EFFECTS OF DELETING MITOCHONDRIAL ANTIOXIDANT GENES ON AGING

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ABSTRACT

EFFECTS OF DELETING MITOCHONDRIAL ANTIOXIDANT GENES ON AGING

Reactive oxygen species (ROS) damage biomolecules, accelerate aging, and shorten life span, whereas antioxidant enzymes mitigate these effects. Because mitochondria are a primary site of ROS generation and also a primary target of ROS attack, they have become a major focus area of aging studies. Here, we employed yeast genetics to identify mitochondrial antioxidant genes that are important for replicative life span. We found that among the known mitochondrial antioxidant genes (*TTR1*, *CCP1*, *SOD1*, *GLO4*, *TRR2*, *TRX3*, *CCS1*, *SOD2*, *GRX5*, *PRX1*), deletion *SOD1* (Cu, Zn superoxide dismutase), *SOD2* (Manganese-containing superoxide dismutase), and *CCS1* (Copper chaperone), shortened the life span enormously under normal conditions. The life span decreased 40% for Δ*sod1* mutant, 72% for Δ*sod2* mutant, and 50% for Δ*ccs1* mutant. When a respiratory carbon source was used in addition to Δ*sod1*, Δ*sod2* and Δ*ccs1*, deletion of *CCP1* (cytocrome c peroxidase) also lead to a decrease in life span which decreased% 79 for Δ*sod1* mutant, 87 % for Δ*sod2* mutant, 51 % for Δ*ccs1* and65 % for Δ*ccp1* mutant. Deletion of the other genes had little or no effect on life span for both conditions. To further investigate the role of these antioxidant genes molecular damages on lipids, proteins, and DNA were detected in mutants. The results showed that level of lipid peroxidation was usually lower when cells were grown under normal conditions. If cells were grown in respiratory substrate glycerol, deletion of *CCS1*, *SOD2*, *GRX2*, *CCP1*, *TRR2* and *PRX1* genes increased cellular lipid peroxidation levels by 87%, 73%, 65, 48%, 30% and 16% respectively. Protein carbonylation levels were 34% higher for Δccp1 and 87% higher for Δgrx2 mutants compared to WT cells when the cells were grown under normal conditions. However, it increased 65% for Δ*ccs1*, 61% for Δ*prx1*, 57% for Δ*glo4*, 55% for Δ*ccp1*, 49% for Δ*sod1*, 37% for Δ*sod2*, 33% for Δ*grx2*, 18% for Δ*trx3*, 17% for Δ*grx5* and 7% for Δ*trr2* when the cells were grown in the presence of glycerol. Q-PCR assay showed that deletion of *CCS1* and *PRX1* lead to DNA damages in mitochondrial DNA. Our overall results showed that some of the antioxidant mitochondrial mutants lived shorter and accumulated extensive molecular damages in the presence of respiratory carbon source.

ÖZET

MİTOKONDRİYAL ANTİOKSİDANT GENLERİN KAYBININ YAŞLANMA ÜZERİNE ETKİLERİ

Reaktif oksijen türleri (ROS) biyomoleküllerde hasar oluşturarak yaşlanmanın hızlanması ve yaşam süresinin kısalmasına sebep olurken antioksidan enzimler bu etkilerin tersini oluşturur. Mitokondriler ROS'ların birincil üretim yerleri olmaları ve reaktif oksijen türlerinden en çok etkilenmeleri sebebiyle yaşlanma çalışmalarında önemlidirler. Çalışmada, replikatif yaşam süresinde etkili mitokondrial antioksidan genlerin tanımlanması için maya genetiği kullanılmıştır. Çalışmamızda mitokondrial antioksidan genler (*TTR1*, *CCP1*, *SOD1*, *GLO4*, *TRR2*, *TRX3*, *CCS1*, *SOD2*, *GRX5*, *PRX1*) arasından, *SOD1* (Cu, Zn superoksit dismutaz), *SOD2* (Mn superoksit dismutaz) ve *CCS1* (Bakır Şaperonin) mutasyonları yaşam sürelerini düşürmüş olup bu düşme Δ*sod1* için 40%, Δ*sod2* için 72% ve Δccs1 için 50% olarak bulunmuştur. Bunun yanında solunumla metabolize edilen karbon kaynağı (gliserol) kullanıldığında Δ*sod1*, Δ*sod2* ve *Δccs1*' nin yanında *CCP1* mutasyonunun da yaşam süresinde kısalmaya sebep olduğu bulunmuş olup mutantların yaşam sürelerindeli kısalma Δ*sod1* için 79%, Δ*sod2* için 87% , Δ*ccs1* için 51% ve Δ*ccp1* için 65% olarak bulundu. Her iki ortam koşulunda da bazı gen mutasyonlarının yaşlanma üzerine çok az veya hiç etkisinin olmadığı bulundu. Mitokondrial antioxidant genlerin oksidatif stres üzerindeki rollerinin daha iyi anlaşılabilmesi için lipid, protein ve DNA'daki hasar oranları belirlendi. Normal şartlarda mutantların WT hücrelere göre daha az lipid peroksitlerini içerdikleri bulundu. Hücrelerin gliserol içeren besiyerinde büyütülmesiyle lipid peroksitlerinin seviyesi *CCS1*, *SOD2*, *GRX2*, *CCP1*, *TRR2* ve *PRX1* mutantları için sırasıyla 87%, 73%, 65, 48%, 30% ve 16% fazla bulundu. Protein karbonilasyonu ölçümlerinde normal besiyerinde büyütülen hücrelerden WT hücrelere kıyasla, Δccp1 mutanlarında 34% ve Δgrx2 mutantlarında 87% yüksek protein karbonil miktarı tespit edildi. Gliserol besiyerinde ise protein karbonil seviyesinin tüm mutanlar için arttığı gözlendi. Q-PCR denemesi sonucunda Δ*ccs1* ve Δ*prx1* için mitokondrial DNA'da hasarının arttığı bulundu. Tüm sonuçlar ışığında, bazı antioksidan gen mutantlarının daha kısa yaşadığı ve solunumla metabolize edilebilen karbon kaynağı varlığında aşırı miktarda moleküler hasar birikimi olduğu bulundu.

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CHAPTER 1

INTRODUCTION

1.1. Oxidative Stress

Oxidative stress can be defined as the imbalance between defence mechanisms which decrease the level of free radicals, and repair and turnover processes that repair or remove oxidatively damaged macromolecules. If there is any deficiency on both or either of these processes can cause increased levels of free radicals and damaged macromolecules. Disesase such as type II diabetes, atherosclerosis, chronic inflammatory processes, and ischemia/reperfusion injury are directly related with oxidative stress (Orrenius et al. 2007). In such diseases, the cells can produce excessive amount of free radicals such as reactive oxygen species (ROS) that can not be tolerated by defence mechanism of the organism. Lifestyle and environment are important events to control our health. From diet to sport activities they all affect the levels of ROS production. Unfortunately, abnormally high level of oxidative stress that could increase their probability of early incidence of decline in optimum body functions can be occured for many people during a normal day life-style conditions. Smoking or being in a place where people smoke (Carnevali et al. 2003), pollution (Kelly and Sandström 2004) pesticides taken by diatery sources can easly increase the levels of oxidative stress.

Despite efforts to control the cellular formation of superoxide, there are examples in which free radicals are generated during enzymatic aerobic metabolism. First reports showed that, superoxide was produced during the oxidation of purines by xanthine oxidase (McCord et al, 1968). In the next years , other enzymes, including nitric oxide synthase (Pou et al. 1992), have been found to generate free radicals as a result of oxidative metabolism. At physiological pH, superoxide rapidly dismutates to hydrogen peroxide. Therefore, in the presence of redox active metal ions, hydroxyl radical can be produced via the metal ion catalyzed Haber-Weiss reaction. The Haber-Weiss cycle consists of the following two reactions:

1. $H_2O_2 + OH^{\cdot} \rightarrow H_2O + O_2^{-} + H^{+}$
2. $H_2O_2 + O_2^{-} \rightarrow O_2 + OH^{-} + OH^{\cdot}$

The second reaction achieved notoriety as a possible source of hydroxyl radicals. However, it has a negligible rate constant. It is believed that iron (III) complexes can catalyse this reaction: First, Fe (III) is reduced by superoxide, followed by oxidation by dihydrogen peroxide. Another reaction that catalizes free radical formation is known as fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

Fenton reaction is the iron-salt-dependent decomposition of dihydrogen peroxide, generating the highly reactive hydroxyl radical, possibly via an oxoiron (IV) intermediate. Addition of a reducing agent, such as ascorbate, leads to a cycle which decreases the damage to biological molecules.

Reactive oxygen species are an important class of free radicals which are produced endogenously. Most commonly known ROS are superoxide ($O^{2\ -)}$ anion, hydrogen peroxide (H_2O_2), and hydroxyl ($OH^{\cdot}$) and peroxyl ($ROO^{\cdot}$) radicals (Sikka et al. 2002). ROS can be derived from numerous sources in vivo. Autooxidation, photochemical and enzymatic reactions, and both endogenous compounds and various xenobiotics can be considered under these source. There are several numbers of enzymes shown to be capable of generating ROS which include the cytochromes P450, various oxidases, peroxidases, lipoxygenases and dehydrogenases. Xenobiotics are another important group for generation that can be particularly important in determining the extent of ROS generated by these enzymes. For example, various quinones can undergo redox cycling, generating large amounts of ROS without themselves being degraded. In addition, NADPH oxidase is well known to generate ROS as part of its antibacterial function in phagocytic cells. However, this enzyme also appears to be present on numerous other cells and may have important signal transduction activities (Kehrer 2000).

Mitochondria can be considered as the most important source for ROS production. To understand the mitochondrial reletionship with ROS production and aging, it is necessary to have an overview of the mitochondrion and its role in the life of biological organisms.

Mitochondria are organelles (“little organs”) found in virtually all eukaryotic cells. There are two membranes which are inner and outer mebranes and two spaces which are intermebrane space and matrix within mitochondria. Outer membrane is highly permeable which contains porins than allow free diffusion of molecules smaller than 1000 daltons. Inner membrane formed by several folds which called as cristea. 70 % of iner membrane consist of proteins that shows the importance of inner membrane on mitochondrial functions. In addition cardiolipins are the mitochondria specific lipids which are localized through inner membrane (Cooper and Hausman 2003).

The number of mitochondria may change from cell to cell as the amount of required energy. The major function of mitochondria is energy production. 90 % of enegy which is stored in ATP is produced by mitochondria (Drew et al. 2003). There are two metabolic pathways which are the tricarboxylic acid (TCA) cycle and electron transport chain (ETC) to produce ATP (Lesnefsky and Hoppel 2006).

An important characteristic is that mitochondria have their own DNA which encodes 12 proteins in yeast and 13 proteins in human cells (Andreoli et al. 2004). Beside the ROS production by ETC, mitochondrial DNA (mtDNA) has a crucial role on aging because of its sensitivity to free radical attack and functions. mtDNA is a circular double-stranded molecule lying in matrix that is avarage 16 kb long for humans 80 kb long for yeast and more than 200 kb long for plants.The number of mt DNA copies changes from cell to cell in diffirent situations (Cooper and Hausman 2004). It encodes two rRNAs, 22 tRNAs and 13 polypeptides. Seven subunits of complex I (NADH dehydrogenase), three subunits of complex IV (cytochrome *c* oxidase), two subunits of complex V (ATP synthase) and cytochrome *b* (a subunit of complexIII) are encoded by mitochondrial genome (Alexeyev et al. 2004).

It is know that 90% of total cellular ROS is produced within the mitochondria (Balaban et al. 2005). Most of the ROS produced by mitochondria is called as superoxide radical which is highly reactive and can convert into other radicals as well. Superoxide radicals are produced during normal respiratory process by ETC. Normally ETC uses oxygen to produce ATP by using the proton gradient between mitochondrial spaces followed by electron flow. From Complex I to V there are five different comlexes required for adoquate flow of electrons and ATP production (Orrenius et al. 2007).

Mitochondrial ROS production occurs mainly on a non-heme iron protein that transfers electrons to oxygen. This occurs primarily at Complex I (NADH-coenzyme Q)

and, to a lesser extent, following the autoxidation of coenzyme Q from the Complex II (succinate-coenzyme Q) and/or Complex III (coenzyme QH_2-cytochrome c reductases) sites. Once again, the precise contribution of each site to total mitochondrial ROS production is probably determined by local conditions including chemical or physical damage to the mitochondria, oxygen availability and the presence of xenobiotics (Kehrer 2000). Today it is know that %2-3 of total oxygen consumed by ETC is converted into ROS. Phagocytic cells damage invading microorganisms by special degenerative products such as reactive oxygen species such as superoxide, hydrogen peroxide, hypohalous acid, and hydroxyl radical (Rosen et al. 1995). Peroxisomes breakdown fatty acid molecules in beta-oxidation reactions. As by products of these reactions, hydrogenperoxide ions are generated which can escape and damage macromolecules (Harman et al. 1956). Cytocrome P450 is an important enzyme aganist toxic chemicals. However during the reactions catalized by cytocrome P 450, oxidant molecules can be formed as a by product (Ahmed et al. 1995).

Mitochondrial ROS production rate can change by the type of available carbon source. If the available carbon source is glucose, yeast cells prefer glycolysis reactions to produce ATP which give 2 ATP yield per mole of glucose. Pyruvate molecules which are the end products are converted into ethanol during fermentation. During substrate level ATP production, NADH is not sent electron transport chain. Instead it is used by alcohol dehydrogenase to reduce acetaldehyde, which is formed by decarboxylation of pruvate by pyruvate decarboxylase, to ethanol. During logaritmic growth phase in the presence of glucose, yeast cells utilize glycolysis (fermentation) to derive energy. When all glucose molecules are used up, cells start to convert ethanol back to pyruvate to channel it throught Krebs cycle to derive more energy. Respiratory substrates such as erhanol, glycerol or lactic acid can be used to produce energy only through Krabs cycle. Thus, respiratory substrates are used to distinguish respiration competible cells from noncompetible ones. They are also used when cells are forced to utilize their mitochondria to derive energy. If glycerol is used as carbon souce, respiration reactions occur in which pyruvate does not used to form ethanol. Instead of that, pyruvate is converted to acetyl CoA which is used by TCA to form ATP, NADH and FADH. NADH and FADH are used by ETC in mitochondria to produce ATP during free radicals are formed. ROS production can cause important damages on lipids, proteins and DNA. It can be infered that usage of glycerol as carbon source is increase the risk of molecular damages.

1.2. Oxidative Damage to Biomolecules

1.2.1. Damages on Lipids

Phospholipids which contain polyunsaturated fatty acids residues are extremely sensitive to oxidation. Fenton reaction is one of the most potent inducer of lipid peroxidation, as it causes peroxide formation (Karbownik and Lewiski 2002). The major effect of lipid peroxidation is changes of membran charctaristics which causes functional declines of them following important age related diseases such as Alzeimer's disease (Wagner et al. 1994). Generally lipid peroxidation affects membranes causing decrease on fluidity and increase on permeability (Kelly et al. 1998). Biochemistry of lipid peroxidation comprise three important cycles which are initiation where activation of O_2 occurs, propagation where lipid hydroperoxides are generated and termination which occurs if peroxy radicals cross-link to form conjugated products which are not radical anymore (Wagner et al. 1994). Lipid peroxides are unstable, and decompose to generate other molecules such as malonaldehyde (MDA) and 4-hydroxyalkenals. Beside these two molecules, some other molecules such as octane, heptane, athene and ethylene can be formed. Measurement of malonaldehyde and 4-hydroxyalkenals has been used as an indicator of lipid peroxidation. This indicators can be detected via spectrophotometric or fluorometric assays (Jo and Ahn 1998). Repair of oxidized lipids is generally carried out by enzymatic activity. Two important repair systems are known which are the sequential action of phospholipase A_2 (PLA_2) with glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase (GPx) (Kelly et al. 1998). In phospholipase A_2 system, PLA hydrolyse the phospholipid hydroperoxides to the hydroperoxy fatty acids which are substates of GPX. The product which is reduced hydroxy groups terminates lipid peroxidation (Sevanian et al. 1983). Phospholipid hydroperoxide glutathione peroxidase system comprise direct reaction of phospholipid hydroperoxide glutathione peroxidase with the esterified phospholipid hydroperoxides which terminates lipid peroxidation (Ursini et al. 1985).

1.2.2. Damages on Proteins

Proteins are one of the major targets of oxidative modification. Oxidation of sulfhydryl groups, oxidative adducts on amino acid residues close to metal-binding sites, reactions with aldehydes, protein cross-linking reactions, and fragmentation reactions are known the results of protein oxidation (Orrenius et al. 2007). The direct oxidation of amino acids, particularly in lysine, arginine, proline, and threonine residues, promotes the formation protein carbonyls. Formation of a protein carbonyl can dramatically alter the tertiary structure of a protein, leading to functional decrease or lost. Maintaining protein pool is an important event after oxidation. After oxidation proteins can be repaired or degraded. Protein repair mechanisms comprise modification of a few amino acid residues. However, special removal systems exist in all cellular compartments for degrading oxidized proteins. Degradation of oxidized protein is carried out by proteasomal system in which enzmatic proteolysis occurs (Friguet 2002). Beside oxidation of certain amino acids to form protein carbonyls, protein methionine residues can be oxidized to methionine sulfoxide residues when exposed to increased intercellular levels of free radicals. methionine sulfoxides can be repaired by protein methionine-S-sulfoxide reductase (PrMSR) and protein methionine-R-sulfoxide reductase which are responsible for re-reducing oxidized methionine residues in the presence of reduced thioredoxin (Ferguson and Burke 1992).Eventhought methionine sulfoxides can be repaired there exists a further oxidation product of methionine, methionine sulfone,which can not be repaired (Poppek and Grune 2005).

1.2.3. Damages on DNA

Oxidative damage on DNA is the most frequently and dangerous type. Any damage on DNA affects the function of related protein which is traslated from the mRNA transcripted from oxidized locus. Especially hydroxyl radical reacts with DNA causing oxidation of bases leading double bound formation, abstraction of an H atom from the methyl group of thymine and each of the C-H bonds of 2'-deoxyribose (David et al. 2002) and fragmentation (Kelly et al. 1998). DNA is the knowledge of the cells and saving DNA from adverse effects has a crictual importance for population to maintain their species.Today it is known as several cellular mechanisms which have

been evoluted to defense DNA from oxidative attack or repair its oxidative damage (Kelly et al. 1998). Base-excision and nucleotide-excision repair are the most important repair mechanisms for oxidatively damaged bases (Jaruga and Dizdaroglu 1996). DNA glycosylase cuts off the mismatched or damaged base forming an apurinic/apyrimidinic (AP) deoxyribose. AP deoxyribose is relased by tandem reactions of AP lyase and AP endonuclease which cleaves 3` and 5` to the AP site, respectively. Then the gap is filled and linked by a new intact base (Sancar 1996). Nucleotide excision repair (NER) system is another important mechanism that repair oxidatively damaged DNA by removing oxidized lesion of DNA. In deed NER is the most important and complicated repair process, comprising more than 30-40 genes encoding protein for this system (Lockett et al. 2005).

1.3. Antioxidant Systems

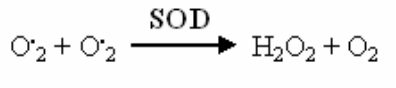
Eventhough the dangerous effects of ROS on lipids, proteins and DNA that cause functional abnormalities, biological organisms developped antioxidant systems againest them. They can be either non-enzymatic or enzymatic. Vitamin C and glutathione (GSH) are most important components of important non enzymatic defence mechanisms which have highly important fuctions on depleting reactive oxygen species. (Kankofer et al. 2004). Both Vitamin C and glutathione are water soluble molecules and their important funtions comprise aquous media where as a lipid soluble vitamin termed as vitamine E has important fuctions to defence organisms aganist oxidants in hydrophobic interiour of membranes (Reiter 1994). Beside non-enzymatic defence mechanism there are several enzymes catalyse the reaction to get rid of oxidant molecules. Superoxide dismutase (SOD) is one of them and may be the most important one which have different types of isoforms such as Mn SOD (SOD2), Cu-Zn SOD (SOD1). It catalyses the reaction that converts superoxide radical into hydrogen peroxide. At the downstream process of the reaction there are some other enzymes differs from cell to cell or from organelle to organelle. Catalase is the most important one for downstream reaction which converts hydrogen peroxide into water and molecular oxygen. Thioredoxin systmem is an enzymatic defence mechanism that they repair several oxidized proteins by oxidation of their –SH residues. Peroxides are also eliminated by glutathione peroxidases which have two isoforms classified as selenium

dependent and selenium independet ones (Valko et al. 2006). Mitochondria as the primary source of ROS and the major organelle which is affected from ROS, contain specialized antioxidant enzymes agains ROS attack.

1.3.1. Mitochondrial Antioxidant Enzymes

1.3.1.1. Superoxide Dismutases (SOD1 and SOD2)

There are different types of superoxide dismutases in different fractions of eukaryotic organisms. Cu, Zn superoxide dismutase (SOD1) and Mn superoxide dismutase (SOD2) are two important dismutases in yeast which are localized in mitochondrial intermembane space and matrix respectively. SOD1 is also localized in cytosol as well (Sturtz et al. 2001). The major function of superoxide dismutases is cleavage of superoxide ions to peroxide ions to reduce the dangerous effects of superoxide ions to a less dangerous form.



There are several researches on superoxide dismutases, only a few studies on proteins which are required for modulation of SODs on life span and oxidative stress. Copper chaperone (CCS1) and cytochrome c peroxidase (CCP1) are examples of them.

1.3.1.2. Copper Chaperone (CCS1)

Copper chaperone is an metallochaperone which incoperate cytosolic copper to SOD1. It is shown that CCS1 is an essential protein for SOD1 activation in mitochondrial intermembrane space (Schmidt et al. 1999). In other words CCS1 is a key protein for scaving superoxide ions in mitochondrial intermembrane space. So far there is no study to show the effect of deleting CCS1 on lifespan. Major of the previous studies are focused on the protein structure and interaction of CCS1 with SOD1.

1.3.1.3. Cytochrome c Peroxidase (CCP1)

Cytochrome c peroxidase is another important protein for mitochondrial antioxidant defense. The function of CCP1 is to convert of H_2O_2 to water. CCP1 is localized in intermembrane space as SOD1 but CCP1 proteins can be isolated from matrix as well. It is because the first step of maturation of CCP1 begins in mitochondrial matrix (Doum et al. 1982). It is shown that aerobic conditions are required for CCP1 activation while the yeast cells are grown in anaerobic conditions (Kawaguchi et al. 1962). It is also shown that CCP1 conveys Pos9 which is a transcription activator for oxidative stress response genes **(**Charizanis et al. 1999**).** Previously, the effect of deleting *CCP1* gene on yeast life span has not determined.

1.3.1.4. Glyoxylase-II (GLO4)

Glyoxylase-II is an enzyme of glyoxylase system. Indeed mitochondrial glyoxylase characterisation II is considered as GLO4 because there is another glyoxylase II enzyme which is localized in cytosol and termed as GLO2. The major function of GLO4 is the hydrolysis of S-D-lactoylglutathione into glutathione and D-lactateas. In the litreture there are few numbers of researchs on GLO4 and they are mainly focused on identification and characterisation of the enzyme (Bito et al. 1997).. This study is the first study that shows the effects of deleting *GLO4* gene on aging and oxidative stress

1.3.1.5. Mitochondrial Thioredoxin System (TRR2 and TRX3)

Mitochondrial thioredoxin system is an important regulator of redox state which is related with several cellular functions. The system consist of thioredoxins and thioredoxin reductases. Thioredoxins are small protein molecules which are responsible to reduce specific oxidized molecules. Thioredoxin contains in its oxidized form (thioredoxin-S_2) a single-redox-active disulfide, formed from the two half-cystine residues of the protein. In the presence of NADPH and a specific thioredoxin reductase this disulfide is opened and the reduced form of thioredoxin (thioredoxin-$(SH)_2$) is formed . Thioredoxin-$(SH)_2$functions as hydrogen donor in ribonucleotide reduction and

other reductive processes. In mitochondria TRX3 acts as thioredoxin and TRR2 acts as thioredoxin reductase..

In the promoter regions of both *TRX3* and *TRR2*, AP-1 sites are localized which is binding site of Yap1 which confers these proteins are expressed when cells exposed to oxidative stress (Pedrajas et al. 1999).

1.3.1.6. Mitochondrial Glutaredoxins (GRX2 and GRX5)

Glutaredoxins are known as thiol disulfide oxidoreductases and important to maintaine redox state of proteins. Two groups of glulatedoxins are GRX1 and GRX2 which contain cysteine pair at their active site and GRX3, GRX4, and GRX5 which contain single cysteine residue at their active sites. GRX2 and GRX5 are localized in mitochondria. Where as GRX2 have both glutathione-dependent disulfide oxidoreductase activity in maintaining redox state of target proteins and glutathione peroxidase activity, (Collinson et al. 2002), GRX5 is a hydroperoxide and superoxide-radical responsive glutathione-dependent oxidoreductaseand involved in the synthesis/assembly of iron-sulfur centers of respiratory complexes (Manzaneque et al. 2002).

1.3.1.7. Peroxiredoxin (PRX1)

Peroxiredoxins are classified under peroxidases. There are two groups of peroxiredoxins which are 1-Cys peroxiredoxin and 2-Cys peroxiredoxin. Mitochondrial peroxiredoxin is 1-Cys peroxiredoxin class enzyme (Pedrajas et al. 2000**).** Eventhough there is a wide range of this class of enzyme, there is only limited information about them but it is showed that prx1 protein is an antioxidant enzyme for scavenging ROS generated from respiration (Wong et al. 2004).

1.4. Aging

Aging is thought as the decrease in functions to under a threshold levels which follows death. Studies of model organisms such as *Drosophila*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae* have revealed that many multifactorial biological events

which cause aging are highly conserved. For years scienctist are focused on to increase human lifespan. The researches on aging which comprise molecular basis started more than one century ago. Wear and Tear Theory which was proposed by Dr. August Weismann in 1891 is one of the oldest theories to describe aging process. After that by means of the development of current molecular techniques several theories have been proposed such as Cross-Linkage Theory proposed by Johan Bjorksten in 1942 (Bjorksten 1967). The Free Radical Theory proposed by Dr. Harman in 1956 (Harman, 1956), Hayflick Limit Theory proposed by Dr. Hayflick and Dr. Moorehead in 1962 (Effros 2004) and Mitochondrial Theory proposed by Dr. Harman in 1972 (Harman, 1972).

Electrons surround atoms in pairs and if an atom loses an electron, unpaired electron causes the atom to act as a free radical. As previously mentioned free radicals are highly reactive and dangerous for all biological macromolecules. The accumulation of free radicals in the cell causes destruction of cellular components. According to the free radical theory of aging, cells produce free radicals continuously, and constant free radical damage eventually causes death of the cell. When death or damaged cells reach a number under a threshold level in an organism, the organism ages (Harman 1956). The mitochondrial theory of aging was first proposed by Denham Harman, who proposed free radical theory, in 1972 (Harman 1972). In 1980 this theory was further developed by Jaime Miquel (Miquel et al. 1980). The mitochondrial theory of aging enlarge free radical theory of aging by comprising mitochondrial components and fucntions which affects lifespan of organisms.

Among all other organisms *Saccharomyces cerevisiae* (yeast) is an invaluable model system with which to explore the molecular basis of aging. The fact that *Saccharomyces cerevisiae* is an eukaryotic cell with a short life cycle, containing a relatively small and well defined genome, expressing numerious genes with human ontologs, that can be rapidly and easily manipulated using established genetic techniques make this organism so important for aging researches. It is a unicellular eukaryotic cell and between yeast and human cells there are many conserved basic facts about molecular processes and metabolism In yeast, two different forms of aging processes are presently being studied which are replicative life span and chronological lifespan. Chronological lifespan is defined as the ability of stationary cultures to maintain viability over time. This aging process simply is the process of deterioration and loss of viability of cells during stationary phase. And replicative lifespan is defined

as the number of times an individual cell divides until it dies. Division of yeast cells occur by budding which is an asymmetric cell division process, the age of a mother cell can simply be determined by counting the number of bud scars on its surface. Basic differentiation of the cellular phsyology and shape can be listed like; increasing bud scar number, increasing cell size granulation, wrinkling of the cell surface and increased generation time can cause ceasing division of yeast cells (Maskell et al. 2003). Whereas for human cells telomer lenght is found an important factor to determine replicative lifespan defined as Hayflict Limit, yeast cells do not suffer from telomer shortening (D'mello and Jazwinski, 1991) Average and maximum life spans are fixed for each yeast strain which makes yeast aging had a strong genetic component. By means of differential hybridization screens 14 genes are differentially expressed over the course of the yeast life span (Sinclair et al. 1998). Beside changing expression pattern, nuclear fragmentation is another event that an important marker for aging. Especially extrachromosomal rDNA circles are increased during aging. Homologous recombination between repeat sequences in the yeast rDNA region results in the formation of extrachromosomal rDNA circles (ERCs). It is found that ERCs are accumulate to overwhelming numbers in aging mother cells. ERCs are formed by the excision of rDNA locus which is locolized in nucleolus. In young cells, only half will be transcriptionally active at any time due to the silencing action of the Sir2 protein. Sir2 protein exhibits NAD-dependent deacetylase activity. Genetic studies have linked aging in this organism to sir (silent information regulator) genes, which mediate genomic silencing at telomeres, mating type loci, and ERC. Sir2 protein determines lifespan by creating silenced rDNA chromatin, thereby repressing recombination and the generationof toxic ERCs (Lin et al. 2000). Recent studies showed that sir2 mediated increase on lifespan is related with another factor which increase life span and called as calory restriction. It is proposed that life span can be extendend by limiting glucose. Calory restriction increase respiration which cause a decrease on NADH levels in the cells. Decreasing NADH levels leads increasing NAD+/NADH ratio which activates Sir2 (Guarente 2005).

There are different factors that can affect replicative life span beside genetic factors. Mitochondrial effects are the most importants amoung them. Both the pathways that controled by mitochondria and free radical formation form mitochondria via respiratory mechanisms increase the importance of mitochondria on aging researches. There are two important features of mitochondria make these organelles so important

for aging researches. The first one is the electron transport chain which produce superoxide radical by %2-3 during normal ATP production patway which uses electron flow that causes a H^+ gradient to produce ATP. Formed superoxide radicals can damages all mitochondrial biomolecules comprising mtDNA, proteins and lipids, directly or indirectly by their secondary products. Produced radicals can escape into cytocol and nucleus as well and can cause more dangerous damages to whole cellular components. The second feature that makes mitochondria so important about aging process is the structure and repair system of mitochondrial DNA (mtDNA). Complexes of ETC are localized throught inner membrane of mitochondria. Thus superoxide radical produced from ETC are directly released into mitochondrial matrix. As the mt DNA is locolized in matrix space, it makes mitochondrial DNA prone for ROS attack (Mandavilli et al. 2002). As previously mentioned all the genes which encodes mRNA, rRNA or tRNA from mt DNA are essential for ETC subunits. Any mutation may affect the function of ETC which increase the portions of produced superoxide radical. In addition the deficiency of histons and introns that may save mitochondria is another event that makes mitochondria so sensitive to ROS attack. Repair system is also not good enough to overcome increased levels of radicals. It is known that mitochondria can repair their DNA by base excision repair (Stuart et al. 2004). However they lack any enzymatic machinery for the removal of bulky lesion from their DNA which makes it impossible to repair their DNA if the damages are strong (Mandavilli et al. 2002). It is also known that eventhough the activity of mitochondrial base excision repair system may increase during aging, the level of activity unable to prevent production of lession in mtDNA (Jaruga and Dizdaroglu 1996).

1.5. Aims

The major scope of this work is to determine mitochondrial antioxidant genes that are important for cellular aging. To do so, growth curves, aging and molecular damage profiles of mutants were analysed.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

A detailed list of commonly used media, buffers, solutions, and their compositions are presented in Appendix A.

2.2. Metods

2.2.1. Yeast Strains

Wild-type (BY4741) and isogenic ten different deletion mutants (Δ*ccp1,* Δ*sod1,* Δ*glo4,* Δ*trr2,* Δ*trx3,* Δ*ccs1,* Δ*grx2,* Δ*sod2,* Δ*grx5,* Δ*prx1*) of *Saccharomyces cerevisiae* were examined. The strains used for the experiment listed in Table 2.1.

Table 2.1. Strains and their genotypes

Strain	Genotype
WT(BY4741)	MATa, *his3*, *leu3*, *met15*, *ura3*
Δ*ccp1*	MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*ccp1*:KAN^R
Δ*sod1*	MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*sod1*:KAN^R
Δ*glo4*	MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*glo4*:KAN^R
Δ*trr2*	MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*trr2*:KAN^R
Δ*trx3*	MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*trx3*:KAN^R
Δ*ccs1*	MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*ccs1*:KAN^R
Δ*grx2*	MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*grx2*:KAN^R
Δ*sod2*	MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*sod2*:KAN^R
Δ*grx5*	MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*grx5*:KAN^R
Δ*prx1*	MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*prx1*:KAN^R

2.2.2. Identification of Mitochondrial Antioxidant Genes

Identification of mitochondrial antioxidant genes were carried out by using an internet based software which is called Mitochondrial Proteome Database (Andreoli et al., 2004).

2.2.3.Yeast Aging Assay

Yeast strains were grown on desired media for 2 days before analysis. Cells were inoculated onto agar plates as a single line. For each strain, avarage 30 individual cells selected and removed from yeast colonies by using a micromanuplator. When new cells formed by these individuals, 20 new daughter cells were collected and lined up by a micromanipulator on agar plates. These 20 cells were termed as starter mothers. New buds (daughters) from these virgin cells were removed and discarded as they formed by controling the plates by 90 minutes intervals. During night plates were stored at 4^{o}C This process continued until cells ceased dividing. Life span was determined as the total number of daughter cells that each mother cell generated.

2.2.4. Yeast Growth Curve Assay

Yeast cells were inoculated in liquid media. Overnight cultures were diluted to OD_{600} 0,05 in fresh media and cell growth was determined by OD_{600}. For 2 hours intervals OD600 values were measured by spectrofotometer for each strain until cells reached stationary phase.

2.2.5. Determination of Lipid Peroxidation and Protein Carbonylation

2.2.5.1. Cellular Extraction

Yeast strains were grown in 250 ml of desired media. After 6 h incubation at 30 ^{o}C cells were precipitated by centrifugation and washed once . 4 ml yeast lysis buffer added into all cell pellets. 1 ml of glassbeats and 500 µl of 100 mM PMSF was added

into suspention Each tube was vortexed for 5 minutes and centrifugated at 3000 rpm for 3 minutes. Supernetants were stored at -80 ^{o}C.

2.2.5.2. Determination of Lipid Peroxidation

50 µl of samples of cellular extracts were taken into microcentrifuge tubes and added 1 µl of TBA reagent (0.25 M HCl, 15% [wt/vol] trichloroacetic acid, 0.375% [wt/vol] TBA). Samples were heated for 15 minutes in a boiling water bath. After cooling, samples were centrifuged at 16,000 g for 1 min in order to remove cell debris. Absorbances of the samples were taken at 535 nm. Concentrations were calculated by the formula obrained from standart curve which is obtained by the absorbance measurement of 0; 2,5; 5; 10 µM 1,1,4,4 tetraethoxypropane at 535. After normalization of the results relative lipid peroxide levels put into graphes for each strain.

2.2.5.3. Determination of Protein Carbonylation

For each strain 200 ml cell extracts were taken two microcentrifuge tubes , one was marked as "test" and the other as "control". 800 µl of 10 mM (DNPH) prepared in 2,5 M HCl was added to the test sample and 800 µl of 2,5 M HCl alone was added to the control sample. The tubes incubated in the dark at room temperature for 1 hour. The tubes were shaken between 15 minutes intervals. 1 ml of 20% TCA (w/v) was added into each tubes and the mixture kept in ice for 10 minutes. The tubes were then centrifuged at 16.000 g for 25 minutes to obtain the protein pellet. The supernatant was carefully aspirated and discarded. This was followed by a second wash with 10 % TCA as described above. Finally the precipitates were washed three times with 800 µl of ethanol:ethyl acetate (1:1, v/v) to remove unreacted DNPH and lipid remnants. The final protein pellet was dissolved in 400 µl of 6 M guanidine hydrochloride and incubated at 37°C for 10 minutes. The insoluble materials were removed by centrifugation. Each sample was read against the control sample (treated with 2.5 M HCl). The carbonyl content was calculated from peak absorption at 370nm using an absorption coefficient (e) of 22,000 M- ^{1} Cm- ^{1}.After normalization of the results relative carbonyl contents put into graphes for each strain.

2.2.5.4. Normalization of Lipid Peroxidation and Protein Carbonylation Measurements

The normalization was carried out by the protein content of the extracts. was carried out by Bradford Assay. 50 µl of protein was added in 200 µl of Bradford reagent. After 5 minutes abrorbances were measured as 595 nm.Protein concentrations were calculated by the formula obtained from standart curve which was obtained by the Bradford Assay in which absorbance at 595 nm measured for 2, 4, 8, 12, 15, 20 µg/ml BSA. Protein concentrations were divided to lowest protein concentration to find normalization coeficients. Both lipid and protein concentration results divided to corresponding coeficients.

2.2.6. Determination of Respiration Compotent Cells

Cells were inoculated into 10 ml of YPD incubated at 30 ^{o}C until the cells reached growth phase. 500 µl of each sample centrifuged at 16.000 g for 5 seconds. The pellets were washed with TBS buffer twice. Washed pellets were resuspended in 500 µl of TBS buffer. 50 µl of 1µM Mitotracker Red Dye was added into each tube. The tubes incubated for 45 minutes at 30 ^{o}C in dark. The cells were washed three times with TBS buffer. Pellets were resuspended in 500 µl of TBS buffer and sonicated for 3 seconds. The level of resprirating compotent cells was measured by measuring the staining efficiency by means of FACS array.

2.2.7. Determination of DNA Damages

2.2.7.1 Detection of Gene Specific DNA Mutations

2.2.7.1.1. DNA Extraction

Yeast strains were grown in both 10 ml of YPG until they reached to exponantial growth phase. The cell suspensions were spined down for 5 minutes at 4000 rpm and resuspended in 1 ml sterile distilled water. The cells were trasfered to microcentrifuge tubes. They were spined down at 16000g for 5 minutes. 200 µl yeast lysis buffer

containing 1 µM DTT , 200µl phenol:chloroform:isoamyl alcohol (25:24:1), and 0,5 ml of acid washed glass beads were added respectively. The samples vortexed for 5 minutes and 200 µl TE buffer added. Tubes were centrifuged for 5 minutes and aqueous phases were transfered to new tubes. 1 ml of 100% EtOH added, and tubes were mixed by inversion. Tubes spined for 1 minuted at 16000 g and supernetant were aspirated. The pellets were resuspended in 400 µl TE buffer containing 4 µl RNase A, and tubes were incubated for 1 hour at 37 ^{o}C to dissolve the pellets. 10 µM of 4M ammonium acetate and 1mL 100% EtOH were added into tubes. Samples were centrifuged for 1 minutes at 16000 g and supernetants were discarded. The pellets were washed with 70% EtOH and resuspended in 50 µl of TE buffer. DNA concentrations were determined by a flourometer.

2.2.7.1.2. PCR Protocol

For 25 ml of PCR reaction 600 µl master mix were prepared. Master mix consisted of 60 µl PCR buffer, 30 µl dNTP, 12 µl DMSO, 6 µl primers, 2µl Accutaq enzyme and 423 µl water. Each reaction recieved equal emount of template DNA and master mix.

By using the appropriate primer pairs (Torres et al. 2000), target DNA sequences *COX1* gene were amplified for 30 cycles, beginning with a pre-denaturation step at 98 °C for 30 seconds, followed by a 15 sec. denaturation step at 94 °C, a 20 sec annealing at 66 °C, and a 12 min extension at 68 °C. At the end 3 min primer extension at 68 °C completed the sequence.

10 µl of PCR each products were loaded onto 1% TAE agarose gel which were prepared with 1% agarose (w/v) and 0,5 µg/ml EtBr by mixing with 4 ml of loading dye.

2.2.7.2. Determination of Spontaneous Mutation Rates of Mutants

Cells were grown in 2 ml of YPD for 24 h at 30 ^{o}C and washed with sterile distilled water. Cells were diluted in 20 ml water and cell numbers were determined by counting under a scope. Certain number of each cells were plated onto each YNB-Arg+Can plate. Cells were incubated at 30 ^{o}C for 4 days and colony numbers were counted. Samples were analysed twice for each cells.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Identification of Mitochondrial Antioxidant Genes

We first listed the mitochondrial antioxidant proteins using Mitop2 program which analyses the mitochondrial targeting sequences on genes and combine the results with certain experimental data to localize the proteins with high accuracy (Andreoli et al., 2004). As shown in Table 3.1. ten different proteins were found to be antioxidant and residing in mitochondria

Table 3.1. List of mitochondrial antioxitant genes, gene products and subcellular localization

ORF (gene)	**Gene Product**	**Subcellular Localization**
SOD2	Mn superoxide dismutase	mitochondrial matrix
GRX5	glutaredoxin	mitochondrial matrix
PRX1	peroxiredoxin	mitochondrion
TRX3	thioredoxin	mitochondrion
CCP1	cytochrome c peroxidase	mitochondrial intermebrane space
SOD1	Cu, Zn superoxide dismutase	cytosol, mitochondrial intermembrane space
GLO4	glyoxylase-II	mitochondrial matrix
TRR2	thioredoxin reductase	mitochondrion
GRX2	glutaredoxin	cytosol, mitochondrion
CCS1	copper chaperone	cytosol, mitochondrial inner membrane

3.2. Growth Rates of Mutants

To investigate whether these genes are important in cell growth, deletion mutants were grown in glucose media (YPD) and in glycerol media (YPG), and growth rate was determined by OD_{600} measurements. As shown in Figure 3.1., most of the mutants did not show significant growth retardation compared to wild-type cells, whereas cells lacking *GRX5*, *SOD1* and *CCS1* genes grew slightly slower under normal conditions.

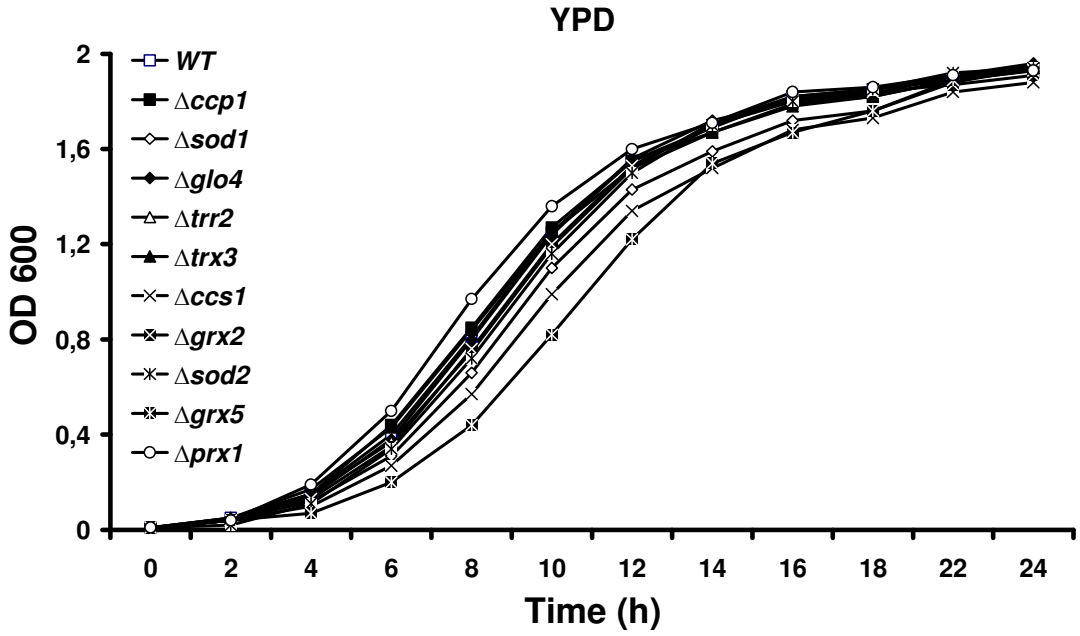


Figure 3.1. Growth rates in glucose media (YPD) .Overnight cultures were diluted to 0.05 OD_{600} in liquid and shaked at 30°C for the indicated time. Growth rate was monitored by OD_{600} measurements.

Similar to YPD growth profiles, none of the mutants showed significant growth retardation compared to wild-type cells in the presence of glycerol. Apart from the results which were obtained from growth in YPD medium, cells lacking *PRX1*, *GRX5*, *SOD1*, *CCP1*, *SOD2* and *GRX2* genes showed slight retardation on growth in glycerol as shown in Figure 3.2.

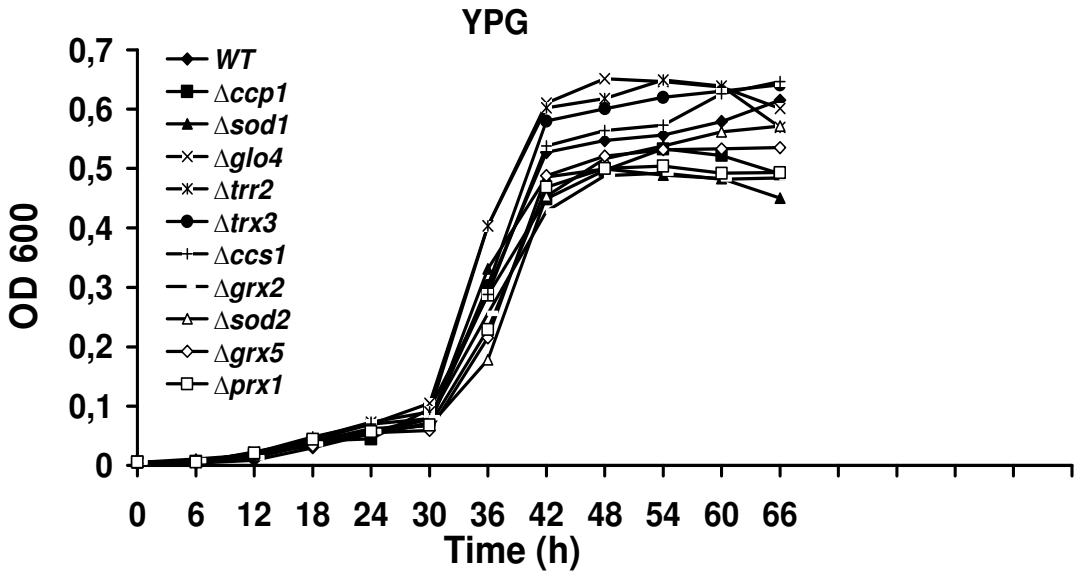


Figure 3.2. Growth in glycerol media (YPG). Overnight cultures were diluted to 0.05 OD_{600} in liquid and shaked at 30°C for the indicated time. Growth rate was monitored by OD_{600} measurements

When challenged with 1 mM of H_2O_2, mutants lacking *GRX5, SOD2, SOD1, CCS1, PRX1, TRR2* and *GRX2* genes showed growth defects suggesting that these mutants are sensitive to oxidative stress as shown in Figure 3.3. Interestingly these results are similar to those of obtained from YPG growth curves. In glucose media yeast cells derive ATP from gylcolysis which does not require mitochondria. In contrast, in glycerol media energy production from glycerol occurs via respiration in which ETC is used. Thus, it is expected to see an increase in the level of oxidative stress in mutant cells when they use glycerol.

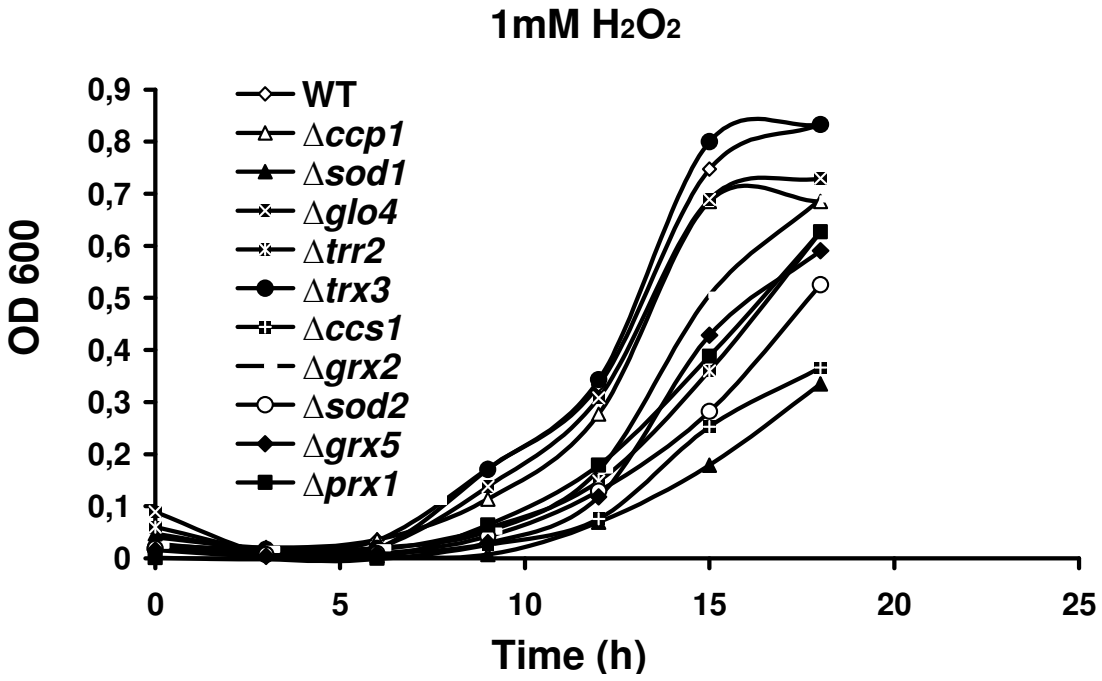


Figure 3.3. Growth in glucose media (YPD) with 1mM H_2O_2. Overnight cultures were diluted to 0.05 OD_{600} in liquid, H_2O_2 was added into aliqouts to final concentration 1mM and shaked at 30°C for the indicated time. Growth rate was monitored by OD_{600} measurements.

When challenged with 1 mM of H_2O_2 gylcerol media mutants lacking, *CCP1, GLO4, SOD2, SOD1, CCS1,TRX3 , TRR2* and *GRX2* genes showed lower growth rate compared to WT cells as shown in Figure 3.4. Results are consistent with those of cells grown in YPD + 1mM H_2O_2.

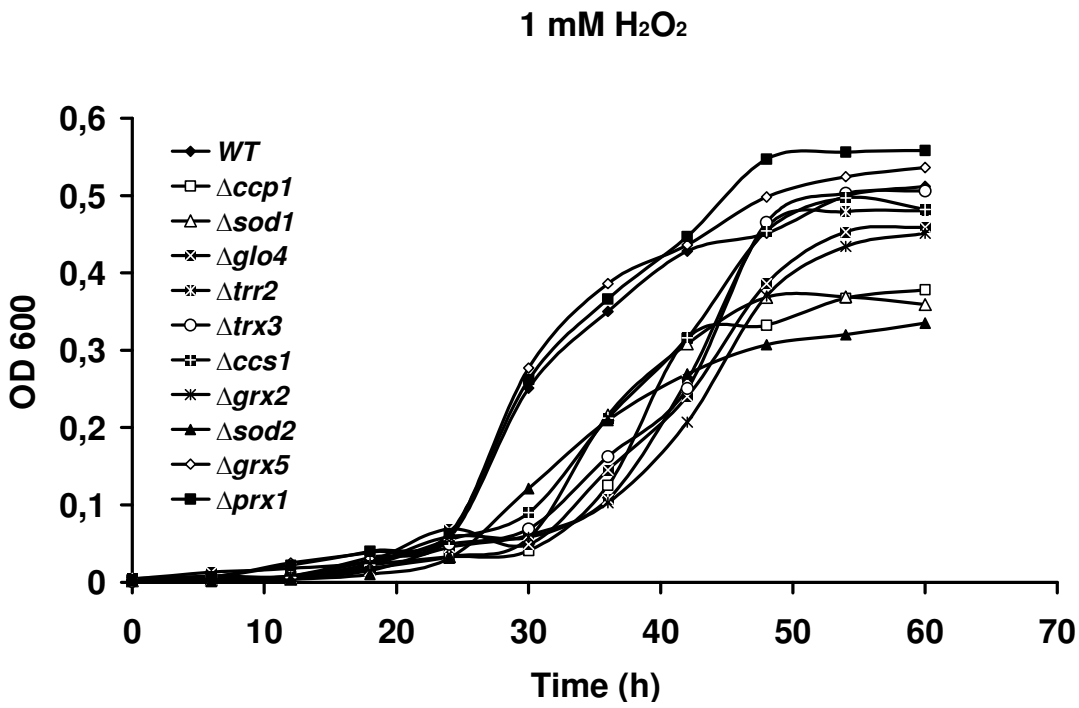


Figure 3.4. Growth in glycerol media (YPG) with 1mM H_2O_2. Overnight cultures were diluted to 0.05 OD_{600} in liquid, H_2O_2 was added into aliqouts to final concentration 1mM and shaked at 30°C for the indicated time. Growth rate was monitored by OD_{600} measurements

3.3. Aging Profiles of Mutants

To see the effect of deleting these genes on the life span, replicative aging profile of each mutant was determined. Deletion of *SOD1*, *SOD2* and *CCS1* genes caused a major drop in both average and maximum life spans as shown in Table 3.2. and Figure 3.5. The life span decrease was % 40 for Δ*sod1* mutant, % 72 for Δ*sod2* mutant and % 50 for Δ*ccs1* mutant. Absence of *GRX2*, *GLO4* and *PRX1* genes also shortened the average life span (10 %), but the extend of decrease was not as significant as deleting *SOD1* and *SOD2* or *CCS1* genes. Surprisingly, deletion of the other mitochondrial antioxidant genes did not lead to a decrease in the life span. Previous studies by others established the role of *SOD1* and *SOD2* in aging. Our results are consistent with the previous findings regarding Δ*sod1* and Δ*sod2* mutants.

Table 3.2. Maximum and average life spans of WT and mutant cells which grown in glucose media (YPD)

	WT	*Δccp1*	*Δsod1*	*Δglo4*	*Δtrr2*	*Δtrx3*	*Δccs1*	*Δgrx2*	*Δsod2*	*Δgrx5*	*Δprx1*
Max Life Span	**29**	**32**	**26**	**32**	**29**	**31**	**18**	**29**	**17**	**31**	**29**
Average Life Span	**22**	**23**	**13**	**20**	**23**	**22**	**11**	**20**	**6**	**23**	**20**

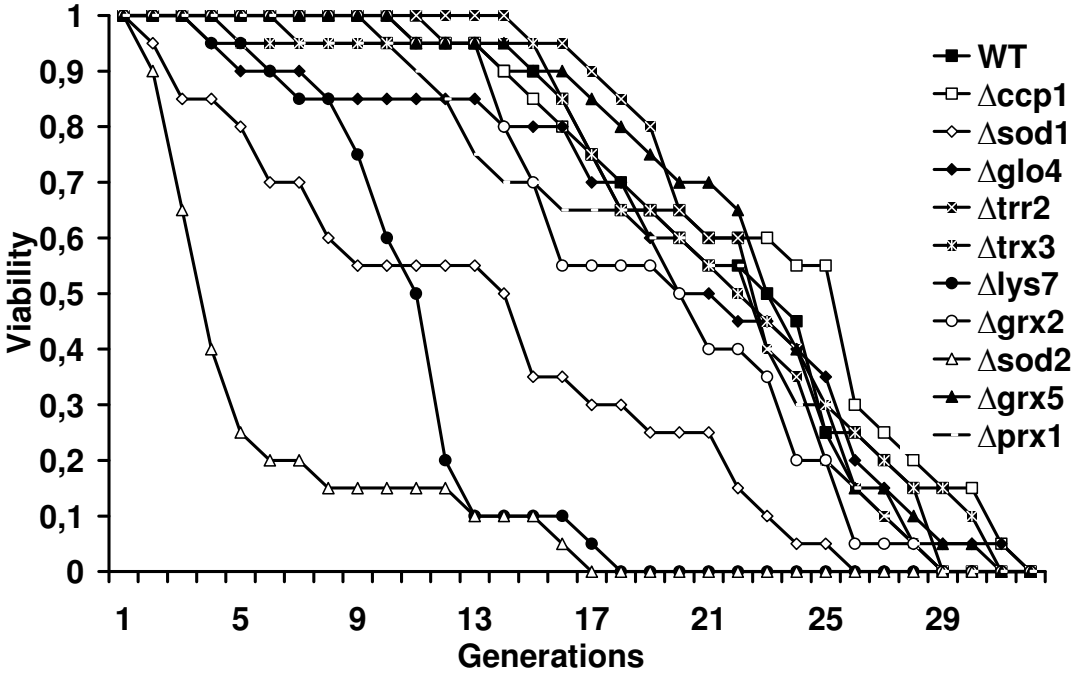


Figure 3.5. Replicative life span analysis of WT and mutant cells which grown in glucose media (YPD)

In the presence of glycerol deletion of *SOD1*, *SOD2, CCS1, CCP1* genes caused a major drop in both average and maximum life spans as shown in Table 3.3. and Figure 3.6. The life span decrease was 79 % for Δ*sod1* mutant, 87 % for Δ*sod2* mutant, 51 % for Δ*ccs1* and 65 % for Δ*ccp1* mutants. Absence of *PRX1*, *TRR2* and *GRX5* genes also shortened the average life span by 8 %, 10 % and 16 % respectively, but the extent of decrease was not as significant as deleting *CCP1*, *SOD1* and *SOD2* or *CCS1* genes.

Surprisingly, deletion of the *GLO4*, *TRX3* and *GRX2* genes did not lead to a change in the life span of cells.

Table 3.3. Maximum and average life spans of WT and mutant cells which grown in glycerol media (YPG)

	WT	***Δccp1***	***Δsod1***	***Δglo4***	***Δtrr2***	***Δtrx3***	***Δccs1***	***Δgrx2***	***Δsod2***	***Δgrx5***	***Δprx1***
Max Life Span	**26**	**20**	**9**	**21**	**20**	**26**	**15**	**24**	**4**	**18**	**20**
Average Life Span	**16**	**6**	**3**	**16**	**14**	**17**	**8**	**17**	**2**	**13**	**15**

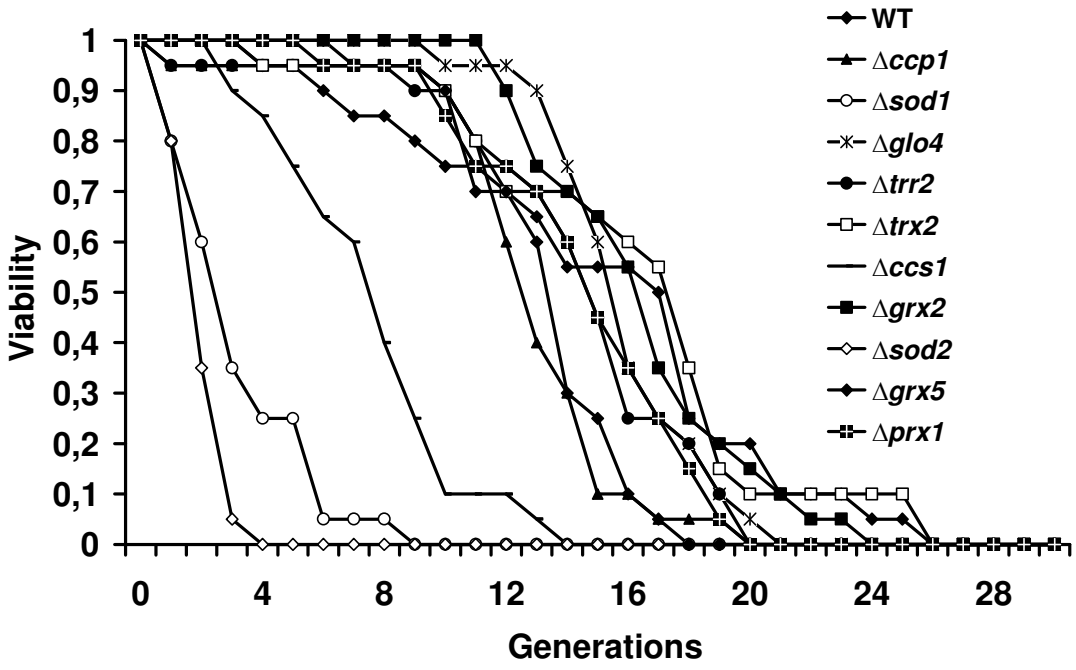


Figure 3.6. Replicative life span analysis of WT and mutant cells which grown in glycerol media (YPG)

3.4. Detection of Molecular Damages in Mutants

3.4.1. Lipid Peroxidation Levels

Lipid molecules can be oxidized by free radicals to form lipid peroxides. In this study MDA levels, which is related with lipid peroxidation levels, were measured to determine mitochondrial and cellular lipid peroxidation level.

Level of MDA was usually lower in mutant cells under normal conditions. Previous studies for Δ*sod1* and Δ*sod2* mutants showed similar results (Pereira et al. 2003). The decrease in MDA levels as a result of single mutations can be explained by the interaction of the genes of interest with other pathways to induce oxidative stress tolerance genes. Deletion of single mitochondrial antioxidant gene may cause an increase on oxidative state which triggers other genes that responsible to regulate oxidative state. It is known that some mitochondrial antioxidant proteins such as SOD1, TRX3, TRR2 can activate oxidative stress response pathway by conveying different transcription activators such as Yap1 and Skn7. Absence of one of these genes can also affect the expression of other mitochondrial antioxidant genes which may increase the cellular defense and repair mechanisms.

As shown in Figure 3.8. when cells grown in glycerol, deletion of *CCS1*, *SOD2*, *GRX2*, *CCP1*, *TRR2* and *PRX1* increase cellular MDA levels 87 %; 73 %; 65 %; 48 %; 30 % and 16 % respectively. Mutants that show higher MDA levels, grow slower in YPG + H_2O_2, that deletion of *CCS1*, *SOD2*, *GRX2*, *CCP1*, *TRR2* and *PRX1* genes cause the cells to become more sensitive to lipid peroxidation.

It is known that increase in lipid peroxidation levels is an impotant event during aging. Especially, recent studies demonstrate that lipid peroxidation in brain is an early event in Alzheimer's disease (Petursdottir et al. 2007). In another research it was found that SOD1 is not necessary for normal motor neuron development and function but it is required under physiologically stressful conditions following injury (Reaume et al. 1996). In our research lipid peroxidation levels were detected without additional stress conditions and the results are consistent with the proposition above. Growth curve assays also support the idea because H_2O_2 pretreated Δ*sod1* mutants showed growth retardation compared to wild-type cells. Further analysis that shows lipid peroxidation levels for the mutants that pretreated with H_2O_2 is need to be carried out to support this

idea. Deletion of *CCS1* gene caused elivated levels of lipid peroxidation compared to WT cells and Δ*sod1* mutants. This result supports the idea about prooxidation event. It is known that CCS1 is need to be expressed for SOD1 activation. We can speculate that Δ*ccs1* mutants may act like double gene mutants for *CCS1* and *SOD1* genes. Thus inactivation of these two genes may increase the rate of lipid peroxidation. Previous studies showed that relationship between SOD activity and life span is not easy to understand. It is known that increased expression of SODs cause increased lipid peroxidation. Studies on bacteria and transgenic animals showed that high levels of SOD actually lead to increased lipid peroxidation and hypersensitivity to oxidative stress (Kowald and Klipp 2004). Eventhough the similarity between the results of lipid peroxidation experiments and aging assays when cells were grown in glycerol media, available data is not enough to propose a relationship between aging and lipid peroxidation. For Δ*ccs1*, Δ*sod2*, and Δ*ccp1* it was shown that there was a decrease on lifespan and increase on lipid peroxidation levels when they cells were grown in glycerol media. Differently, eventhough there was a considerable increase on lipid peroxidation in Δ*trr2*, Δ*grx2* and Δ*prx1* mutants, there wasn't anyeffect on life span. Thus, decrease on lifespan because of deletion of antioxidant genes can not be evaulated just only with the increase of lipid peroxidation. We can speculate that there may be other effects which decrease lifespan of Δ*ccs1*, Δ*sod1*, Δ*sod2*, and Δ*ccp1*.

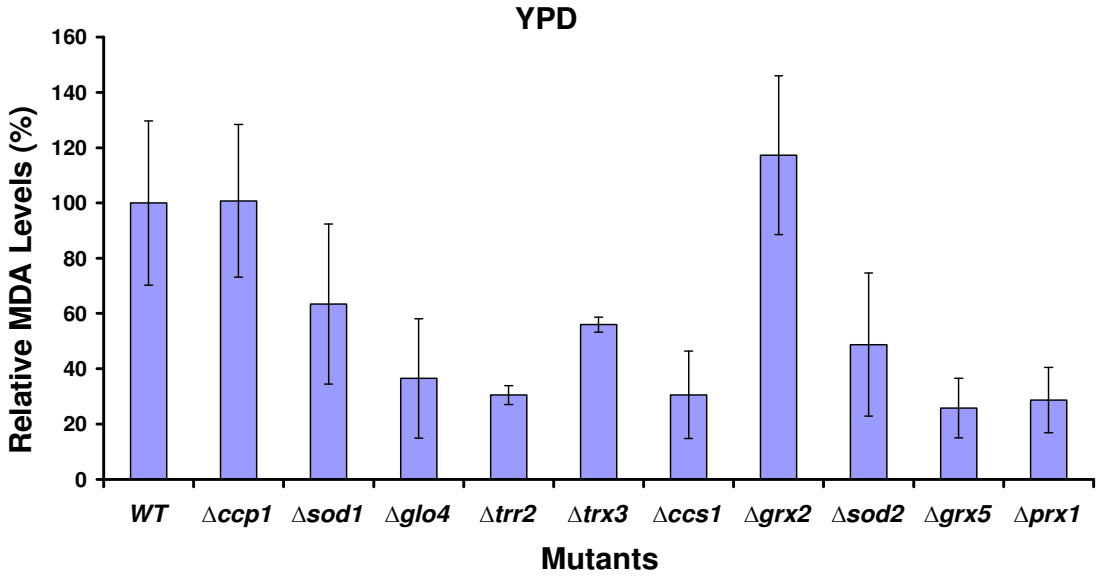


Figure 3.7. Cellular relative lipid peroxidation levels for WT and mutant cells which were grown in glucose media (YPD)

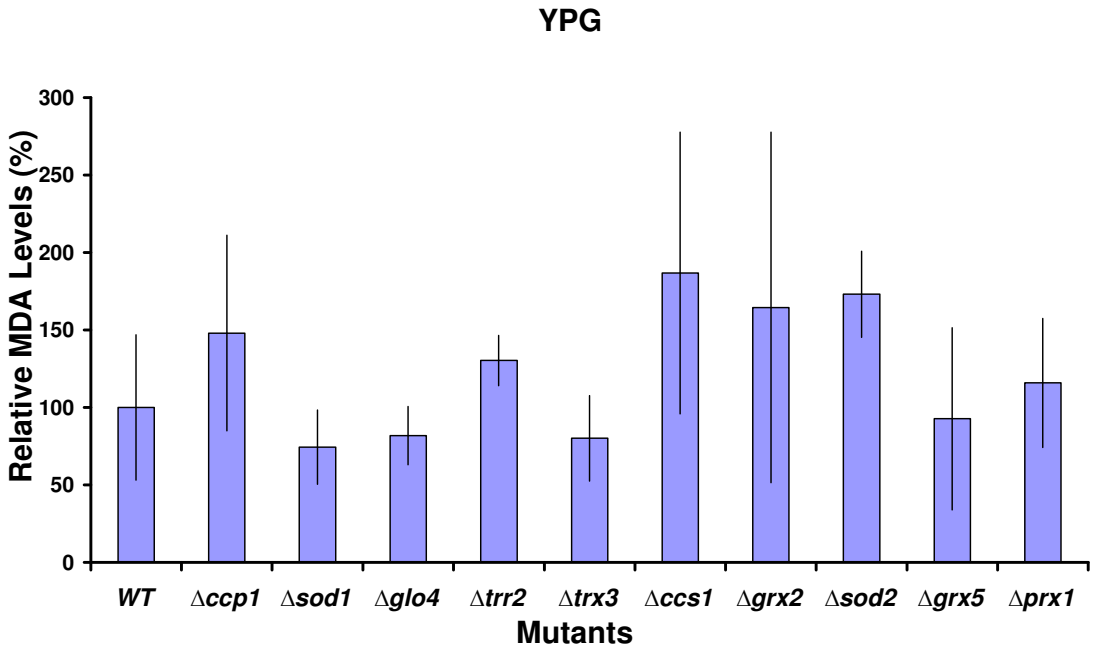


Figure 3.8. Cellular relative lipid peroxidation levels for WT and mutant cells which were grown in glycerol media (YPG)

3.4.2. Protein Carbonylation Levels

As shown in Figure 3.9., Δ*ccp1* and Δ*grx2* mutants showed 34 % and 87 % higher carbonylation levels compared to WT cells. In another research similar results were obtained for mitochondrial protein carbonylation levels in Δ*sod1* and Δ*sod2* mutants, previously (O'Brien et al. 2004).

Like those of lipid peroxidation, the mutants show higher level of protein carbonylation compared to WT cells when they are grown in glycerol media instead of glucose media. The results showed that all of the single mutations cause an increase on cellular protein carbonylation. The increases were 65 % for Δ*ccs1*, 61 % for Δ*prx1*, 57 % for Δ*glo4*, 55 % for Δ*ccp1*, 49 % for Δ*sod1*, 37 % for Δ*sod2*, 33 % for Δ*grx2*, 18 % for Δ*trx3*, 17 % for Δ*grx5* and 7 % for Δ*trr2* as shown in Figure 3.10.

When cells were grown in glucose media, carbonylation results were similar to those of MDA measurements when cells grown in glucose media. For both *Δgrx2* mutants were more sensitive to molecular damages. Grx2 is the major thiol system for the protection

of proteins against H_2O_2 induced carbonylation. It is also known that Grx2 accounts for most of the glutaredoxin activity during exponential growth (Luikenhuis et al. 1998). This means deletion of *GRX2* may cause the loss of major glutaredoxin activity which is especially important for protection of proteins againist oxidation. Δ*ccp1* mutants also showed higher levels of protein carbonylation. So that we can speculate that as both of GRX2 and CCP1 show peroxidase activity, loss of peroxidase activity increase molecular damages on lipid and especially proteins.

When cells were grown in glycerol media all of the mutants showed elivated levels of carbonylation compared to WT cells. Deletion of CCS1 showed the highest carbonylation level compared to other mutants and WT cells which is similar to results of lipid peroxidation levels. This result support the idea that Δ*ccs1* mutation may effect the cells like double gene mutantion for *CCS1* and *SOD1* genes.

Beside of Δ*ccs1*, Δ*sod1*, Δ*sod2*, and Δ*ccp1* mutants, other mutants, of which lifespan did not changed compared to WT cells, showed increased levels of protein carbonylation. Studies have shown that protein carbonylation increases with the age and in some cases such as age related disease like Parkinson's disease, Alzheimer's disease, carbonylation has been linked to age-dependent wear and tear of specific enzymes, such as aconitase and the nucleotide translocator ANT (Nyström 2005). In addition, some mutations showed that specific carbonylation patterns which means carbonylation of specific proteins can be expected for different mutants. *Δgrx5* mutants showed that at least one band appeared to be specifically oxidized which was not oxidized for other *GRX* mutants (Manzaneque et al. 1999). In deed, what caused the specific protein carbontlation is not known. Thus we can speculate that there may be specific carbonylation patterns that causes decrease on lifespans of Δ*ccs1*, Δ*sod1*, Δ*sod2*, and Δ*ccp1* mutants. Western Blot analysis need to be carried out for all mutants to identify the specific protein carbonylation idea.

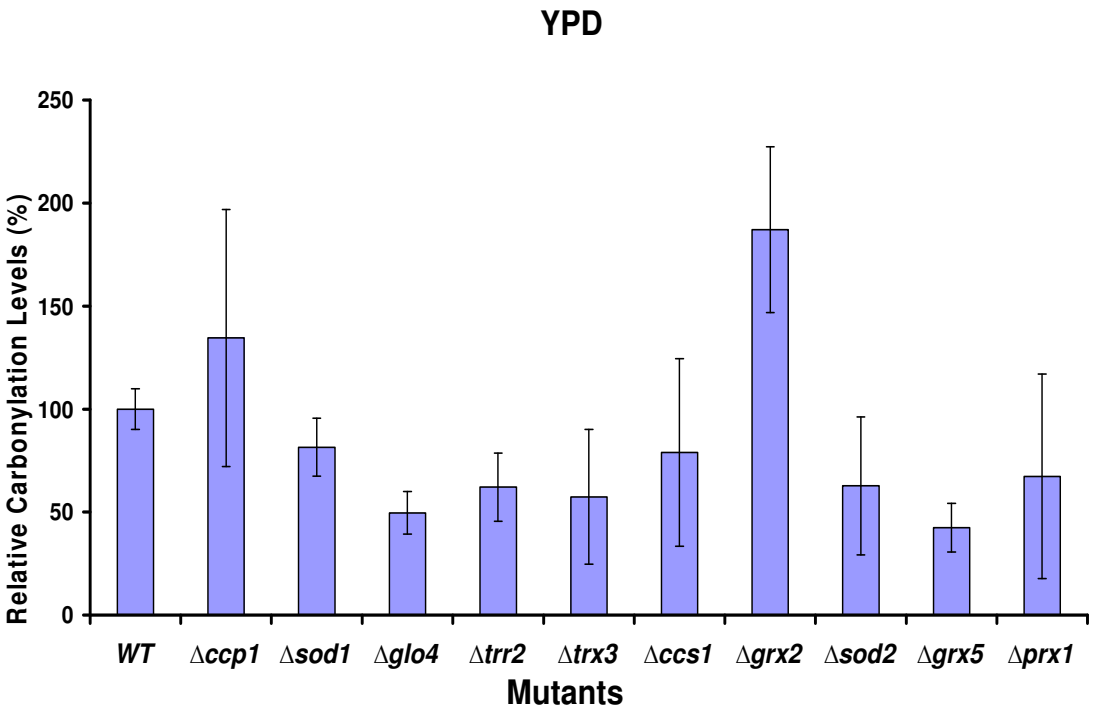


Figure 3.9. Cellular relative protein carbonylation levels for WT and mutant cells which were grown in glucose media (YPD)

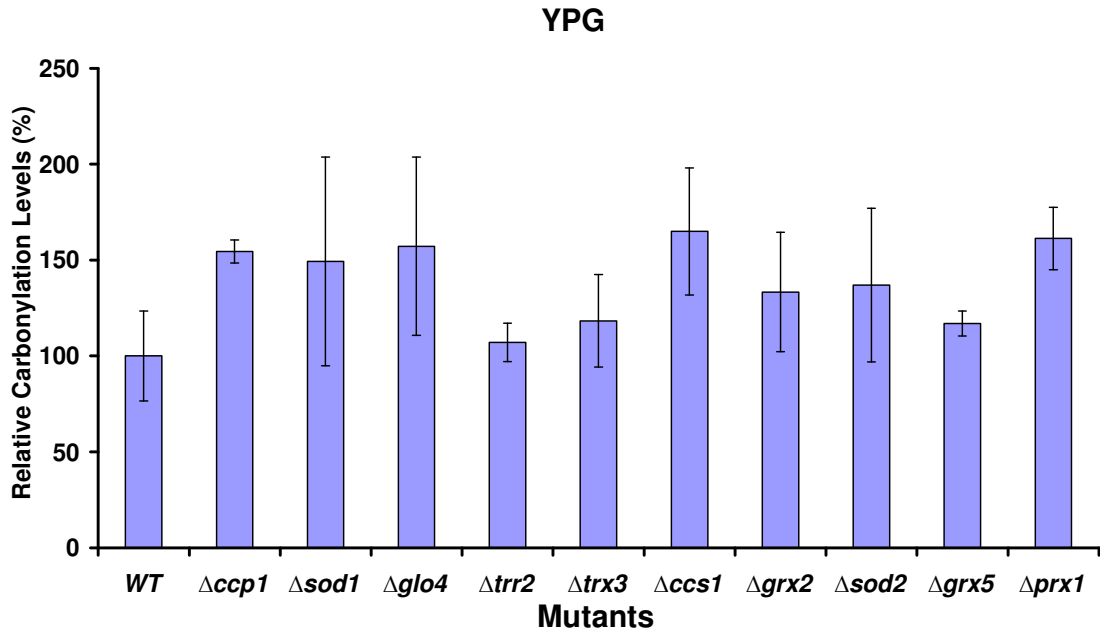


Figure 3.10. Cellular relative protein carbonylation levels for WT and mutant cells which were grown in glycerol media (YPG)

3.4.3. Determination of Respiration Compotent Cells

As a by product of respiration, superoxide radicals are formed which is followed by an increase on conseqiuent free radical formation. Cells lacking mitochondrial antioxidant genes may have impaired mitochondrial functions.

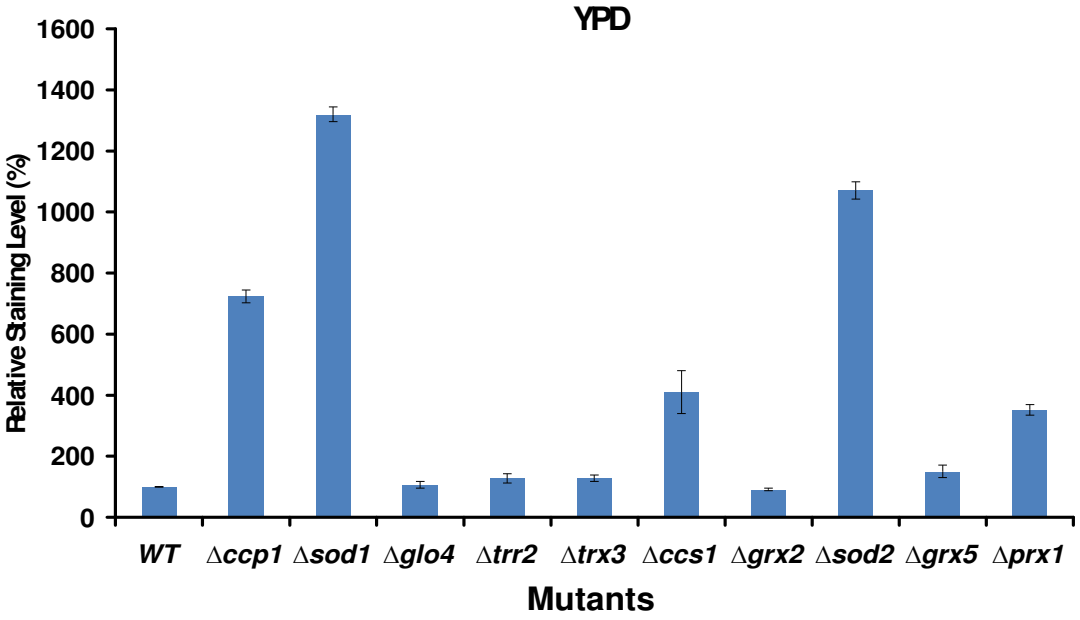


Figure 3.11. Relative levels of respiration compotent cells in glucose media (YPD)

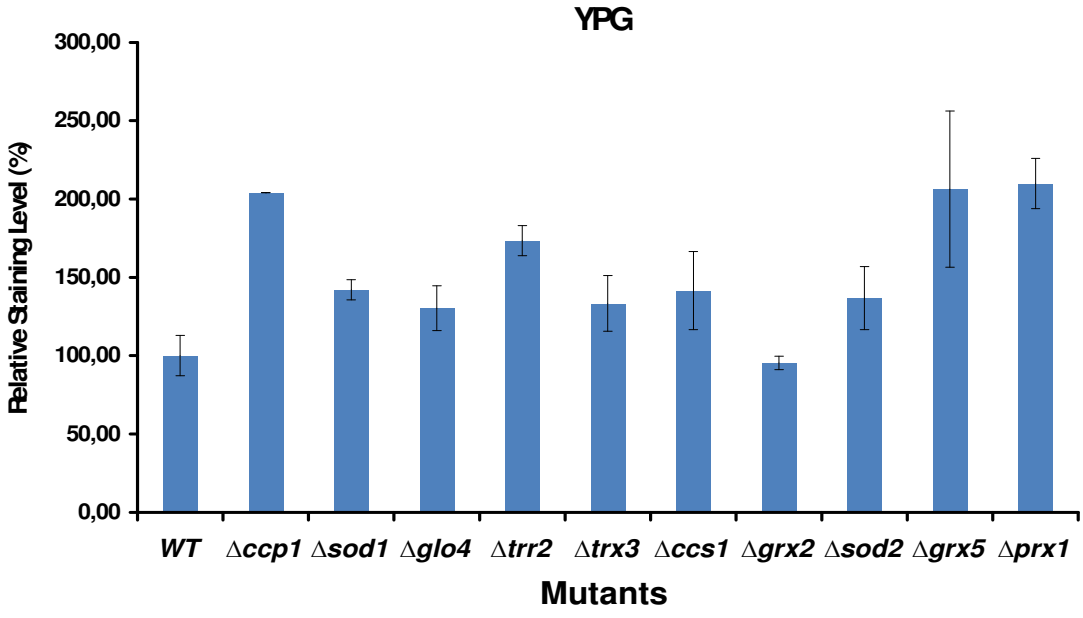


Figure 3.12. Relative levels of respiration compotent cells in glycerol media (YPG)

We wanted to know whether mutant cells have actively respiring mitochondria. To distinguish respiratory compotents cells from noncompotent cells, samples were stained with Mitotracker Red, which becomes flourescent upon oxidation by electron transport chain. Then cells were analysed by FACS array system to asses the ratio of respiratory compotent cells. In the presence of glucose flourescence intensity (Figure 3.11.) was low for most of the cells including WT, but Δ*prx1*, Δ*sod2*, Δ*ccs1*, Δ*sod1*, and Δ*ccp1*mutants gave higher level of signals which may reflect the oxidation potentials of these mutants. Deletion of *SOD1* increase level of respiration compotent cells 13,20 fold, deletion of the *SOD2* increase respiration compotent cells 10,71 fold, deletion of the *CCP1* increase respiration compotent cells 7,24 fold, deletion of the *CCS1* increase respiration compotent cells 4,10 fold and deletion of the *PRX1* increase respiration compotent cells 3,52 fold. Figure 3.12. shows the level of respiration compotent cells when the cells were grown in glycerol media. Level of respiration compotent cells were increased 110 % for Δ*prx1*, 106 % for Δ*grx5*, 104 % for Δ*ccp1*, 73 % for Δ*trr2*, 42 % for Δ*sod1*, 42 % for Δ*ccs1*, 37 % for Δ*sod2*, 33 % for Δ*trx3* and 30 % for Δ*glo4*, and decreased 5 % for Δ*grx2* when compared to WT cells. These data confirms that glycerol activates respiration rate in yeast. It also shows that deletion of antioxidant genes increases the oxidation potantial of the cells.

3.4.4. DNA Damage

In this study two different approaches were carried out to figure out the level of DNA damage in mutants. The first one is Quantitive PCR amplification method in which COX1 gene used as template. By comparing the intensity of the bands of PCR amplification products between different mutants and WT cells any strand break or damages that prevent amplification of template, which corresponds to damage on DNA, can be detected. Amplification of COX1 gene and flanking sequences (6,9 kb) covers 1/10 of total mitochondrial genome and it is a good measure of assessing mtDNA damages.

Q-PCR results showed that deletion of *CCS1* and *PRX1* totally prevents amplification of *COX1* gene which indicates that deletion of these two genes increase the sensitivity of mitochondrial DNA compared to WT and other mutant cells. In

addition Δ*trx3*, mutants showed sensitivity on mtDNA for which amplification levels reduced to 25 %, 41 %, 58 % respectively, as shown in 3.13. and 3.14.

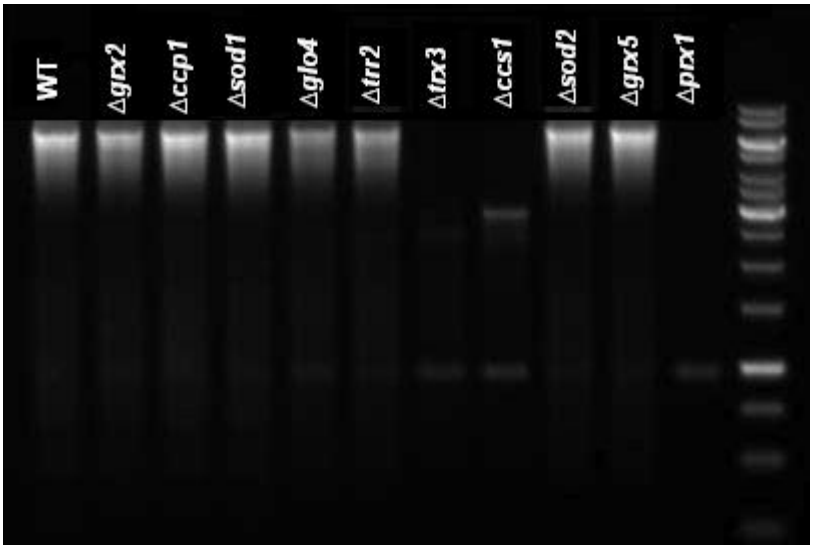


Figure 3.13. Q-PCR analysis of a 6,9 kb fragment of mitochondrial DNA including *COX1* gene. Cells were grown in glycerol media (YPG)

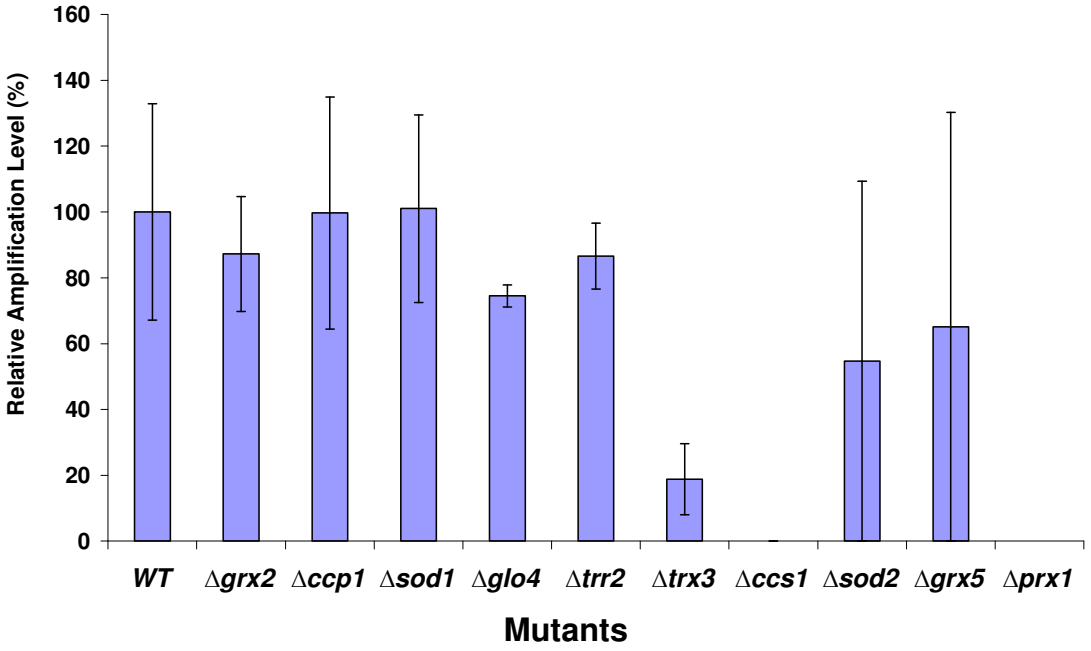


Figure 3.14. Density analysis for Q-PCR gel electrophoresis for the cells that grown in glycerol media (YPG)

It is possible that cells experiencing oxidative stress may have higher oxidative stress. Thus, we analyzed spontaneous mutation rates or genomic instability of mutants.

CAN1 gene encodes a plasma membrane arginine permease. Cells take up arginine via arginine permease and canavanine competitively inhibits arginine uptake. Resistance to canavanine is associated with loss of arginine permease function. High-level resistance to canavanine occurs exclusively because of mutation at *CAN1* locus. Because canavinine is a competitive inhibitor, arginine must be excluded from media for the experiments. Normally if the selective media is supplied with canavanin it competietively inhibits arginine and stops translation. Cells can not grow if *CAN1* gene activity is normal. We aimed to show random mutations by monitoring the *CAN1* activity by supplying the media with canavanin. Mutations in *CAN1* gene let cells grow in the presence of canavanin. Figure 3.15 shows the results of the cells which were grown in the presence of canavanin.

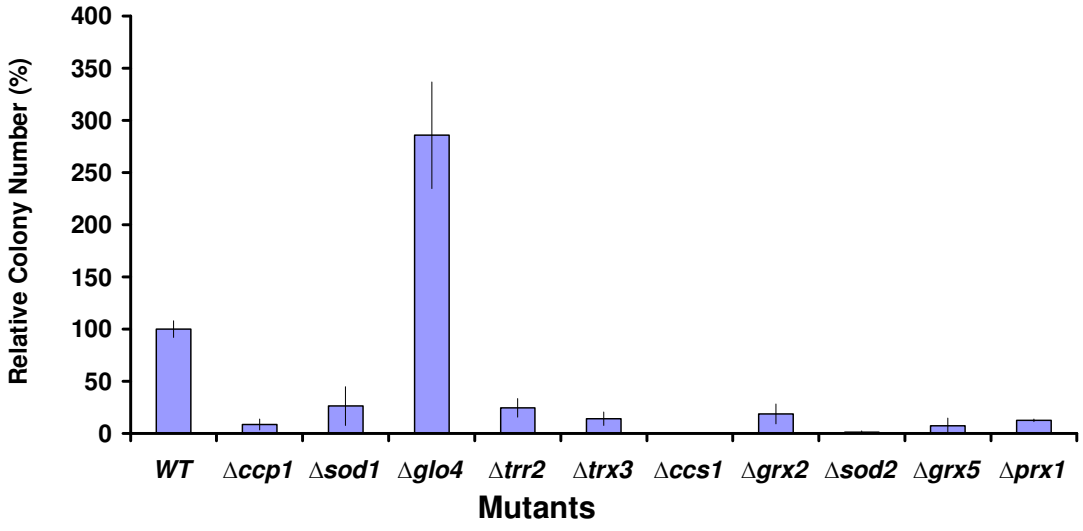


Figure 3.15. Relative colony numbers for WT and mutant cells which were grown in YNB supplied with canavanin.

While we were expecting to see CAN1 inhibition due to expected higher mutation rate in Δ*ccs1*, Δ*sod1*, Δ*sod2* mutants, only Δ*glo4* mutants yielded higher spontaneous rates among all mutants. This experiments was repeated twice and similar results were observed. Thus, mutants except Δ*glo4* have lower level of spontaneous mutation rate.

This phenomoneon may be explained by that absence of antioxidant genes may situmulate the expression of other antioxidant genes which protect cells from deleterious effects of ROS.

CHAPTER 4

CONCLUSION

The aim of this study was to show the effect of deleting mitochondrial antioxidant genes on aging. For this purpose we first identified the antioxidant genes and found ten different genes which are all expressed from genomic DNA and function in mitochondria. The life span profiles showed that the deletion of *SOD1*, *SOD2*, *CCS1* decreased life span in both glucose and glycerol media. In addition deletion of *CCP1* gene which encodes cytocrome c peroxidase decreased life span just only in YPG. We also carried out experiments to determine molecular damages in lipids, proteins and DNA for the mutants and wild type cells to find if there is any relation between the decrease of life span and increase of damages on macromolecules. In agreement with the free radical theory of aging, some of the mitochondrial antioxidant gene mutants lived shorter, but they did not accumulate extensive molecular damages. It is known that deletion of antioxidant genes can activate antioxidant systems through the activation of certain transcription factors (Allen and Tresini 2000). We speculate that, in our single mutants absence of each antioxidant gene may have shanged the redox status of cells and lead to an induction of other antioxidant enzymes to protect the cells. Thus we did not detect consistent and extensive molecular damages in macromolecules. Experiments with multiple mutants lacking antioxidant and redox transcriptional factors may be required to evaluate the effects of each mitochondrial antioxidant gene deletion on molecular oxidation levels.

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APPENDIX A

1. Media

a. Glucose (YPD) medium, per liter

1% yeast extract, 2% peptone, 2% glucose (Media sterilized by autoclaving at 121 ^{o}C for 15')

b. Glucose (YPD) agar medium, per liter

1% yeast extract, 2% peptone, 2% glucose, 2% agar (Media sterilized by autoclaving at 121 ^{o}C for 15')

c. Glucose (YPD) medium with H_2O_2, per liter

1% yeast extract, 2% peptone, 2% glucose, (Media sterilized by autoclaving at 121 ^{o}C for 15') 1 mM H_2O_2 (added just before inoculation)

d. Glycerol (YPG) medium, per liter

1% yeast extract, 2% peptone, 3% glycerol (v/v) (Media sterilized by autoclaving at 121 ^{o}C for 15')

e. Glycerol (YPG) agar medium, per liter

1% yeast extract, 2% peptone, 3% glycerol(v/v), 2% agar (Media sterilized by autoclaving at 121 ^{o}C for 15')

f. Glycerol (YPG) medium with H_2O_2, per liter

1% yeast extract, 2% peptone, 3% glycerol (v/v), (Media sterilized by autoclaving at 121 ^{o}C for 15') 1 mM H_2O_2 (added just before inoculation)

g. Supplemented Minimal Medium(YNB-*Arg*+Can), per liter

6,79g/L YNB with ammonium sulfate, 2% glucose, 2 ml methionine (1 g/100 ml), 3 ml leucine (1 g/100 ml), 10 ml uracil (0,2 g/100 ml), 2 ml histidine(1g/100 ml), 2% agar (Media sterilized by autoclaving at 121 ^{o}C for 15'), 3 ml of 20 mg/ml canavanine sulfate

2. 0,5 M EDTA per liter

186,1 g EDTA added into 800 ml water.pH adjusted to 8.0 with NaOH. Solution sterilized by autoclaving at 121 ^{o}C for 15 minutes.

3. Yeast Lysis Buffer

4 ml Triton -100, 20 ml 10% SDS, 4 ml 5M NaCl, 400 µl 0,5 EDTA, 2 ml 1M Tris (pH 8) 0,5 ml 400mM DTT were added into 169,1 ml distilled water.

4. PMSF (phenylmethylsulfonylfluoride)

100 mM PMSF was dissolved in isopropanol and filter-sterilized. It was stored at -20°C to be used at 1mM working concentration when required.

5. TBA reagent

0,25 M HCl, 15% [wt/vol] trichloroacetic acid, 0.375% [wt/vol] TBA

6. Bradford reagent

10 mg Coomassie Brilliant Blue G-250 was dissolved in 5 ml 95% ethanol and 10 ml 85% (w/v) phosphoric acid added.Solution diluted to 100 ml when the dye has completely dissolved, and filter through Whatman #1 paper just before use.

7. 1X TE buffer

10 mM Tris HCl(pH 8.0), 1mM EDTA

8. 50XTAE Buffer

242 g Tris base and 37.2 g Na2EDTA (2H2O) was dissolved in 900 ml deionized water. After adding 57.1 ml glacial acetic acid, the volume was adjusted to 1 liter with water.

9. Primers

6,9-kb mitochondrial fragment in *COX1* gene:

Sense : 5'-GTG CGT ATA TTT CGT TGA TGC GT-3'

Antisense: 5'-GTC ACC ACC TCC TGC TAC TTC AA-3'