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Copper supplementation increases yeast life span under conditions requiring respiratory metabolism

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Abstract

To further exploit yeast as a model for cellular aging we have modified the replicative life span assay to force respiration, by replacing glucose with the non-fermentable carbon source glycerol. The growth rates of several different strains varied greatly, with doubling times ranging from 2.7 to 7 hours. Life spans of all strains were lower on media containing glycerol than on media containing glucose. However, supplementation of glycerol-containing media with copper resulted in increases in life span of between 17 and 72%; life spans equivalent to or beyond those obtained on glucose media. Addition of copper to glucose medium had no effect on life span. Microarray analysis showed that genes responsible for high affinity import of copper display reduced expression upon addition of copper, while most genes showed no change in expression. No differences in growth rate, oxygen uptake, or the levels of subunit II of the copper-containing cytochrome c oxidase were found between cultures of yeast grown with or without copper supplementation. Copper supplementation greatly extended the life span of *sod1* and *sod2* strains, suggesting that addition of copper may reduce the generation of superoxide. Forcing yeast to respire places an emphasis on mitochondrial function and may aid in the identification of factors involved in aging in other respiratory-dependent organisms.

Keywords

Aging; mitochondria; yeast; copper; respiration; microarray

Introduction

The budding yeast *Saccharomyces cerevisiae* is used as a model for cellular aging. One method of measuring yeast life span involves counting the number of buds individual cells produce and is referred to as the replicative life span. Measurement of replicative life span has been done almost exclusively on media containing 2% glucose as a carbon source. Reducing the concentration of glucose in media or limiting the uptake of glucose by cells results in extension of life span (Jiang et al., 2000; Lin et al, 2000). This effect is apparently due to an increase in respiratory activity and is also dependent on signals from the mitochondria to the nucleus (Jiang et al., 2000; Lin et al., 2002). Yeast life span extension on reduced glucose is proposed to mimic the effects of caloric restriction seen in higher organisms.

The fact that lowering glucose concentration results in a change in metabolism highlights the fact that yeast use fermentation as the major source of energy production, even in the presence of oxygen. The exact ratio between the use of fermentation and respiration in yeast is likely

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strain dependent, but fermentation accounts for a majority of the glucose consumed, even in strains with fully functional mitochondria (Gancedo and Serrano, 1989).

Even though yeast respire very little in the presence of glucose, experimental evidence has shown that mitochondrial function is an important determinant of aging in yeast grown on glucose media (Jazwinski, 2005). The *UTH1* gene is a determinant of yeast longevity and has been linked to mitochondrial biogenesis and degradation (Camougrand et al., 2004). The retrograde response, a signal from mitochondria to the nucleus indicating mitochondrial dysfunction, increases life span when activated (Kirchman et al., 1999; Borghouts et al., 2004). Prohibitins, mitochondrial proteins encoded by *PHB1* and *PHB2*, are responsible for proper segregation of mitochondria and, if deleted, cause decreases in life span (Piper et al., 2002; Kirchman et al., 2003). A mutation in *ATP2*, which encodes a subunit of the mitochondrial ATPase, was shown to influence the proper segregation of mitochondria to newly budded cells (Lai et al., 2002). Finally, enzymes that limit the damage caused by reactive oxygen species, such as superoxide dismutases, catalase, and methionine sulfide reductases have each been linked to yeast longevity (Wawryn et al., 1999; Van Zanduycke et al., 2002; Koc et al. 2004). These results were all obtained on media containing glucose, even though respiratory metabolism is limited on glucose.

While results do indicate that mitochondria are important in determining the life span of yeast, the model is not ideal since respiratory metabolism is largely bypassed. A yeast model that requires cells to rely more heavily on respiration might provide a better model for understanding the role of mitochondria in the aging process. Here we describe a modified method for analyzing yeast replicative life span that requires yeast to utilize only mitochondrial respiratory metabolism. The addition of the non-fermentable carbon source glycerol in place of glucose forces yeast cells to use only mitochondrial respiration to generate energy, more closely mimicking the situation in higher organisms. We measured the growth rates and life spans of several strains of yeast on media containing glycerol. We serendipitously found that the addition of copper to the media resulted in an increase in life span. The extent of copper's influence on yeast life span under respiratory conditions was strain dependent. Copper had no effect on yeast life span when strains were grown on media containing glucose. However, copper supplementation greatly extended the life spans of *sod1* and *sod2* strains on glycerol containing medium.

Materials and Methods

Yeast strains and growth conditions

S. cerevisiae strains W303-1A (*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*), S288c (*MATa SUC2 gal2 mal mel flo1 flo8-1 hap1*), BY4741 (*MATa SUC2 gal2 mal mel flo1 flo8-1 hap1 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), BY4742 (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*), BY4743 (diploid of BY4741 and BY4742), *sod1Δ* (BY4742 *sod1::KanMX*), *sod2Δ* (BY4742 *sod2::KanMX*), YPK9 (*MATa, ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52*) and SP-22 (*MATa/a leu1 arg4*) were grown in YPG medium (1% yeast extract, 2% peptone, 3% glycerol, Nanopure water) or YPD medium (1% yeast extract, 2% peptone, 2% glucose, Nanopure water). All BY strains and their derivatives were obtained from Open Biosystems. SP-22 is related to SP-4 (Bilinski et al., 1978). For growth rate and life span analyses, copper sulfate was added to a final of 62 μM. Plates used for life span analyses contained 2% agar. Oxygen uptake was assayed as described (Creanor, 1978).

Life span analysis

Life span analyses were conducted as previously described (Kim et al., 1999). Briefly, 1μl of logarithmically growing cells from YPG liquid media was placed onto YPG plates. Individual

unbudded cells were then separated from the rest of the population by the use of a micromanipulator. These cells were allowed to produce buds. The buds were removed and used as the cohort for life span analysis. Each time that a bud was removed from an individual cell the cell was counted as one generation older. Cells were grown at 30°C during the day, and overnight at 12.5°C if on glycerol medium and 4°C if on glucose medium. Growth of cells at low temperatures does not affect replicative life span (Muller et al., 1980). Statistical analyses of life span were performed using the nonparametric Mann-Whitney test.

Media metal analysis

Analysis of medium for content of metals was conducted by Jupiter Environmental Laboratories on a Thermo Excel ICP-MS using EPA Method 200.8.

PCR screening of the *CTR3* promoter

To screen for the presence of a Ty2 insertion in the promoter of the *CTR3* the following primers were used. To detect an intact *CTR3* promoter, primer P1 - 5' CAGAAACGCAAAACCAGAAGA3' was paired with primer P3 - 5' ACAAGTCGCTTTTTTGGCAG3'. To detect a Ty2 insertion, the primer P2 - 5' TTGCAGATTCCCTTTTATGGA3' was paired with P3. Chromosomal DNA was prepared using the Easy-DNA kit (Invitrogen). Amplification was performed for 30 cycles with the following temperatures and times: 94C for 30 seconds; 57C for 30 seconds; 72C for 30 seconds. The products were separated on 2% agarose.

Removal of TY2 from the *CTR3* promoter

To remove the TY2 insertion from the promoter of *CTR3*, the non-disrupted promoter and most of the gene was amplified from SP-22 using the following primers: CTR3-6489 - 5' CGAAGCATTTGGATATGGAC3' and CTR3+721r - 5' ACGTCTAGAGCAGCATTTGCGATCATCA3'. The PCR product was directly ligated into pGEM-T Easy (Promega) and used to transform *E. coli*. The amplified fragment was then excised with *EcoRI* and *XbaI*, ligated into pRS406 (Sikorski and Hieter, 1989) that had been digested with the same enzymes. The ligation was used to transform *E. coli*, yielding p406CTR3. This plasmid was linearized with *HpaI* and used to transform yeast strain BY4742 to uracil prototrophy. Transformants were then grown on nonselective medium to allow excision of the integrated plasmid and flanking regions, followed by growth on medium containing 5-FOA to select for uracil auxotrophs. Strains were then screened as described above for loss of the Ty2 insertion in the *CTR3* promoter.

Microarray analysis

RNA was prepared from 20 ml samples harvested at an OD₆₀₀ of 0.4 using hot acidic phenol as described (Collart and Oliviero, 2006). In vitro transcription, cDNA synthesis, sample clean-up and labeling of approximately 2 µg of total RNA was accomplished using the Affymetrix GeneChip One-Cycle Target Labeling Kit as directed by the manufacturer. Labeled samples were hybridized to the Affymetrix GeneChip (Affymetrix Inc., Santa Clara, CA) Yeast Genome 2.0 Array, using standard Affymetrix protocols. The microarray allows the interrogation of approximately 99% of the annotated genes from both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The GeneChip Arrays were pre-hybridized with 1X Hybridization Buffer for 10 minutes at 45°C. The labeled samples were added to the GeneChip Arrays and hybridized for 16 hours at 45°C. The arrays are stained and washed according to Affymetrix Fluidics Station 450 protocol EukGEWS2v5_450. Intensity values were collected from the GeneChips by scanning the arrays with a GeneChip Scanner 3000 7G. Biological triplicates were conducted for each unique treatment and each replicate was hybridized to a separate microarray. All microarray data met quality control standards for overall intensity,

scaling factors, and 3' to 5' ratios for control genes (Hubbell et al., 2002). The intensity values were normalized using the GC-RMA algorithm and the data was filtered to remove hypothetical open reading frames and all probes that achieved an average intensity value of less than 200 across all samples.

Western blot analysis

Yeast cells for western blot analysis were harvested at an OD₆₀₀ of 0.5. Cell pellets (2.5 ml) were washed 1 time with ice cold water and then pellets were quick frozen in a dry ice ethanol bath. Cells were prepared for electrophoresis as described (Horvath and Riezman, 1994) and separated on 12% SDS-PAGE gels (PageGel Inc.). Proteins were transferred to nitrocellulose using a semi-dry method and probed with antiserum as described (Scopes, 2006). Monoclonal antibodies to yeast Cox2 (4B12) and yeast mitochondrial porin (16G9) were obtained from Invitrogen and used as recommended by the supplier. Goat anti-mouse alkaline phosphatase conjugate was obtained from Promega and used at a dilution of 1:7,500. Color was developed using Western Blue substrate (Promega).

Results

Growth rates and life spans vary greatly under respiratory conditions

Growth rates were measured in YPG liquid medium for 6 yeast strains. Strains YPK9, W303-1A, BY4741, and BY4742 have previously been used for life span analyses on fermentable carbon sources (Kirchman et al., 1999; Kaeberlein et al., 2004). Strain S288c was selected because it is a prototroph and strain SP-22 was selected for its robust growth under respiratory conditions (Zagulski pers. comm.). 20 ml cultures were grown in YPG shaking at 250 RPM and optical density reading were used to determine doubling time (Table 1). Because of its extremely poor respiratory growth, YPK9 was eliminated from further analysis. Life span analyses on YPG agar were conducted on the remaining strains.

While BY4741 grew well in liquid medium, life span analysis proved impossible on YPG medium due to the frequent inability to remove daughter buds from the mother cell. Several trials of the life span analysis were performed on the 4 remaining strains; W303-1A, S288c, BY4742, and SP-22. Representative results of the life span analyses are shown in figure 1. The growth rates of the strains on solid medium correlated with that obtained in liquid medium, with SP-22 growing the most rapidly and W303-1A dividing the slowest. However, life span did not correlate with growth rate. While the mean life span of the more quickly dividing SP-22 was the longest, the slowest grower, W303-1A had a longer mean life span than S288c or BY4742 on glycerol medium (Figure 1).

Copper increases life span of respiring cells

One repeat of the life span assay showed an increase in life span that was significantly longer than in previous experiments (not shown). It was discovered that, because of a plumbing error, the water used for preparing the media was tap water instead of Nanopure water. Analysis of the medium for metals showed that both copper and calcium were present at elevated levels in the media made with tap water compared to media made with Nanopure water (Table 2). The levels of 20 other metals, including manganese, zinc, magnesium, and iron did not differ by more than 20% and were not tested further (data not shown). Growth analysis in media supplemented with calcium or copper showed no change in the growth rate (not shown), or a decrease in growth rate at high copper levels (gt;300 μ M). Several copper levels were analyzed and a concentration of 62 μ M (0.01g CuSO₄/liter) was chosen as optimal. No change in growth rate was detected with this concentration of copper, however life spans were extended.

Supplementation of solid media with copper resulted in an increase in life span compared to controls grown on media with no copper supplementation. In one representative experiment, the degree of the extension in W303-1A was 17%, while the life span of the S288c strain increased 42%, BY4742 was increased 38%, and that of the SP-22 strain increased 72% when copper was added to the medium (Figure 2). These results were similar in repeated trials.

The *CTR3* promoter is disrupted in S288c but not SP-22.

The promoter of the gene for the high affinity copper transporter, *Ctr3*, has been shown to contain an insertion of the transposon Ty2 in many laboratory strains, including S288c and BY4742, a derivative of S288c (Knight et al., 1996). This insertion separates the promoter and start point of transcription from the *CTR3* gene by 6 kb. To explore one possible reason for the differential effect of copper on the life spans of S288c, and SP-22, we analyzed the *CTR3* promoter of both strains for the presence of a Ty2 insertion. Primer pairs were designed to yield products 460 bp in length in the absence of a Ty2 insertion, and 275 bp in length if the insertion was present (Figure 3). As expected, amplification of S288c chromosomal DNA yields a 275 bp product indicative of a Ty2 insertion. Amplification of chromosomal DNA from SP-22 resulted in the 460 bp product expected only if the *CTR3* promoter is not interrupted by an insertion of Ty2 (Figure 4).

Repair of the *CTR3* promoter disruption does not influence life span

We hypothesized that the shorter life spans of S288c and BY4742 relative to SP-22, both in the presence and absence of copper supplementation, might be due in part to a diminished ability to import copper, caused by the TY2 insertion in the promoter of *CTR3*. We removed the TY2 insertion from the promoter of strain BY4742 using a replacement strategy involving integration of the intact gene and promoter and subsequent removal of the disrupted sequence (Scherer and Davis, 1979). Life span analyses were performed on two independent isolates of BY4742 from which the TY2 insertion in the *CTR3* promoter had been removed. No difference was found in the life spans of BY4742 strains in which the promoter for *CTR3* had been repaired (not shown). The presence of the TY2 insertion in the promoter is not responsible for the shorter life spans of BY4742 and S288c relative to SP-22.

Copper does not increase the life span of cells grown on glucose medium

Since life span analyses have traditionally been performed on media containing glucose, we analyzed the effect of copper supplementation on glucose containing YPD medium. Since S288c, BY4742, and SP-22 showed the greatest increases when supplemented with copper analyses were performed on these three strains. The addition of copper to YPD medium had no effect on the life spans of either S288c, BY4742, or SP-22 yeast compared to controls grown on YPD (Figure 5).

Copper transporter genes *CTR1* and *CTR3* expression is reduced when cells are supplemented with copper, but stress responses or *COX* genes are not induced

Microarray analysis of yeast strain SP-22 grown in liquid YPG or YPG supplemented with copper revealed that expression of the genes for the high affinity copper transporters *CTR1* and *CTR3* is greatly reduced when copper is added to the medium as is expression of several genes involved in copper and iron import (Figure 6). Since import of iron is dependent on copper containing enzymes, the down-regulation of genes responsible for iron import in the presence of added copper is not unexpected, however cells were not likely starved for iron in our experiments since expression of *FET3*, which is known to be induced under conditions of iron starvation (Yamaguchi-Iwai et al., 1995) was unchanged (not shown). Two genes responsive to high copper levels, *CRS5* and *CUP1-2*, showed increased transcript levels in cells grown in YPG supplemented with copper, although levels were only approximately 2-fold higher than

in cells grown in YPG. The concentration of copper used in this experiment (60 μ M) was more than 100-fold below levels known to be toxic to yeast (Shanmuganathan et al., 2004). Expression levels of most genes, including genes involved in stress response and respiration, were unchanged. Most notably, *SOD1*, which codes for copper-zinc superoxide dismutase, showed no change in expression. *SOD1* expression has previously been shown to increase 2.5 fold when copper is added to growth medium (Gralla, et al., 1991), however that induction was noted between copper-free synthetic medium and synthetic medium supplemented with 50 μ M copper, whereas copper is present in our unsupplemented YPD medium (Table 2). Interestingly, the single gene not known to be involved with copper or iron metabolism that showed altered expression in the presence of copper was *HXT2*, which codes for the high affinity hexose transporter. Deletion of another gene involved in hexose metabolism, *HXK2*, which codes for hexokinase, has been shown to increase yeast life span presumably by a mechanism resembling caloric restriction (Lin et al., 2000). Why *HXT2* would be expressed in our assay, and why its expression would be decreased by copper supplementation is unknown.

Copper supplementation does not alter cytochrome c oxidase levels or oxygen consumption rate

Although expression levels of most genes remained unchanged by the addition of copper we investigated the possibility that the levels of respiratory enzymes or respiration rate might still be altered by the addition of copper. Cytochrome c oxidase (Complex IV) of the electron transport chain contains both iron and copper molecules. If either of these cofactors are limiting, cytochrome c oxidase assembly can not be completed (Herrmann and Funes, 2005). Incompletely assembled mitochondrial complexes are degraded by AAA proteases (Arnold and Langer, 2002). We performed western blot analyses on total protein from yeast grown with or without copper supplementation to detect subunit II (Cox2) of cytochrome c oxidase. No difference in the amount of Cox2 was detected between cells grown with or without copper supplementation (Figure 7). Analysis of oxygen consumption rate also showed no detectable difference between cultures grown with or without copper supplementation (not shown). Respiratory rate and the level of cytochrome c oxidase are unchanged in growing cultures, which does not eliminate the possibility that added copper has its effect only on old cells.

Copper supplementation restores the life span of superoxide dismutase mutants

To examine the possibility that copper was reducing oxidative stress in cells we assayed the effect of copper supplementation on the life span of superoxide dismutase mutants. Mutants with a deletion of either the *SOD1* gene, which codes for Cu/Zn SOD, or the *SOD2* gene, which codes for Mn SOD, were assayed on YPG medium with and without copper supplementation. As expected, strains containing a deletion of either *SOD1* or *SOD2* had extremely short life spans. In one representative experiment the mean life span of the *SOD1* mutant was 3.4 generations, only 20% of the BY4742 control, while the mean life span of the *SOD2* mutant was 6.5, only 38% of the control. Copper supplementation increased the life span of BY4742, as in previous experiments, and also increased the life spans of the *SOD1* and *SOD2* mutants to means of 16.5 and 21.5 generations respectively (figure 8). This result indicates that copper supplementation is acting to increase longevity by reducing the production or increasing the removal of superoxide.

Discussion

We have initiated a modified system for the analysis of replicative life span in yeast that emphasizes respiratory metabolism. By replacing glucose with glycerol as a carbon source, yeast are forced to derive energy through respiration as opposed to fermentation. This change opens the possibility for finding novel effects of mitochondrial metabolism on longevity using

yeast. The advantages of using yeast lie in its extremely short life span and the powerful genetic tools available. By forcing yeast to respire their metabolism more closely models other multicellular organisms.

While the short life span of yeast relative to other organisms is desirable and advantageous in terms of the rapidity with which experiments can be completed, yeast strains with short life spans may be problematic as models of aging. As pointed out recently by Kaerberlein et al. (2005), manipulations that extend the life span of strains with short life spans may simply be suppressing mutations already present in the strain. The SP-22 strain has an extremely long life span, but life span analyses do not take significantly longer due to the more rapid division of this strain. This strain is also a diploid, a factor that makes this strain more similar to higher eukaryotes. Because the strain is a MAT a/a diploid it is still able to mate. The ability to mate allows us to transfer in different mitochondrial DNA mutations, by cytoduction with a *kar1* mutant (Conde and Fink, 1976). We are currently using this strain to assay the effects of mitochondrial DNA point mutations on longevity. The fact that the strain is diploid should reduce the effects of nuclear point mutations on life span, making the effects of mitochondrial mutations more pronounced and/or detectable.

The fact that the life spans of strains S288c, BY4742, and SP-22 on media containing glycerol as a carbon source were shorter than on media containing glucose is what would be predicted by theories of aging involving oxidative damage. Our results support the observation that yeast primarily ferment in the presence of glucose. The finding that the addition of copper to the medium allowed cells to live significantly longer might be explained by two different mechanisms. First, it is possible that the addition of copper increases the antioxidant properties of the metallothionein Cup1. Overexpression of the *CUP1* gene in the presence of copper was shown to compensate for phenotypes associated with deletion of *SOD1*, although the concentration of copper and the increase in *CUP1* expression were much higher than in our experiments (Tamai et al., 1993). Another possibility is that added copper allows for increased respiration, by allowing more efficient electron transfer to cytochrome oxidase, which contains copper. Increasing the flow of electrons through the respiratory chain might be expected to increase the production of reactive oxygen species (ROS), shortening life span. However, it has been shown that treatments that decrease the uptake of glucose and increase respiration extend yeast life span (Lin et al., 2002). This is supported by our finding that copper-supplemented respiring yeast live even longer than cells grown on glucose containing medium. Increasing the respiratory rate with uncoupling agents has recently been shown to increase the life span in both yeast and mice (Barros et al., 2004; Speakman et al., 2004). In mice the effect seems to be due to a decrease in the mitochondrial membrane potential. Decreasing membrane potential correlates with a decrease in ROS production (Nicholls, 2004). In yeast the extension of life span by an uncoupling agent was proposed to be mediated by the genes involved in the retrograde response, a signal from the mitochondria to the nucleus indicating mitochondrial dysfunction. If these genes are responsible it seems that they must be acting through a different form of activation, since an uncoupling agent would increase respiratory activity, just the opposite of the normal activation signal for the retrograde response (Butow and Avadhani, 2004).

The large variation in life span extension provided by copper supplementation may be due to a variety of factors. The fact that the life span of W303-1A was not increased to a large extent may mean that metabolism is not the limiting factor in determining life span in this strain. If this strain carries a mutation that is limiting its life span, these treatments may be masked. One mutation that may be responsible for the difference is the *his3-11* mutation carried by W303-1A. Histidine mutants are known to be more sensitive to copper toxicity (Pearce and Sherman, 1999). This may explain why the addition of copper to the media had less of an effect on W303-1A than on S288c and SP-22, which are histidine prototrophs. W303-1A is therefore

not a good choice for assaying life span under respiratory conditions. Another histidine mutant allele, *hisΔ200*, in addition to increasing sensitivity to copper also reduces the stability of the mitochondrial DNA, due to a partial deletion of the promoter for the divergently transcribed *MRM1* gene, which encodes mitochondrial rRNA methyltransferase (Struhl, 1985; Sirum-Connolly and Mason, 1993). The results of a recent study examining copper levels, Cu/Zn SOD activity, and yeast longevity may be attenuated due to the presence of the *hisΔ200* mutation in the strains used (Harris et al., 2005). Assaying longevity on media that forces yeast to respire makes them a better model for human aging. Optimized growth conditions and proper strain selection are also needed so that life spans are not artificially limited. Cells that die due to poor media conditions, such as the lack of copper, or mutations unrelated to aging, such as *his⁻* mutations, may mask results pertaining to cellular aging.

The question of why life span was increased by the addition of copper to the medium remains open. Copper is needed as a cofactor by several enzymes in yeast. Since the addition of copper had no effect on life span when cells were grown on media containing glucose, and copper restored the life span of SOD mutants, the effect is likely exerted through respiratory metabolism. However, the increased copper concentration is not likely to be working by providing copper to Cu/Zn SOD, since copper greatly increased the life span of an *SOD1* mutant. It has been demonstrated that yeast contain a pool of copper in the mitochondrial matrix that is not associated with any protein (Cobine et al., 2004). This pool may be used to buffer copper concentration in the cell, or to provide copper to cytochrome c oxidase and the fraction of Cu/ZnSOD present in the intermembrane space (Sturtz et al., 2001). Assembly of cytochrome c oxidase is sensitive to heme deficiency and recovery of cytochrome c oxidase levels after heme deficiency is slower in old fibroblasts (Atamna et al., 2001). A similar effect might occur with copper deficiency. Although we found no difference the levels of Cox2, which would indicate lower levels of complex IV, our assays were performed on growing cultures of yeast. These results and the fact that the growth rate of yeast cells is unchanged when copper is added to the medium indicating that the added copper may affect only older cells, which make up a very small fraction of an exponentially growing liquid culture and would not change the growth rate or oxygen uptake rate of a liquid culture (individual cells of generation n are 1 out of every 2^{1+n} cells). As cells age perhaps the mechanism used to accumulate copper in the mitochondrial matrix slows or stops and cells survive until the pool is exhausted.

Copper affects the life span of *Podospora anserina*, another fungal model of aging, in quite a different way. In this system copper depletion within the cell results in the loss of cytochrome c oxidase function. When deprived of copper, *P. anserina* makes use of an alternate pathway of respiration that bypasses complex III and IV (Borghouts et al., 2002). The extension of life span in *P. anserina* may be due to a decrease in the production of ROS from complex III, which, along with complex I, is believed to be a major source of ROS production in the most cells (Balaban et al., 2005). *Saccharomyces cerevisiae* and other organisms in which aging is studied do not have this alternate pathway and must rely on the passage of electrons through complexes III and IV.

Organisms that require complexes III and IV to complete respiration have no choice but to use this pathway, even if it is functioning at less than optimal efficiency. If copper is limiting then the flow of electrons may be slowed due to a lack of functional cytochrome c oxidase. Inhibiting the flow of electrons is known to result in an increase in ROS. Inhibitors of electron transport that increase the pool of reduced coenzyme Q result in increased production of ROS (Brand, 2000). On the other hand, when electron flow is increased, by expression of uncoupling protein in mice (Speakman et al., 2004) or by the addition of a chemical uncoupler in yeast (Barros et al., 2004) life span is increased. Increasing the rate of electron flow may result in decreased production of ROS, by giving electrons a place to go other than going prematurely to oxygen.

While we have attempted to create a situation that more closely models respiratory metabolism in higher organisms, the model is still not ideal. While we have forced yeast to respire, the respiratory chain of yeast is not identical to higher organisms in that yeast NADH dehydrogenase is a single subunit that does not pump protons (Velazquez and Pardo, 2001). Further, cells that have high energy requirements derive energy from glucose through glycolysis and then mitochondrial respiration. To more closely model this in yeast would require reengineering the metabolism. It does appear that this may be possible and simpler than one might expect. By creating a strain with a chimeric hexose transporter as the only glucose transporter in yeast cells, Otterstedt et al. (2004) succeeded in switching the metabolism of yeast fully to respiration. This strain will be useful for industrial purposes and may also prove useful in studies of aging.

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Figure 1.

Life spans of 4 different strains grown under conditions requiring respiration. The mean life spans were as follows: W303-1A (●) 21.8; S288c (○) 18.3; BY4742 (▽) 16.4; SP-22 (▼) 31.5.

Figure 2.

Copper supplementation increases life span under respiratory conditions. (A) The mean life span of W303-1A was increased from 20.2 on YPG medium (●) to 23.6 on YPG supplemented with copper (○) ($p=0.01$). (B) The mean life span of S288c was increased from 19.0 on YPG medium (●) to 26.9 on YPG supplemented with copper (○) ($pl<0.001$). (C) The mean life span of BY4742 was increased from 18.2 on YPG medium (●) to 25.2 on YPG supplemented with copper (○) ($pl<0.001$). (D) The mean life span of SP-22 was increased from 33.4 on YPG medium (●) to 57.4 on medium supplemented with copper (○) ($pl<0.001$). Cohorts were composted of 50 cells in each experiment. Note the differences in scale between the x axes in each graph.

Figure 3.

Relative positions of primers used to analyze the *CTR3* promoter. Primer P1, when paired with primer P3 yields a product 460 bp in length in the absence of a Ty2 insertion in the *CTR3* promoter. Primer P2, when paired with primer P3, yields a product 275 bp in length in the presence of a Ty2 insertion. The Ty2 insertion separates primers P1 and P3 by more than 6 kb, resulting in no product under the extension conditions employed (30 seconds).

Figure 4.

The *CTR3* promoter of SP-22 does not contain a Ty2 insertion. Amplification of chromosomal DNA using primers to detect a Ty2 insertion in the *CTR3* promoter confirms that strain S288c contains a disruption of the promoter, due to the presence of the 275 bp band when primers 2 and 3 are used. The presence of the 460 bp band obtained when SP-22 DNA was amplified using primers 1 and 3 shows that the *CTR3* promoter is not disrupted by Ty2 in this strain.

Figure 5.

Copper supplementation does not increase life span under conditions allowing fermentation. (A) The mean life span of S288c on YPD medium (●) was 29.0 and was unaltered ($p=0.37$) on medium containing copper (○) (mean 27.3). (B) The mean life span of BY4742 on YPD medium (●) was 19.6 and was unaltered ($p=0.46$) on medium containing copper (○) (mean 18.5). (C) The mean life span of SP-22 on YPD medium (●) was 47.0 and was unaltered ($p=0.33$) on medium containing copper (○) (mean 44.0). Cohorts were composed of 50 cells in each experiment.

Fold induction vs. control

Figure 6.

Genes responding to copper supplementation under respiratory conditions. RNA was isolated in triplicate from SP-22 grown in YPG medium or YPG supplemented with copper. The figure shows the heat map of selected genes from three replicate samples grown in YPG supplemented with copper when compared to the average signal from the controls.

Figure 7.

The Cox2 subunit of cytochrome c oxidase is unchanged in cells supplemented with copper. Equal amounts of total protein from cells grown in YPD and YPD supplemented with copper was probed with antiserum to yeast Cox2. Mitochondrial porin levels were used to control for variation in sample loading.

Figure 8.

Copper supplementation extends the life span of superoxide dismutase mutants. The mean life span of BY4742 was increased from 17.0 on YPG medium (●) to 28.5 on YPG supplemented with copper (○) (p<0.001). The mean life span of BY4742 *sod1* was increased from 3.4 on YPG medium (▼) to 16.5 on YPG supplemented with copper (▽) (p<0.001). (C) The mean life span of BY4742 *sod2* was increased from 6.5 on YPG medium (■) to 21.5 on YPG supplemented with copper (□) (p<0.001). Cohorts were composed of 40 cells in each experiment.

Table 1

Doubling time of yeast strains in YPG liquid medium.

Strain	Doubling time (hours)
SP-22	2.7
S288c	3.3
BY4741	3.3
BY4742	3.2
W303-1A	4.4
YPK9	7.0

Table 2

Media Metal Analysis. Concentrations of calcium and copper in YPG media made with Nanopure water or tap water.

	Calcium (micromolar)	Copper (micromolar)
Nanopure	140	0. 41
Tap	420	10