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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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ENTITLED Construction and Characterization of Saccharomyces

Cerevesiae Catalase Null Mutants

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF Bachelor of Science in Biochemistry

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CONSTRUCTION AND CHARACTERIZATION OF *SACCHAROMYCES CEREVESIAE* CATALASE NULL MUTANTS

BY

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THESIS

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TABLE OF CONTENTS

Introduction	3
Methods and Materials	7
Results	9
Discussion	11
Acknowledgements	13
Figures	14
References	17

INTRODUCTION

Free atmospheric oxygen (O_2) has become, since its emergence around two billion years ago, both an indespensible benefit and and unpredictable nuisance. Oxygen can be oxidized both intra- and extracellularly to produce three highly reactive species: superoxide ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\cdot}$). These oxidants have been found to be the cause of damage to DNA, proteins, and lipids. Oxidation of DNA by these oxidants is linked to mutagenesis, which may lead to tumorigenesis (12, 18, 22, 36). The accelerated oxidation of proteins has been shown to be a factor in the genetic disorders as progeria and Werner's disease (32), and oxidative protein damage has been linked to Down's syndrome, Alzheimer's disease, and even the overall aging process (10). Cytotoxicity of oxygen free radicals is mainly attributed to the degradation of lipids in membranes by the peroxidation of fatty acids (14). This peroxidation increases the permeability of the membrane, thereby short-circuiting the gradient potential on the membrane. The peroxidation of lipids in low density lipoproteins (LDLs) may be a factor in atherogenesis (35).

The superoxide radical is generated by the oxidation of O_2 and is the natural by-product of several cellular enzymes such as xanthine oxidase, oxygenases and peroxidases, as well as hemoglobin (26). $O_2^{\cdot}$ is a charged molecule and does not permeate membranes easily (39); this and the fact that $O_2^{\cdot}$ also causes hydroperoxidation to lipids suggests that $O_2^{\cdot}$ plays a role in lysosomal phagocytic activity in macrophage ingestion (19). Some cells may use $O_2^{\cdot}$ as a metabolic or defensive tool. For instance, in periods of oxygen starvation follwed by a reoxygenation of endothelial

cells, xanthine oxidase will produce a burst of superoxide radical production (51). This effect may be the major cause of cellular damage immediately following heart attacks.

Hydrogen peroxide is a naturally generated oxidation product of $O_2^{\cdot}$; H_2O_2 is perhaps the most studied oxygen free radical metabolite due to its ability to permeate membranes easily and because it is a sluggish oxidant. It is produced by the same oxygenases, flavoproteins, and peroxidases as $O_2^{\cdot}$. However, $O_2^{\cdot}$ and H_2O_2 can react in a Haber-Weiss reaction to form the hydroxyl radical, $OH^{\cdot}$; this reaction is accelerated in the presence of free transition metals such as Fe^{3+} in a Fenton reaction (4).

$OH^{\cdot}$ is probably the least known and most destructive of the three radicals due to its fleeting existence and severe reactivity. $OH^{\cdot}$ can arise from the above reactions, and it is generated from the ionization of water by X-rays (27). Because it is so reactive, it will oxidize the first molecule it encounters (23, 11). Protein damage may result from this oxidation, cleaving the proteins at specific residues, such as tyrosine or histidine (21). Its effects can be seen by indirectly as oxidative damage to intracellular molecules, production of radical scavenging molecules as uric acid and ascorbate, and spin trapping methods using aprotic solvents (47).

The cell has a number of defenses against these oxidants. Low molecular weight radical scavengers, such as uric acid, ascorbate, pyrimidines, and nicotinamide dinucleotide (NAD^+), play a minor role (3, 27). Compartmentalizing the enzymes which produce radicals has been suggested as a passive defense (many are found within organelles such as peroxisomes and lysosomes) (13, 46, 49). However, the major line of defense lies with the antioxidant enzymes; that is, enzymes which metabolize oxygen free radicals. In *Saccharomyces cerevesiae* there are five such enzymes.

Cu/Zn superoxide dismutase (SOD1) is found in the cytosol and catalyzes the reaction $2\,O_2^{\cdot} \rightarrow H_2O_2 + O_2$. MnSOD (SOD2) is a nuclear gene whose protein product is found in the mitochondrial matrix. It performs the same task as SOD1.

SOD2 is thought to be a "higher form" of superoxide dismutase than SOD1 or bacterial FeSOD, because it is both imported into the mitochondria from the cytosol and is induced transcriptionally by heat shock, oxidative stress, and high redox potential molecules such as NO_3^-, SO_4^{-2}, and $Fe(CN)_6^{-3}$, while SOD1 is expressed constitutively (2, 34, 44). Although NO_3^- can be used as a terminal electron acceptor, SO_4^{-2} and CN^- cannot; it is thought that SOD2 is not regulated by a generic respiratory control system but by a separate oxidative-damage induction system (6, 38).

Cytochrome C peroxidase (CCP) is also encoded in the nucleus, but its product is imported into the mitochondrial intermembrane space. It utilizes H_2O_2 to oxidize cytochrome C and provides cytochrome C with a proton. H_2O_2 arises from O_2^- produced by ubiquinol and ubisemiquinone in the electron transfer chain within the mitochondria. During rapid growth, CCP is induced (presumably by oxygen and heme levels) and is so efficient virtually no H_2O_2 builds up in the mitochondria (7).

Two different catalase enzymes are found in two cellular locations and forms; the cytosolic enzyme is known as catalase T (CTT) and the peroxisomal enzyme is catalase A (CTA). Their function is to catalyze the reaction $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$. In low concentrations of H_2O_2, however, catalase catalyzes the peroxidatic reactions $H_2O_2 + AH_2 \rightarrow A + 2\ H_2O$, where AH_2 represents a two-electron donor (such as ethanol or formic acid), and $H_2O_2 + 2\ AH \rightarrow 2\ H_2O + 2\ A$, where AH represents a one electron donor (such as sodium nitrite). In high concentrations of hydrogen peroxide, AH_2 is H_2O_2. Glutathione peroxidase catalyzes the peroxidatic reactions in eukaryotic cells, but *S. cerevesiae* does not possess this enzyme (1, 11, 48); it has been suggested that since normal cellular levels of H_2O_2 in yeast is below 10^{-6} M, the peroxidatic reactions may be the major function of catalase (33).

Catalase is controlled by carbon source, O_2, and heme levels in yeast. Its translation is completely repressed by high glucose levels, while its translation is

activated by heme, which is in turn activated by oxygen (15). It is thought to be controlled by an overall system that enables yeast to switch from anaerobic to aerobic growth.

In order to assess the global effect of CTT and CTA activity, and since these two enzymes act interdependently with the other anti-oxidant enzymes, it is interesting to ask whether a cell would grow in the functional absence of either CTT or CTA, or both. The goal of this thesis was to construct null mutants of CTT and CTA and characterize the phenotype of these mutants. To accomplish this, a knockout mutation was made in CTT and CTA by replacing their coding sequences with selectable auxotrophic markers. These constructs were used in a one-step gene disruption to create CTT and CTA null mutants in *S. cerevesiae*. Similar constructs of SOD2 and CCP have been made in hopes of crossing the null mutants to produce double or triple antioxidant null mutants.

METHODS AND MATERIALS

Reagents and Chemicals

All chemicals used were reagent grade. All enzymes, kits, and materials were used to suppliers' specifications. Standard cloning and DNA manipulation procedures were as described (24) unless otherwise stated.

Plasmids and Bacterial Strains

pGEM3Z$^-$ (Promega) was used as a vector for plasmids containing SOD2 (pGTSB1), CTT (pGSCT1), and CTA (pGTCA1) (constructed and mapped in the Kaput lab); DH5α (*F-, Φ80d, lacZM15, (lacZYA-argF), U169, recA1, endA1, hsdR17 (rK-, mK+), supE44, Lambda-, thi1, gyrA, relA1*) and RR1 (*hsdS20 (rB-, mB-), supE44, ara14, Lambda-, galK2, lacY1, proA2, rpsL20, xyl-5, mtl-1, recA+....Restriction: (r-, m-), mcrA(+), mcrB(-)*) strains were used for selection, transformation, and subsequent amplification of constructed plasmids. The sequence for SOD2 was obtained from (25). The plasmids and sequences for ctt::URA3 and cta::URA3 were obtained courtesy of H. Ruis (9, 16, 43).

Yeast Strains

The isogenic wild type strains, *Saccharomyces cerevesiae* DBY 746 (Mat α) and DBY 747 (Mat a), were used in haploid form. The genotype for DBY 746 and 747 are *ura3-52, leu2-3, 112 his3-Δ1*, and *trp1-289am*; 747 is also *gal1can1* for selection. Thus, a total of four auxotrophic markers can be used in creating double and triple antioxidant null mutants. An ADE2 blaster was constructed and used to create a fifth auxotrophic mutation.

Transformation of yeast was done by pretreating the cells with LiCl, incubating the cells with linearized plasmid constructs in the presence of sonicated fish sperm DNA in ~25% PEG, heat shocking at 40^{0}C, and recovering at 30^{0}C in nonselective media (37). The transformed cells were selected on synthetic media agar plates containing essential amino acids and nucleosides minus the selected auxotrophic marker products. Rates of transformation were near normal (data not shown).

RESULTS

Plasmid Construction

In order to create antioxidant null mutants, the method of one-step gene disruption was employed. This involved making knockout mutations within the gene encoding the antioxidant enzyme and replacing these sequences with an auxotrophic marker. The marker gene is thus flanked with the ends of the antioxidant gene locus. Homologous recombination in yeast results from base pairing of the ends of linear DNA with wild-type gene in the chromosome. Hence, the functional gene is replaced with the construct (37). The plasmids containing SOD2, CTT, and CTA were digested and the fragments containing the ends of the gene locus were isolated. The auxotrophic markers URA3, HIS3, and ADE2 were then individually ligated to the ends of the above fragments, respectively (see figure 1 and 2). First attempts to construct the plasmids by creating blunt ends before ligation were unsuccessful. However, insertion of the auxotrophic markers was done by using complementary restriction sites in the vector and insert. The plasmids, pGTSB1::URA3, pGSCT1::HIS3 and pGTCA1::ADE2 (pGTSB1 = SOD2, pGSCT1 = CTT, pGTCA1 = CTA, in pGEM3Z-), containing ampicillin resistance genes, were transformed into bacteria, and selected on plates containing 0.100 g/L ampicillin. The correct plasmids were determined by restriction mapping (see figures 3, 4, and 5).

Characterization

Tests of actual Chromosomal gene disruption were done using Southern blotting of endonuclease digested total yeast chromosomal DNA (42). ^{32}P-labelled probes were made by the random priming method using EcoR1 digested fragements of either CTT or

CTA as templates. Wild-type plasmid DNA was cut, blotted, and probed along side potential null mutants as a control.

The fragment encoding CTA was larger than the auxotrophic marker URA3 inserted in its place; hence the disrupted allele will be smaller (see figure 6). The fragment containing the disrupted CTT allele, however, contained an EcoR1 site within the auxotrophic marker. Since EcoR1 was used to digest the total yeast DNA, the wild-type DNA would hybridize to two DNA fragments, while the null mutant DNA hybridized in a single, higher band. The transformant DNA for the ctt::URA3 null mutant showed this pattern, but pGSCT1::HIS3 showed both null mutant and wild-type genotype (figure 7). It was assumed that under both the increased oxygen stress and selective pressure the transformed haploid cells mated and became diploid cells, thus giving rise to a diploid cell containing one wild-type and one disrupted allele. A third transformation of yeast cells was done except that the recovery time following transformation was increased and the null mutants were grown on selective media under anaerobic conditions. These conditions were chosen to reduce the stress on the cells. Southern blots for the potential mutant strains have not been completed as yet.

DISCUSSION

The plasmids pGTSB1::URA3, pGTCA1::ADE2, and pGSCT1::HIS3 have been constructed and mapped, and the yeast strains deficient in CTT and CTA have been characterized. Two other plasmids, ccp::URA3 and sod2::TRP1, have been made and mapped. Using these plasmids, four auxotrophically different yeast strains, each deficient in one antioxidant enzyme, can be created. Back-crossing to wild-type haploid will allow for sporulation and asci counts and may be necessary to prove the viability of the null mutants. If the strains are viable, mating the different haploid strains will produce double and triple null mutants, capable of being selected on synthetic media.

Mutants deficient in SOD2, CCP, CTT, and CTA have been characterized and used in the past, but they were either point mutations or null mutants with URA3 as an auxotrophic marker. Single null mutants were not inhibited to a great extent by aerobic conditions. However, yeast deficient in SOD2 showed amino acid biosynthetic defects (8). Yeast defective in CCP showed an increase in petite formation, which suggested an increase in mitochondrial damage. In other tests SOD2 was shown to play a more crucial role in cell survival than CTT or CTA. However, loss of one enzyme may be complemented by the activities of the other antioxidant enzymes (5, 17, 30, 50).

The effect of mutations in two or more enzymes are cellular physiology will be determined using a wide range of genetic and biochemical assays. Northern blots (RNA transcripts-DNA probes) will be used to quantify transcriptional responses of SOD2, CCP, or catalases A and T under different oxidative stress levels. Viability, possible cell cycle arrest, and mating efficiencies will also be determined under oxidative stresses. Because SOD2 and CCP are located in the mitochondria, effects to the mitochondria may result in petite formation, mtDNA damage, and ability to import cytoslic proteins. Damage to the nucleus may be estimated by reversion frequencies of point mutations,

and via the Ames test of oxygen radical generators such as paraquat (29). Lipid peroxidation will be assayed by measuring the intracellular levels of malonaldehyde, a product of alkenyl hydroperoxide decomposition, as well as by thiobarbituric acid or fluorescence assays.

These assays will lead to a deeper understanding of the actions and interactions of antioxidant enzymes and their overall effects on a cell's physiology. Results will paint a more thorough picture of the relation of oxygen to living systems in general. These findings may also help to uncover possible causes of such universal conditions as cancer, aging, and DNA mutations.

ACKNOWLEDGEMENTS

This project originally began as an uncertain desire to "do research" in biochemistry; gratitude goes to Dr. Kaput for allowing it to evolve into a thesis project. There must also be thanks and praise for the guiding hands of Wes Jennings and Marietta Piattoni, as well as all the advice and help from Sandy Kirchner, Denise Eckberg, Tim Elliot, and Debbie Swartz. Notice should not be denied to all the members of MSB 4th floor for putting up with occasional fits of impatience.

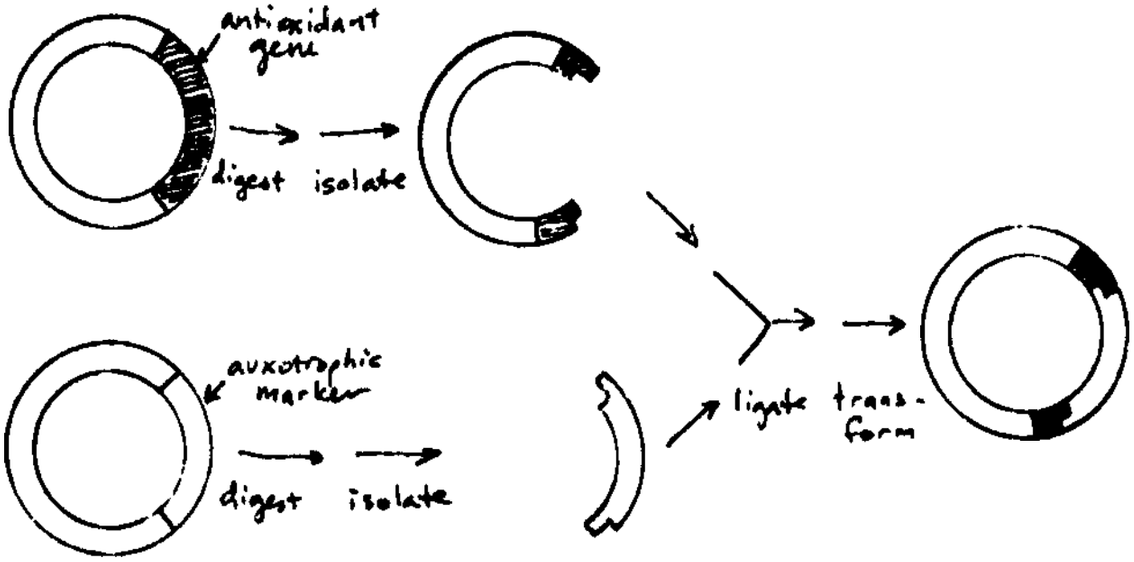

Figure (1): Schematic showing generic method of constructing knockout mutations in antioxidant genes with auxotrophic markers.

VECTOR	INSERT	FRAGMENT DELETED[α]	MARKER	FIGURE
pGTSB1::URA3	SOD2	+6 to +1234[β]	URA3	3
pGTCA1::ADE2	CTA1	-6 to +1560[γ]	ADE2	4
pGSCT1::HIS3	CTT1	+252 to +1507[δ]	HIS3	5

α Numbers refer to nucleotides deleted with the initiating A of the first ATG as nucleotide +1.
β SOD2 is 699 nucleotides long.
γ CTA1 is 1545 nucleotides long.
δ CTT1 is 1719 nucleotides long.

Figure (2): Table containing all plasmids constructed for this thesis.

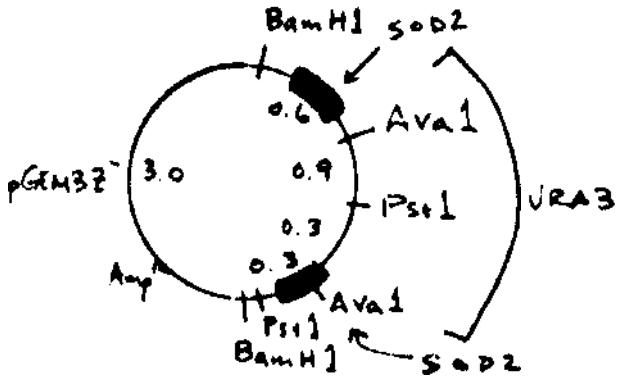

Figure (3): Map of pGTSB1::URA3.

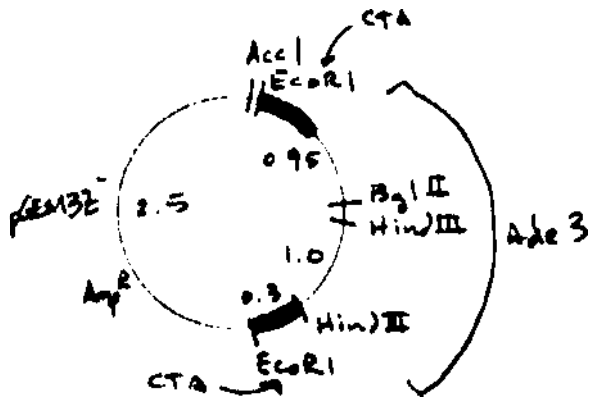

Figure (4): Map of pGTCA1::ADE2.

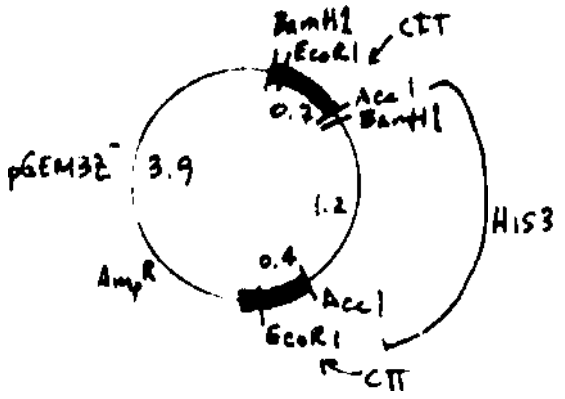

Figure (5): Map of pGSCT1::HIS3.

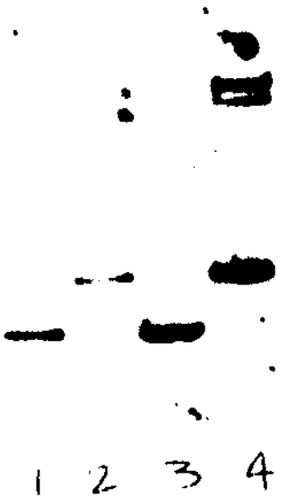

Figure (6): Southern blot of cta::URA3, hybridized with the EcoR1 fragment of CTA. Lanes 1 and 3 are cta::URA3 null mutants, lane 2 is a ctt::URA3 null mutant, and lane 4 is a pGSCT1::HIS3 null mutant.

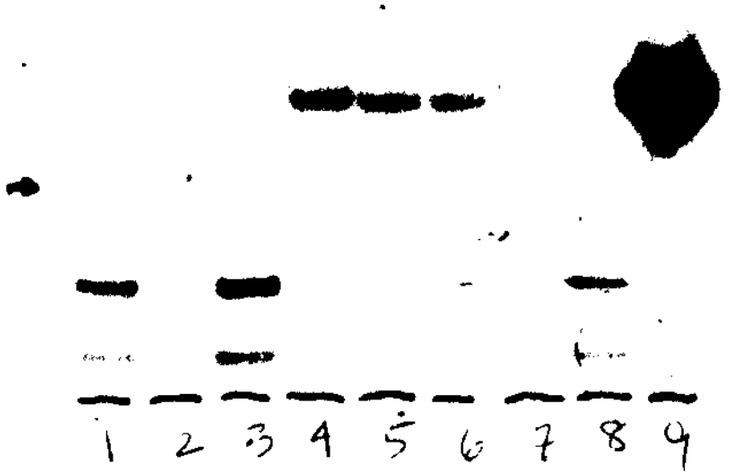

Figure (7): Southern blot of ctt::URA3 and pGSCT1::HIS3 null mutants, hybridized with the EcoR1 fragment of CTT. The ctt::URA3 single band can be seen faintly across from the arrow, while both the single band and wild type for pGSCT1::HIS3 null mutants are obvious. Lanes 1 and 3 are cta::URA3 null mutants, lane 2 is a ctt::URA3 null mutant, lanes 4, 5, and 6 are pGSCT1::HIS3 null mutants, and lanes 7 and 8 are wild-type controls. Lane 10 is the plasmid pGSCT1::HIS3 digested with EcoR1 as a control.

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