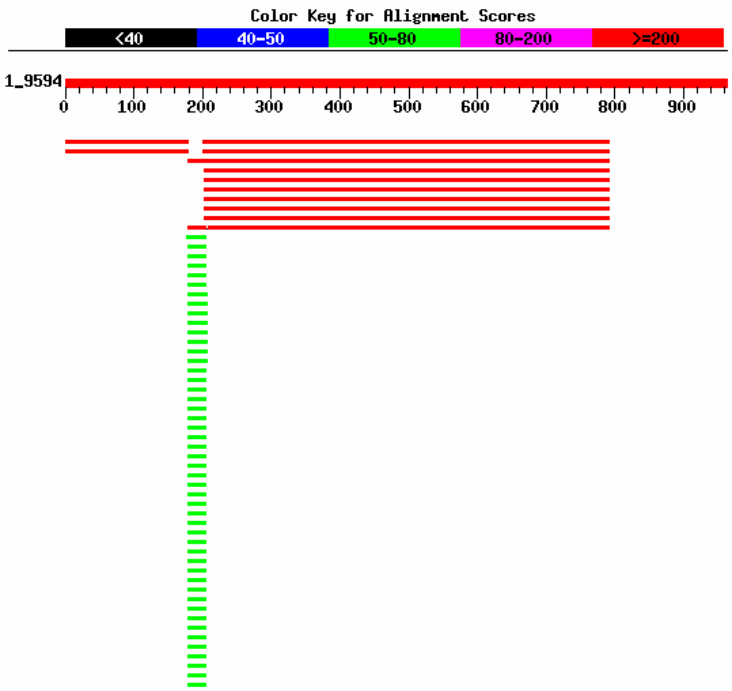
Characterization of pKP2 clones
Transformed *E.coli* cells were analyzed for the presence of the pKP2 plasmids by PCR reactions with primers SP16 and SP17. Successful transformants (lanes B-G) can be identified by the presence of a 219 base pair amplification product. Amplification products were analyzed on a 1% gel.

Lane A: DNA Standard Marker X
Lanes B-G: PCR products from six different potential pKP2 transformants

B.

BLAST analysis of DNA sequencing results

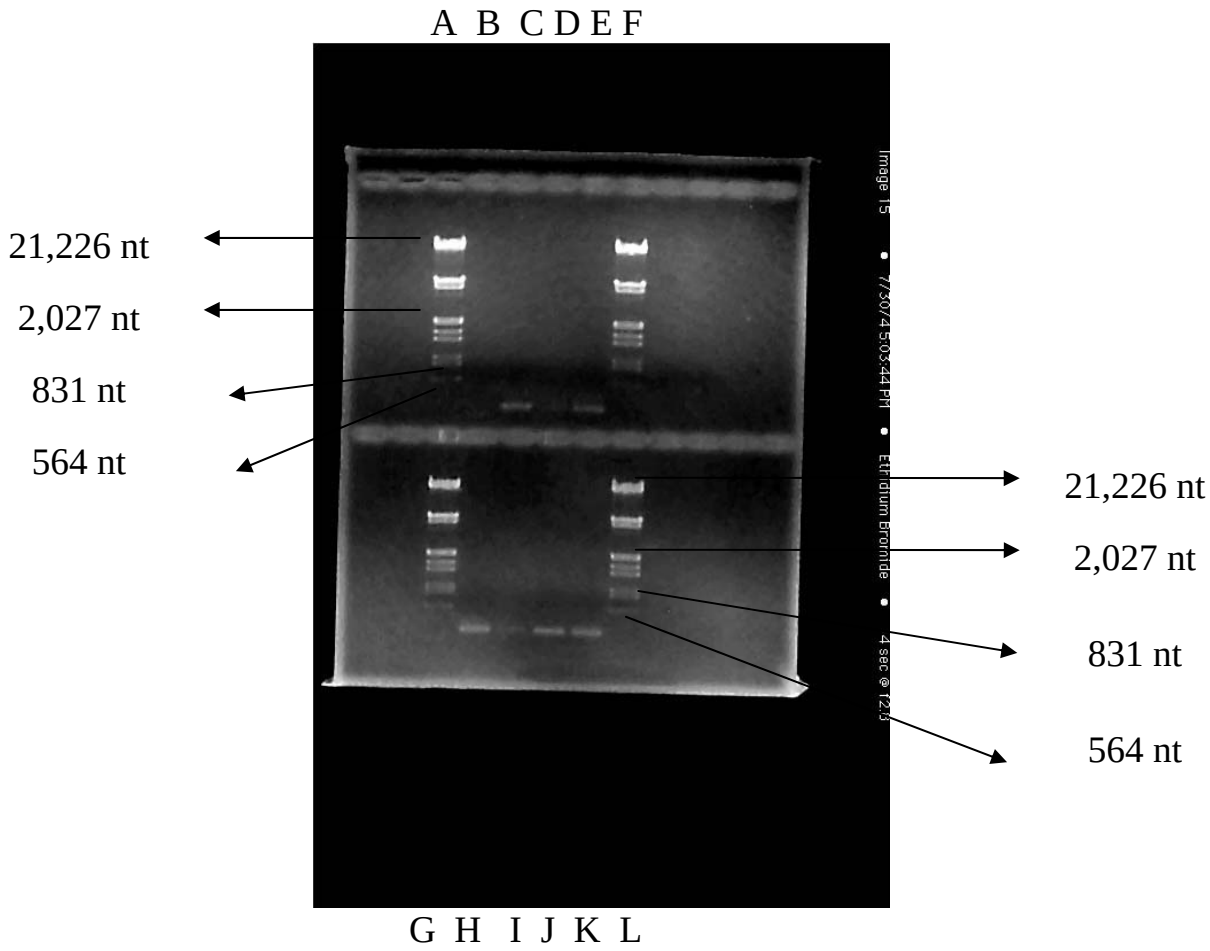
The sequence of plasmid pKP2 was determined using the dideoxy nucleotide chain termination method, and the sequence was compared to the non-redundant DNA database using the BLAST algorithm. Both the *S. pombe ura4*$^{+}$ gene and cosmid c29E6 share regions of sequence identity with pKP2 as shown by their BLAST scores (asterisks).



Sequences producing significant alignments:		Score (bits)	E Value
gi|5133|emb|X13976.1|SPURA4	Schizosaccharomyces pombe ura4 ..	1114	0.0 *
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=search&term=5133[NUID]			
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=search&term=5133[NUID]			
gi|3646441|emb|AL031603.1|SPCC330	S.pombe chromosome III co...	1114	0.0
gi|22347652|gb|AF531173.1|	Cloning vector pDblet, complete ...	1112	0.0
gi|37654597|gb|AY303168.1|	Degron tagging vector pSMUG2+ be...	1110	0.0
gi|37654593|gb|AY303167.1|	YFP Integration vector pSMUY2+ b...	1110	0.0
gi|37654589|gb|AY303166.1|	CFP Integration vector pSMUC2+ b...	1110	0.0
gi|19682862|emb|AJ439454.1|CVE439454	Cloning vector pSMUG+d...	1110	0.0
gi|13928064|emb|AJ306911.1|IVE306911	Integration vector pSM...	1110	0.0
gi|6453570|emb|AJ250107.1|IVE250107	Integration vector pSMU...	1110	0.0
gi|31044413|gb|L25928.3|YSPSEQB	Shuttle vector pJK210, comp...	1096	0.0
gi|1044926|emb|Z66525.1|SPAC29E6	S.pombe chromosome I cosmi...	339	8e-90 *
gi|6723883|emb|AL136538.1|SPAC30	S.pombe chromosome I cosmi...	339	8e-90 *
gi|51832323|gb|AY710294.1|	Synthetic construct Maa7/X IR ar...	58	5e-05

Figure 10

A.



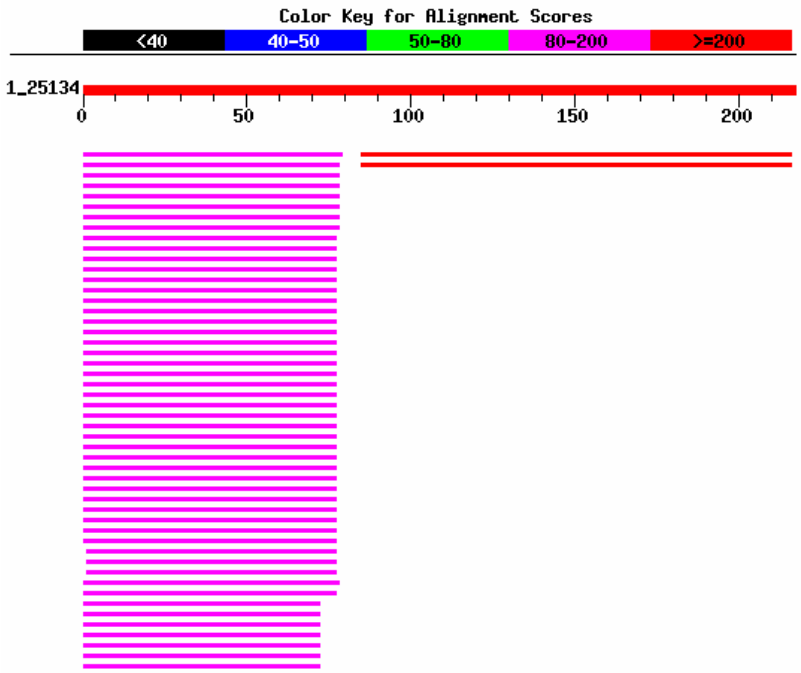
Characterization of pSD1 clones
Successfully transformed colonies carrying pSD1 were identified using PCR and separation of the PCR products on a 1% agarose gel. The expected size of the amplification product is 238 base pairs.

Lanes A, F, G, and L: DNA Standard Marker III
Lanes B-E, H-K: PCR products from eight different potential pSD1 transformants

B.

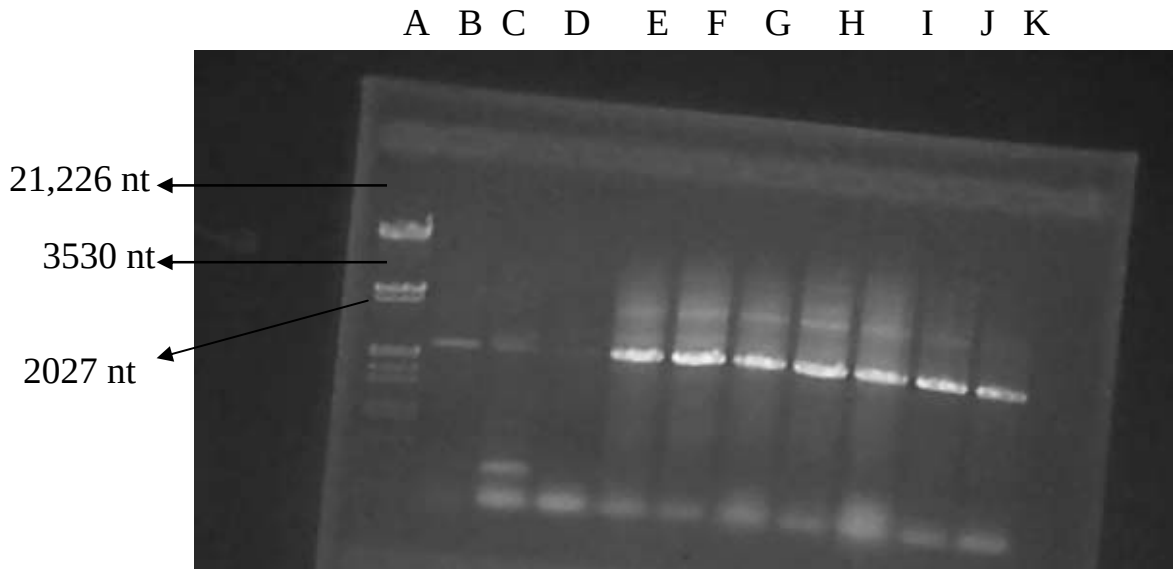
BLAST analysis of DNA sequencing results

DNA sequencing was performed on plasmid pSD1 using the dideoxy nucleotide chain termination method, and the sequence was compared to the non-redundant DNA databases using the BLAST algorithm. The plasmid insert showed an excellent match to the expected region of the *S. pombe* cosmid c29E6 (asterisks).



Sequences producing significant alignments:		Score (bits)	E Value	
gi|1044926|emb|Z66525.1|SPAC29E6	S.pombe chromosome I cosmi...	262	3e-67	*
gi|6723883|emb|AL136538.1|SPAC30	S.pombe chromosome I cosmi...	262	3e-67	*
gi|18651492|gb|AF406099.1|	HIV-1 clone 5M2-3 from USA nonfu...	151	8e-34	
gi|58423648|emb|CS000922.1|	Sequence 1 from Patent EP1500663	149	3e-33	
gi|25265349|emb|AJ518307.1|UBA518307	Unidentified bacterium...	149	3e-33	
gi|25265337|emb|AJ518301.1|UBA518301	Unidentified bacterium...	149	3e-33	
gi|32526537|emb|AJ494839.1|SAU494839	Stigmatella aurantiaca...	149	3e-33	

Figure 11



Verification of plasmid pKP4
A 1% agarose gel was used to separate the amplification products from a PCR screening of *E.coli* colonies transformed according to the TOPO cloning procedure. Cells were transformed with the ligation products from a reaction between the purified right arm from plasmid pSD1 and plasmid pKP2. The expected size of the amplification products is 2.16 kilobases.

Lane A: DNA Standard Marker III
Lanes B-K: PCR products from ten different potential pKP4 transformants

ura4$^+$ gene as approximately 2.16 kb. The gel results verified the correct size of the PCR product, and the plasmid was labeled pKP4. For yeast transformation, primers SP16 and SP19 were used to amplify the *ura4*$^+$ gene with both of the *msrA* flanking sequences from pKP4 using the Qiagen Purification of DNA from a PCR reaction.

MsrB: The cloning strategy used for working with the *msrB* gene is shown in Figure 12. Cosmid c216 (Figure 5) was used as a template in PCR reactions designed to amplify sequences flanking the *msrB* gene. The cosmid was obtained from the Sanger Centre, and the primers were obtained from the MWG Bioctech Company. The downstream flanking sequence, the "left arm," was composed of nucleotides 6901 to 7176 of cosmid c216. The primers used to generate this fragment were SP20 and SP21 (shown in Figure 7), resulting in an amplification product 276 base pairs (bp) in length. The SP20 primer has an added linker containing a *KpnI* restriction enzyme recognition site, and the SP21 primer has an added linker containing a *XhoI* recognition site. The upstream flanking sequence, the "right arm," was composed of nucleotides 7812 to 8018 of cosmid c216. The primers used to generate this fragment were SP22 and SP23 (shown in Figure 7), resulting in an amplification product 207 bp in length. The SP22 primer has an added linker containing a *KpnI* restriction enzyme recognition site, and the SP23 primer has an added linker containing a *XhoI* recognition site.

The DNA from c216 was isolated from overnight liquid cultures using the Qiagen Midi Prep. Two PCR reactions were set up using the purified cosmid c216 DNA, one using the left arm primers (SP20 and 21), and one using the right arm primers (SP22 and SP 23). The size of the amplified "left" arm was verified by electrophoresis on a 1% agarose gel (Lane F, Figure 8). The amplified left arm was then cloned into the TOPO vector using the TOPO TA cloning kit, thereby creating the plasmid pHD1 (see cloning strategy found in Figure 12).

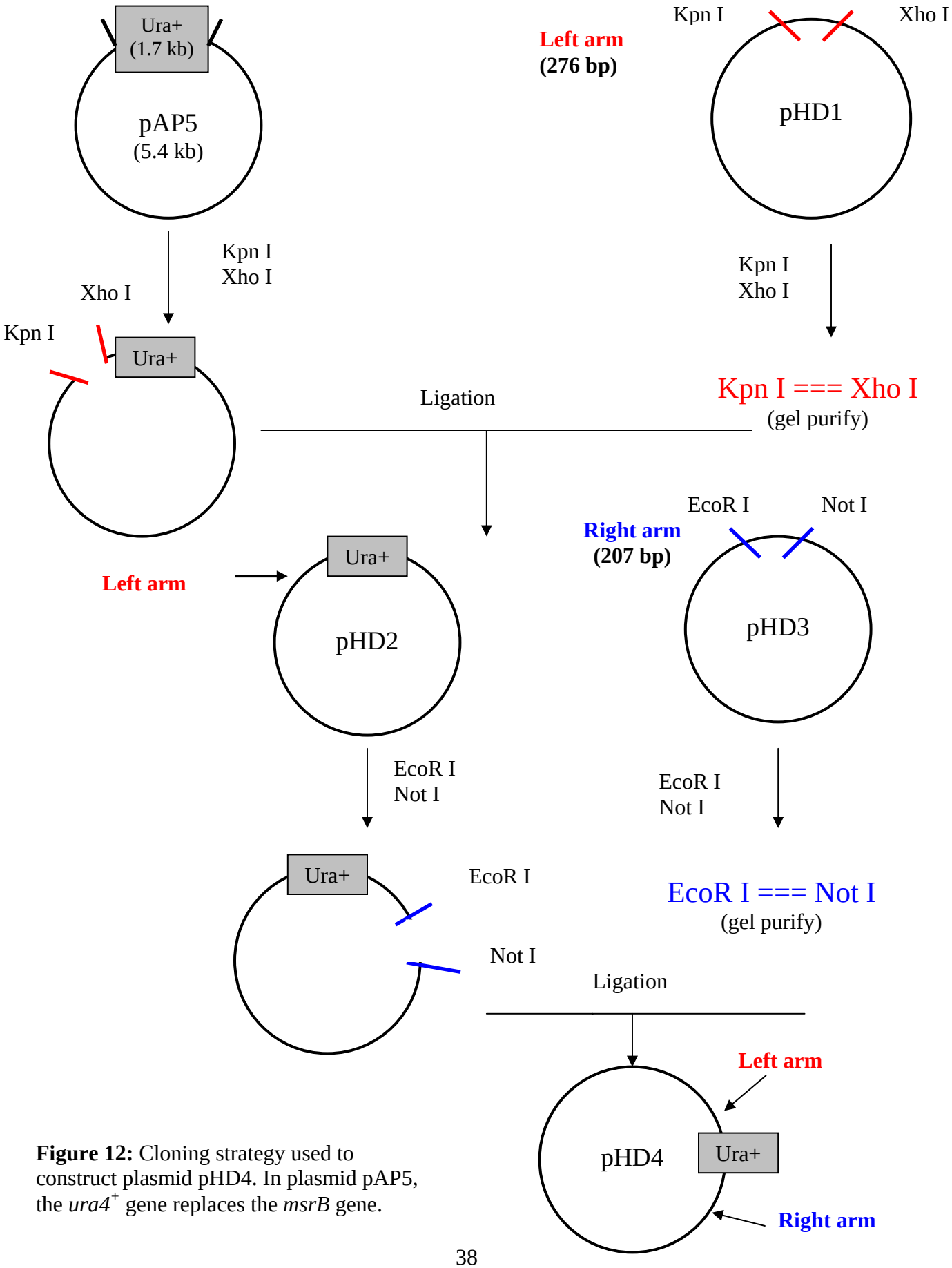


Figure 12: Cloning strategy used to construct plasmid pHD4. In plasmid pAP5, the *ura4*$^{+}$ gene replaces the *msrB* gene.

Plasmids were purified from the pHD1-transformed cells using the Qiagen Spin Miniprep Kit. Purified pHD1 was then digested with the *KpnI* and *XhoI* restriction enzymes to liberate the left arm, which was then isolated on a 1% agarose gel prior to ligation with pAP5 in the next step of plasmid construction. The pAP5 plasmid, containing the *ura4*$^{+}$ gene, was isolated from the transformed cells using the Qiagen Spin Midiprep Kit. This plasmid DNA was then digested with the *KpnI* and *XhoI* restriction enzymes; followed by phenol-chloroform extraction to inactivate the restriction enzymes. An overnight ligation reaction was performed with the purified left arm and digested pAP5, using T4 DNA ligase and a 10:1 molar excess of the left arm insert to the pAP5 double cut vector. The ligation reaction was carried out in a 16°C water bath overnight. The ligation products were transformed into the TOP-2 cells, and successful transformants were identified by PCR screening of ampicillin-resistant *E.coli* colonies, using the SP20 and SP21 primers. Small aliquots of the PCR products were analyzed on a 1% agarose gel to verify that the left arm (276 bp) had indeed been inserted into the AP5 plasmid, as shown in Figure 13.

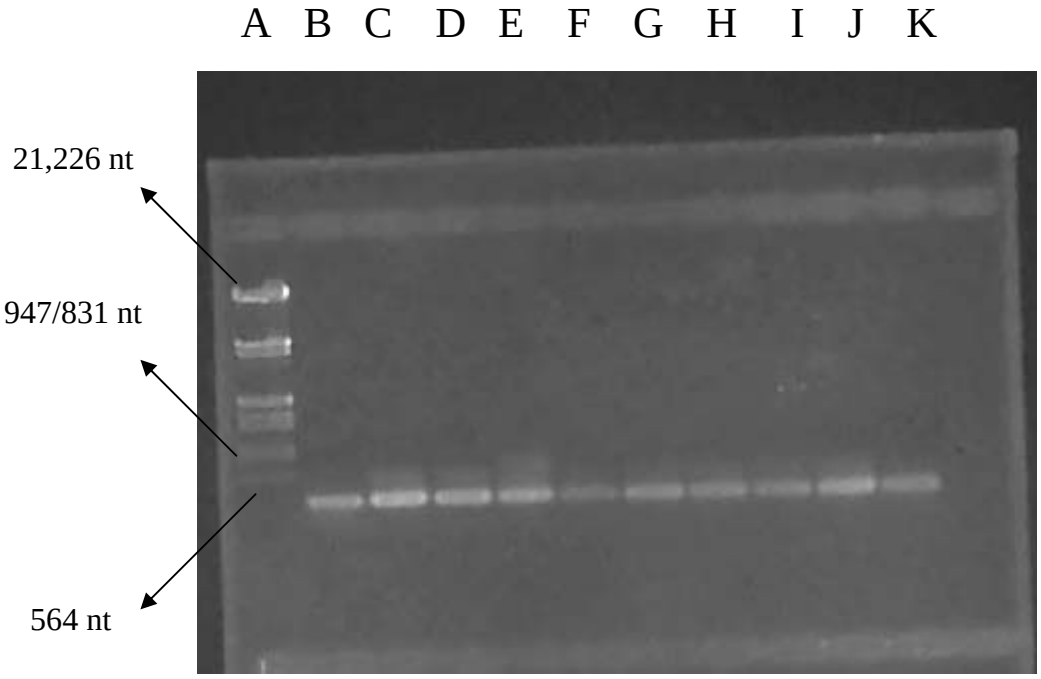
The PCR product of the right arm, amplified using primers SP22 and SP23, as seen in Lane H of Figure 8, was then cloned into the pCR 2.1-TOPO vector using the TOPO TA cloning kit, thereby creating the plasmid pHD3 (see cloning strategy found in Figure 12). The pHD3 was then digested with the *NotI* and *EcoRI* restriction enzymes, and the right arm (insert from pHD3) was purified following electrophoresis on a 1% agarose gel using Primm Columns. In the last step of the construction, the pHD2 plasmid containing the *ura4*$^{+}$ gene was digested with the *NotI* and *EcoRI* restriction enzymes and ligated overnight with the gel-purified right arm. Ligation products were transformed into TOP-2 cells, and successful transformants were identified by colony PCR, using the SP20 and SP23 primers. The pHD2 plasmid (with the *ura4*$^{+}$ gene and the

pHD1 left arm insert) is 5.0 kb in size, and the *ura* gene itself is 1.7 kb, while right arm (pHD3 right arm insert) is 207 bp, giving a total size of the right and left flanking sequences with the *ura4*$^{+}$ gene as approximately 2.18 kb. Successful construction of plasmid pHD4 was analyzed using an agarose gel (Figure 14A). This plasmid was then sequenced using the dideoxy nucleotide chain termination method and the sequence was compared to the non-redundant DNA database using the BLAST algorithm. As shown in Figure 14B, significant homology was observed between pHD4 and both the *S. pombe ura4*$^{+}$ gene and cosmid SPBC216.

II. Transformation into the FWP16 S. pombe strain

The FWP16 (*h*$^{-}$*, leu 1-32*) strain of *S. pombe* was designated as the wild-type strain into which the *msrA* and *msrB* replacement sequences would be transformed. The *msrA* and *msrB* replacement sequences were amplified from pKP4 and pHD4 using SP16 and SP19 as primers for *msrA* and SP20 and SP23 as primers for *msrB*, respectively. Twenty mL cultures of FWP16 at a density of 10^{7} cells/mL were used for transformation. One FWP16 culture was transformed with the purified pKP4 product (the left and right flanking regions of *msrA* on either side of the *ura4*$^{+}$ gene), and one FWP16 culture was then transformed with the purified pHD4 product (the left and right flanking regions of *msrB* on either side of the *ura4*$^{+}$ gene). This transformation procedure was done using salmon sperm carrier DNA, which was mixed with either pKP4 or pHD4, and then incubated with permeabilized FWP16 cells to allow the transformation, and homologous recombination, to take place. The transformed cells were then grown in liquid YEL media, before being plated with sterile glass beans on PM media lacking uracil. These plates were grown, and any growth was assumed to come from cells that were properly transformed

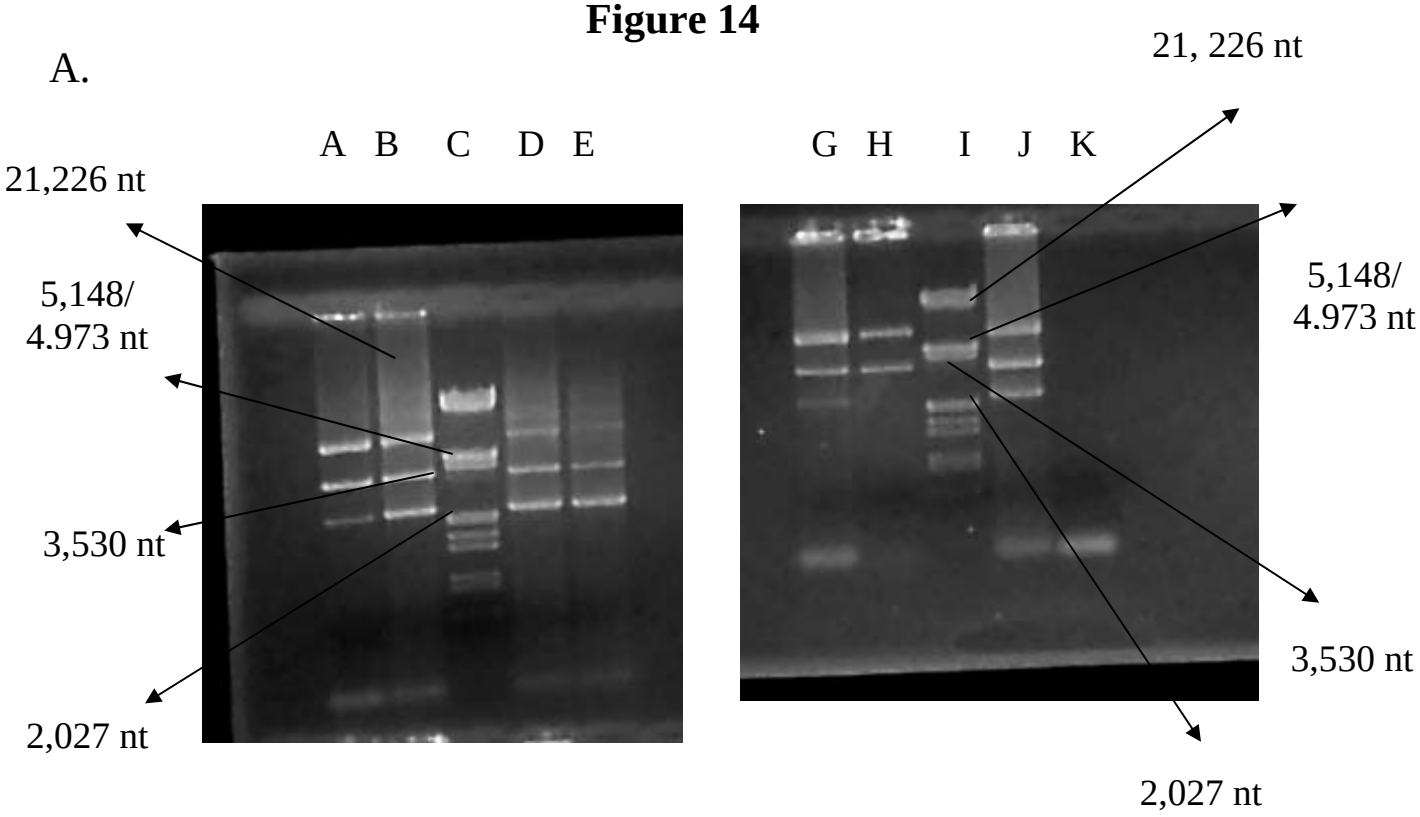
Figure 13



Verification of plasmid pHD2 construction
A 1% agarose gel was used to analyze the results from a PCR screening of *E.coli* colonies transformed with the ligation products of the purified left arm of *msrB* from plasmid pHD1 and plasmid pAP5. Primers SP20 and SP21 were used in the PCR reaction. The expected size of the PCR product is 276 base pairs.

Lane A: DNA Standard Marker III
Lanes B-K: PCR products from ten different potential pHD2 transformants

Figure 14



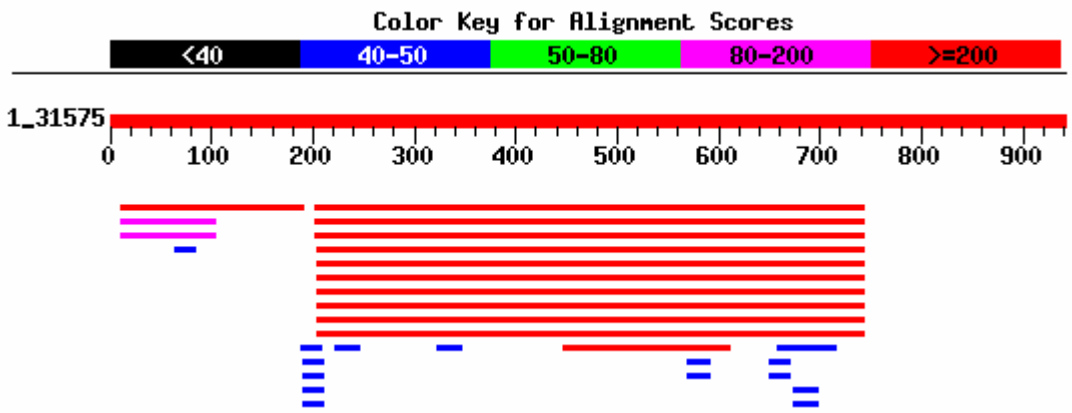
Verification of plasmid pHD4 construction
A 1% agarose gel was used to analyze the results from a PCR screening of *E.coli* colonies transformed with the ligation products of the purified right arm of *msrB* from plasmid pHD3 and plasmid pHD2.

Lanes A, B, D, E, G, H, J, and K: PCR products from ten different potential pHD4 transformants
Lanes C, I: DNA Standard Marker III

B.

BLAST analysis of DNA sequencing results

The BLASTN algorithm was used to compare the sequence of plasmid pHD4 with the non-redundant DNA database. Significant homology was observed with the *S. pombe ura4*$^{+}$ gene and cosmid c216 sequences (asterisks).



Sequences producing significant alignments:		Score (bits)	E Value	
gi|31044413|gb|L25928.3|YSPSEQB	Shuttle vector pJK210, comp...	842	0.0	
gi|5133|emb|X13976.1|SPURA4	Schizosaccharomyces pombe ura4 ...	842	0.0	*
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=search&term=5133[NUID]				
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=search&term=5133[NUID]				
gi|3646441|emb|AL031603.1|SPCC330	S.pombe chromosome III co...	842	0.0	
gi|22347652|gb|AF531173.1|	Cloning vector pDblet, complete ...	841	0.0	
gi|37654597|gb|AY303168.1|	Degron tagging vector pSMUG2+ be...	839	0.0	
gi|37654593|gb|AY303167.1|	YFP Integration vector pSMUY2+ b...	839	0.0	
gi|37654589|gb|AY303166.1|	CFP Integration vector pSMUC2+ b...	839	0.0	
gi|19682862|emb|AJ439454.1|CVE439454	Cloning vector pSMUG+d...	839	0.0	
gi|13928064|emb|AJ306911.1|IVE306911	Integration vector pSM...	839	0.0	
gi|6453570|emb|AJ250107.1|IVE250107	Integration vector pSMU...	839	0.0	
gi|4581501|emb|AL049558.1|SPBC216	S.pombe chromosome II cos...	359	9e-96	*
gi|237253|gb|S60039.1|S60039	3' end of ura4 {3' region} [Sc...	289	7e-75	

with either pKP4 or pHD4. Frozen glycerol stocks of these potential mutants were then made and stored in the freezer.

III. Proving the existence of the mutant strains

To determine if the *ura4*$^+$ gene had replaced the *msr* genes in transformants, colony PCR was performed with primers specific for the two *msr* genes. The primers corresponded to coding sequence of the *msr* genes, so would not be expected to primer amplification reactions in mutant strains of pKP4 (Δ*msrA*) and pHD4 (Δ*msrB*). Three normal zymolase colony PCR reactions were set up for each strain, two of which contained different *msrA* primer sets and the third of which contained a *msrB* primer set (seen in Figure 15). The purpose of this negative result experiment was to produce a PCR product only in genes that were not deleted, i.e. a Δ*msrA* strain would generate an amplification with *msrB* primers. The deleted gene would not produce a product with its own primers; thereby indirectly verifying that the homologous recombination event needed to replace the *msr* gene with the *ura4*$^+$ gene had taken place. The *msrB* primer set (SP38 and SP44) used with the potential Δ*msrA* (pKP4) strains produced a PCR product of 416 bp, and no PCR product with either of the *msrA* primer sets (SP43 and SP36, or SP43 and SP37). This is shown in Lanes H-M in Figure 16A, where no PCR product is visible for the Δ*msrA* strains when the PCR was performed with the *msrA* primers, and a PCR product of the correct size for the *msrB* gene is visible for the PCR with the *msrB* primers. One *msrA* primer set for the potential Δ*msrB* (pHD4) strains produced a PCR product of 564 bp in some cases, a second PCR product of 506 bp with the alternate primer set, and no PCR product with the *msrB* primer sets. This is shown in Lanes F-G in Figure 16A and in Lanes D-E and I-J in Figure 16B, where no PCR product is visible for the Δ*msrB* strains when the PCR was performed with the *msrB*

primers, and a PCR product of the correct size for the *msrA* gene is visible for the PCR with the *msrA* primers. Based on this analysis, HD4 strains 5 and 6 were tentatively identified as Δ*msr* mutants and KP4 strains 1, 3, and 8 were tentatively identified as Δ*msrA* mutants.

IV. Hydrogen Peroxide Sensitivity Test

The three Δ*msrA* strains (labeled KP4-1, KP4-3, and KP4-8) and the two Δ*msrB* strains (HD4-5 and HD4-6) identified in Figure 16 were then selected for the hydrogen peroxide sensitivity test. The wildtype *S.pombe* strain FWP10, or 972 (h^-) was used as the control to test for normal yeast growth under the oxidative stress conditions presented by the addition of hydrogen peroxide (H_2O_2) to the growth media. Each strain was grown in YEA media overnight, and then the cells were diluted to the same starting concentration (2×10^6 cells/mL). A 0.1 M H_2O_2 solution was added to each culture to achieve a final concentration of 5 mM H_2O_2 and cells were grown at 30°C. At each of four different time points (including time zero, right after the addition of the hydrogen peroxide solution) three 10-fold serial dilutions were made for each strain, and the dilutions were plated on YEA plates in 5 μL spots, including one undiluted spot for each time point Plates were grown at 30°C for three days at which time plates were photographed. The results are shown in Figure 17. These results show that for all time points, the control strain (972) grew to a greater cell density at later time points and at larger dilution factors, when compared with the five mutant strains (three of Δ*msrA* and two of Δ*msrB*).. All five mutant strains seem to have approximately the same amount of decrease in growth as compared with the control. As the time after the addition of the hydrogen peroxide increases, the concentration of cells in each dilution decreased (most visible in the spots for the 1:100 and 1:1000 dilutions). There was a significantly larger decrease seen in all five of the mutant strains

Figure 15 : Negative Test *msrA* and *msrB* primers

MsrA (SPAC29E6)

SP43 → Forward: nucleotides 8292 to 8313 (24447 to 24469 for alternate cosmid SPAC30)

5'-ATG CAG ATT GCT ATT ATT GCT GC-3'

SP36 → Reverse: nucleotides 7753 to 7775

5'-AAA ACA TTC CAA CGC AAA AAA TC-3'

SP37 → Alternate Reverse: nucleotides 23905 to 23923

5'-CTC AAA AAC ATT CCA ACG C-3'

msrB (SPBC216)

SP38 → Forward: nucleotide 7666 to 7689

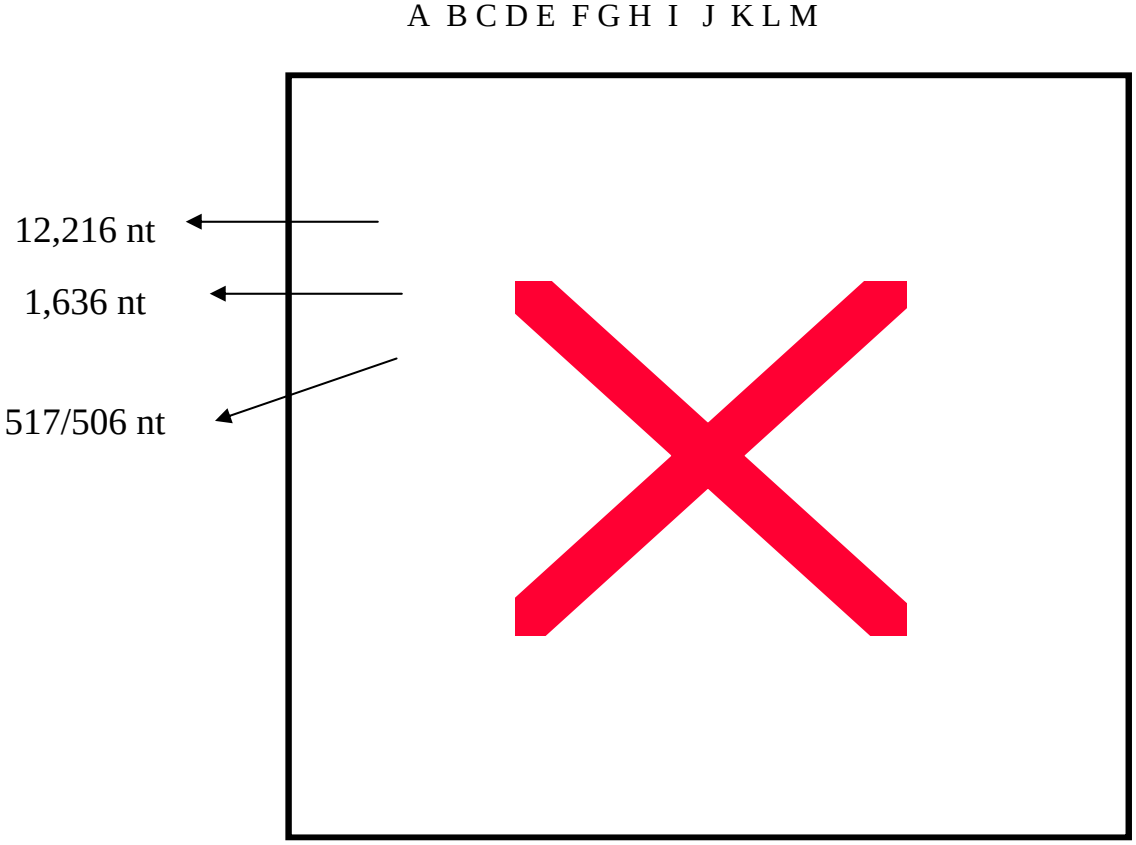
5'-ATG GGA TTT CCA TTG GAA AAA AGC-3'

SP44 → Reverse: nucleotides 7276 to 7300

5'-ATT GTC ATT TGT TGC CTC ATT ATG G-3'

Figure 16

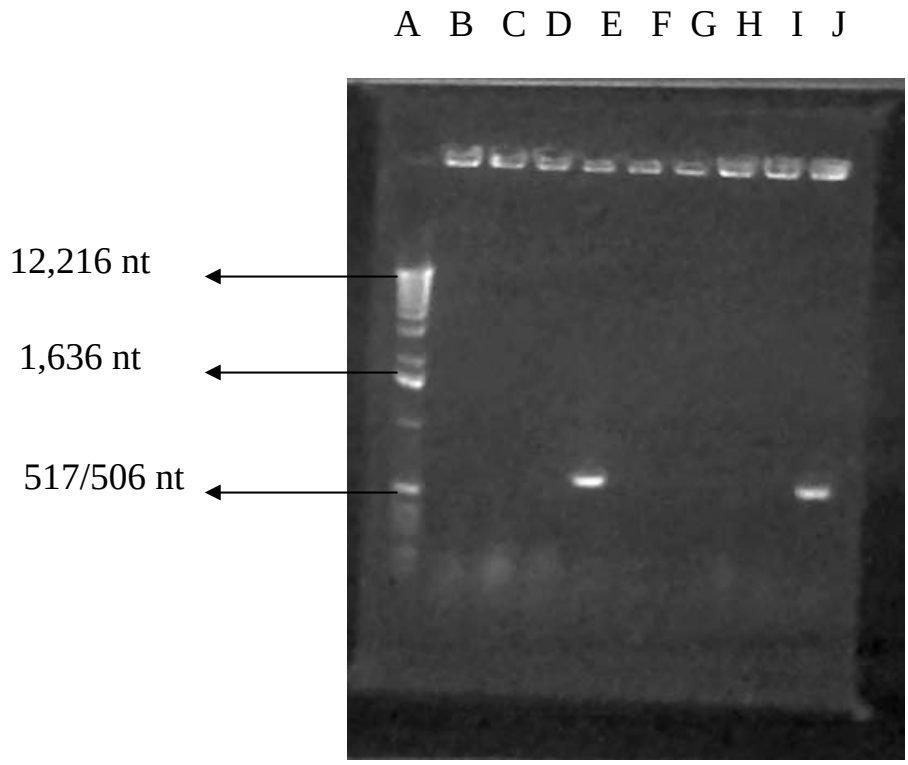
A.



Colony PCR of potential *msr* deletion strains
A 1% agarose gel was used to separate the amplification products obtained from a lyticase colony PCR procedure using primers designed within the coding region of either the *msrA* or *msrB* genes. Both sets of primers were used on all potential mutant strains. Strain KP4 was confirmed as a Δ*msrA* strain and strain HD4 was confirmed as a Δ*msrB* strain. The expected amplification product with *msrA* primers SP36 and SP43 is 564 base pairs. The expected amplification product with *msrB* primers SP38 and SP44 is 416 base pairs.

Lane A: DNA Standard Marker X
Lane B: Potential HD4 (#1) with *msrA* primers
Lane C: Potential HD4 (#1) with *msrB* primers
Lane D Potential HD4 (#2) with *msrA* primers
Lane E: Potential HD4 (#2) with *msrB* primers
Lane F: Potential HD4 (#3) with *msrA* primers
Lane G: Potential HD4 (#3) with *msrB* primers
Lane H: Potential KP4 (#1) with *msrA* primers
Lane I: Potential KP4 (#1) with *msrB* primers
Lane J: Potential KP4 (#2) with *msrA* primers
Lane K: Potential KP4 (#2) with *msrB* primers
Lane L: Potential KP4 (#3) with *msrA* primers
Lane M: Potential KP4 (#3) with *msrB* primers

B.

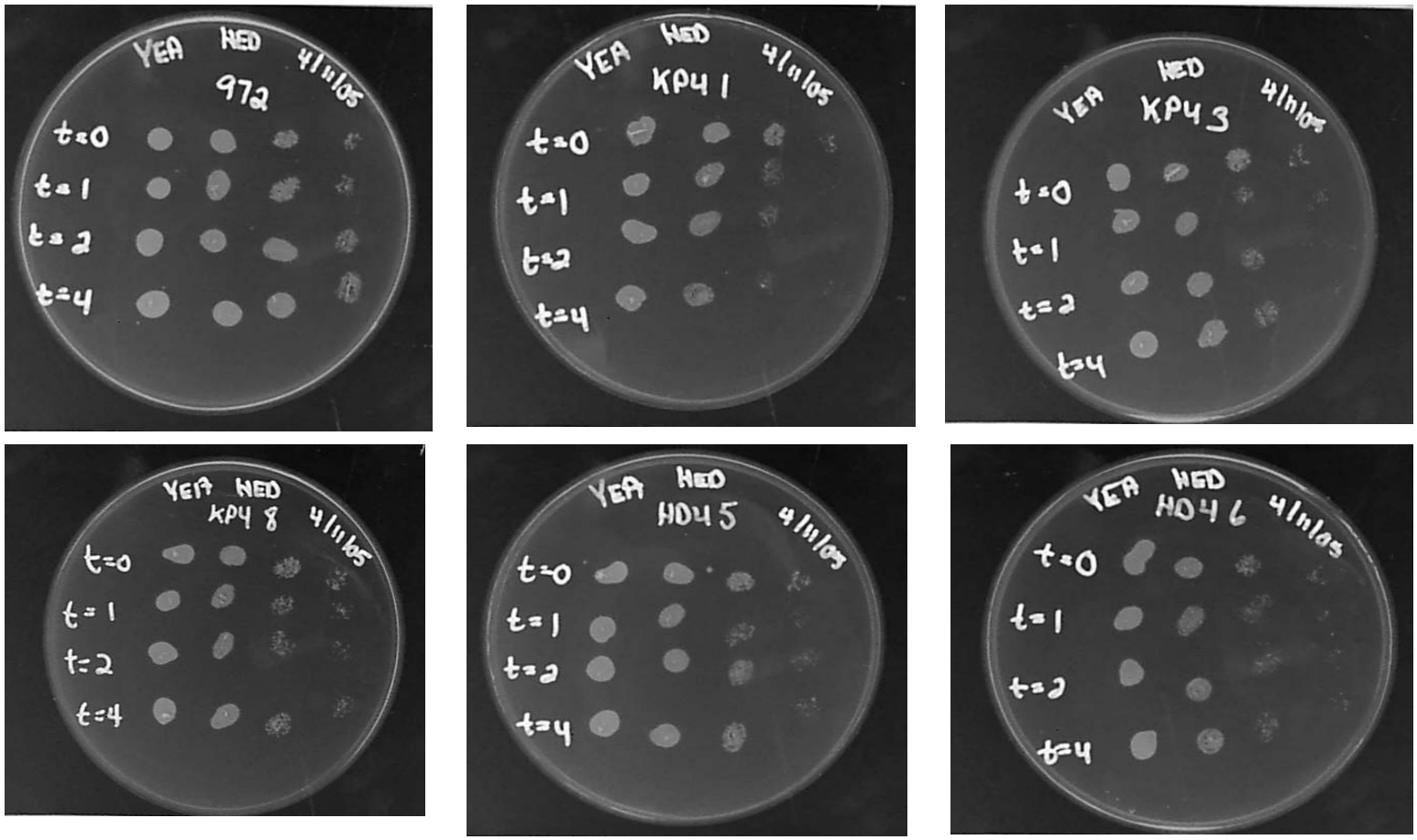


Colony PCR of potential *msr* deletion strains
A 1% agarose gel was used to separate the amplification products obtained from a lyticase colony PCR procedure using primers designed within the coding region of either the *msrA* or *msrB* genes. Both sets of primers were used on all potential mutant strains. Strain KP4 was confirmed as a Δ*msrA* strain and strain HD4 was confirmed as a Δ*msrB* strain. The expected amplification product with *msrA* primers SP36 and SP43 is 564 base pairs. The expected amplification product with *msrB* primers SP38 and SP44 is 416 base pairs.

Lane A: DNA Standard Marker X
Lane B: Potential HD4 (#4) with *msrA* primers
Lane C: Potential HD4 (#4) with alternate *msrA* primers
Lane D Potential HD4 (#4) with *msrB* primers
Lane E: Potential HD4 (#5) with *msrA* primers
Lane F: Potential HD4 (#5) with alternate *msrA* primers
Lane G: Potential HD4 (#5) with *msrB* primers
Lane H: Potential HD4 (#1) with alternate *msrA* primers
Lane I: Potential HD4 (#2) with alternate *msrA* primers
Lane J: Potential HD4 (#3) with alternate *msrA* primers

as compared with the 972 control strain. In addition, in a comparison of the decrease in cell concentration at the 1:100 and 1:1000 dilutions of the Δ*msrA* and Δ*msrB* mutants were equivalent, with no significant difference between the populations of the two mutant strains.

Figure 17



Hydrogen peroxide sensitivity test results

The spot plates represent four serial dilutions, from left to right in each row; 1:1, 1:10, 1:100, 1:1000. Spot plates were prepared for one control strain (972, top left corner), three possible pKP4, or Δ*msrA*, mutants (middle and top right corner, bottom left corner), and two possible pHD4, or Δ*msrB*, mutants (middle and bottom right corner). The cultures were treated with a 0.1 M hydrogen peroxide solution to a final concentration of 5 mM H_2O_2. Dilutions were made at each of four time points, and spots were then made for each dilution on YEA plates.

Chapter 4: Discussion and Future Work

The majority of this project was spent generating the appropriate clones for homologous recombination with the *msrA* and *msrB* genes in a wildtype strain of *Schizosaccharomyces pombe*. Flanking regions downstream and upstream of each gene were isolated and amplified, and then cloned into a vector plasmid containing the *ura4*$^+$ gene. The flanking regions were insert on either side of the *ura4*$^+$ gene, which would replace the *msr* gene in the wildtype yeast strain, and also act as a selectable marker to show whether the homologous recombination event had occurred, thereby generating a knockout of the *msr* gene in question.

The fact that the several of the potential knockout strains grew on PM media lacking uracil (after the transformation with the salmon sperm carrier DNA) indicated that these strains were capable of uracil biosnythesis. The only way these strains would be able to synthesize uracil would be if a recombination even had occurred, inserting the *ura4*$^+$ gene from the vector plasmid into the wildtype strain chromosome. These recombination events could be either homologous or nonhomologous situations, with only the homologous recombination situations producing the desired *msr* knockouts. The negative PCR experiment using both the corresponding and non-corresponding primers for each gene was a way to test for the existence of either homologous or nonhomologous recombination events. In Lane C of Figure 16A, the presence of a product for a potential Δ*msrB* knockout strain using the *msrB* primers indicates a nonhomologous recombination event. The strain did grow on PM media lacking uracil, which indicates the presence of the *ura4*$^+$ gene, but the PCR results also indicate the presence of the *msrB* gene, indicating that homologous recombination did not occur. Many of the other potential mutant strains (for both *msrA* and *msrB*) give products only with the opposite set of primers,

indicating that it is likely that a homologous recombination event did occur. These strains were then selected as likely candidates for knockout strains of the respective strains.

The hydrogen peroxide sensitivity test gave further proof that the five selected mutant strains were indeed knockout mutants for either *msrA* or *msrB*. As seen in Figure 17, all of the knockout strains of both *msrA* and *msrB* showed a significant decrease in cell concentration with increasing length of exposure to a 5 mM concentration of hydrogen peroxide solution, as compared with a wildtype control strain. An important fact is that the decrease in cell concentration between the Δ*msrA* and Δ*msrB* strains was approximately equal. According to previous research, the MsrA protein is the major contributor to the repair of damage to methionine residues in proteins with MsrB playing only a minor part. The results of this one study indicate that a loss of the *msrA* gene had a significant affect on the viability of the yeast *S.cerevisiae*, causing a 26% decrease in the longevity of the cells. The loss of the *msrB* gene had little affect on the viability of the yeast, and a Δ*msrA*Δ*msrB* double mutant produced a larger reduction in viability than the loss of *msrA* alone (Koc et al., 2004). This information would indicate that the Δ*msrA* strains should show a larger decrease in cell concentration than the Δ*msrB* strains, which was not the result found in the peroxide sensitivity experiment. The results of the clearly contradict this previous research, showing an equal reduction in the viability of the yeast for both Δ*msrA* and Δ*msrB* mutants. This discrepancy could be due to the fact that previous researchers used a different method of placing oxidative stress on their knockout yeast strains.

In addition, another study stated that, in mice, only when the *msrA* gene had been disrupted the significance of the *msrB* gene was apparent, possibly reflecting an unmasking or up-regulation of *msrB* in the absence of *msrA*. This study also showed that MsrB protein activity

in the liver and kidney was only 35% of the wildtype level of activity when tested in a Δ*msrA* mutant, and only 75% of the wild type activity in the brain and lungs (Moskovitz et al., 2001). This is further evidence that the Δ*msrA* and Δ*msrB* mutant strains should not show the same level of decrease in cell concentration in response to oxidative stress, but according to the results of the peroxide sensitivity experiment in Figure 17, this was not found to be the case. Obviously more research needs to be done, perhaps including a repeated of the hydrogen peroxide sensitivity experiment as well as working with other oxidative stress situations, in order to prove the validity of these results and the potential discrepancies they indicate.

One of the most important next steps in this project is to verify that the *ura4*$^+$ gene has replaced the endogenous *msr* genes in knockout strains. This was attempted using PCR with the leftmost and rightmost primer of each arm for each *msr* gene. The results produced only primer dimers, however, and did not allow us to verify the absence of the *msr* genes. Successful homologous recombination would be detected by a PCR fragment of 2.16 kilobases (for replacement of *msrA*) or 2.18 kilobases (for replacement of *msrB*) respectively, consistent with the substitution of the larger *ura4*$^+$ gene for the smaller endogenous *msr* genes. So, a first step would be to work on creating a set of primers, possibly outside of the flanking regions used to generate the clones for the homologous recombination knockout, that allows for size verification of the recombination event. An alternative would be to use Southern blotting to verify the presence/absence of the *msr* and *ura4*$^+$ genes in various strains.

Another important area for further investigation is to continue the phenotyping of the Δ*msrA* and Δ*msrB* knockout strains. A more detailed phenotypic analysis would determine if there are any effects beyond those elucidated in the hydrogen peroxide sensitivity experiment. Another important future project would involve mating the two knockout strains (Δ*msrA* and

Δ *msrB*) to create a double mutant strain (Δ *msrA* Δ *msrB*) of *S.pombe*, in order to observe the effects of a loss of both methionine sulfoxide reductase activities. This experiment would provide important information about whether the MsrA and MsrB activities show redundancy and the ability to compensate for the loss of one another's activities.

Another future direction, in a project that is currently underway, is to create *msrA* and *msrB* overexpressor strains of *S.pombe*, in order to study the effects of an excess of the methionine sulfoxide reductase genes on the life cycle and longevity of the yeast. The plasmids used for the overexpressors will also be useful in rescue experiments with Δ *msrA* and Δ *msrB* strains.

It is obvious from previous research done in several model organisms, including budding and fission yeast, fruit flies, and mice, that the methionine sulfoxide reductase genes serve an important function in the protein repair process. As these genes are continually studied and characterized, the relevance of this information to humans cannot be overlooked. In this respect, recent studies have linked a decrease in methionine sulfoxide reductase activity with Alzheimer's disease, and overexpression of the Msr protein is reported to increase the lifespan of human lymphocytes (Gabbita et al., 1999; Moskovitz et al., 1998). This fission yeast presents a simple model for analyzing metabolic relationships of Msr activities and other proteins, such that results obtained with this model will aid our understanding of human health.

References

Chenna, Ramu, Sugawara, Hideaki, Koike,Tadashi, Lopez, Rodrigo, Gibson, Toby J, Higgins, Desmond G, Thompson, Julie D. Multiple sequence alignment with the Clustal series of programs. (2003) *Nucleic Acids Res 31 (13):3497-500 PubMedID: 12824352*

Gabbita, S.P., M.Y Aksenov, M.A. Lowell, and W.R. Markesbery. 1999. Decrease in Peptide Methionine Sulfoxide Reductase in Alzheimer's Disease Brain. *Journal of Neurochemistry.* 73: 1660-1666

Grimaud, R., B. Ezraty, J.K. Mitchell, D. Lafitte, C. Briand, P.J. Derrick, and F. Barras. 2001. Repairs of oxidized proteins: identification of a new methionine sulfoxide reductase. *The Journal of Biological Chemistry.* 276: 48915-48920

Koc, A., A.P. Gasch, J.C. Rutherford, H. Kim, and V.N. Gladyshev. 2004. Methionine sulfoxide reductase regulation of yeast lifespan reveals reactive oxygen species-dependent and –independent components of aging. *Proceedings of the National Academy of Sciences of the United States of America.* 101: 7999-8004

Kumar, R.A., A. Koc, R.L. Cerny, and V.N.Gladyshev. 2002. Reaction Mechanism, evolutionary analysis, and role of zinc in *Drosophila* methionine-*R*-sulfoxide reductase. *The Journal of Biological Chemistry.* 277: 37527-37535

Moskovitz, J., E. Flescher, S.B. Berlett, J.A. Azare, M. Poston, and E.R. Stadtman. 1998. Overexpression of peptide-methionine sulfoxide reductase in *Saccharomyces cerevisiae* and human T cells provides them with high resistance to oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America.* 95(24): 14071-14075

Moskovitz, J., S. Bar-Noy, W.M. Williams, J. Requena, B.S. Berlett, and E.R. Stadtman. 2001. Methionine sulfoxide reductase (MsrA) is a regulator of antioxidant defense and lifespan in mammals. *Proceedings of the National Academy of Sciences of the United States of America.* 98: 12920-12925

Ruan, H., X.D. Tang, M. Chen, M.A. Joiner, G. Sun, N. Brot, H. Weissbach., S.H. Heinemann, L. Iverson, C. Wu, and T. Hoshi. 2002. High-quality life extension by the enzyme peptide methionine sulfoxide reductase. *Proceedings of the National Academy of Sciences of the United States of America.* 99: 2748-2753

Sambrook, J., D.W. Russell. 2001. *Molecular Cloning: A Laboratory Manual*. J. Argentine, D.Brown, S. Curtis, N. Irwin, K.A. Janssen, N. McInerny, S. Schaefer and M. Zierler, editors Cold Spring Harbor Laboratory Press, Cold Harbor Spring, New York.

Acknowledgements

First and foremost, I would like to thank Dr. Clare O'Connor for giving me the amazing opportunity to work in her research laboratory for the past two years. She is truly a great scientist and an amazing teacher, and I feel privileged to have had the chance to work with her.

I would be remiss if I did not extend my deepest thanks to Linda Tanini, without whom there is no way I would have survived the past two years. She taught me what it truly means to be a scientist in every sense of the word, including having confidence in my abilities and skills in the laboratory. I would also like to thank Dean Wagner, who has been a constant source of guidance and inspiration. This project would not have been possible without his assistance.

To Krissy Pattin and Scott Davis – thank you for your help in the cloning of *msrA*; it was an adventure, but we finally did it! To "Stabilized" Jim Casey, Ashley Walther, Alisha Cutler, and of course, my partner Meghan Rice – thank you for making my last semester in the lab something really special.

Words can never properly express my gratitude for the unconditional love and support of my mother, Sharon DeFoer. Without her presence and light in my life every day, I would not be the person I am, and I would never have the confidence to work to become the person I want to be.

Last, but certainly not least, I would like thank the most amazing group of women I have ever met, or ever will meet in my lifetime. I came into Walsh 306 our sophomore year filled with trepidation and anxiety, but I'm leaving the third floor of Gabelli feeling like the luckiest person in the world to have shared the last three years with the seven of you. So, thank you to Joanna Sweeney, Alyssa Hadley, Jacqueline Croteau, Beth Sullivan, Katie Desbois, Breanna Finneron, and Emily Dendinger – you have all truly changed my life.