Extension of Yeast Chronological Lifespan by Methylamine

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Abstract

Background: Chronological aging of yeast cells is commonly used as a model for aging of human post-mitotic cells. The yeast Saccharomyces cerevisiae grown on glucose in the presence of ammonium sulphate is mainly used in yeast aging research. We have analyzed chronological aging of the yeast Hansenula polymorpha grown at conditions that require primary peroxisome metabolism for growth.

Methodology/Principal Findings: The chronological lifespan of H. polymorpha is strongly enhanced when cells are grown on methanol or ethanol, metabolized by peroxisome enzymes, relative to growth on glucose that does not require peroxisomes. The short lifespan of H. polymorpha on glucose is mainly due to medium acidification, whereas most likely ROS do not play an important role. Growth of cells on methanol/methylamine instead of methanol/ammonium sulphate resulted in further lifespan enhancement. This was unrelated to medium acidification. We show that oxidation of methylamine by peroxisomal amine oxidase at carbon starvation conditions is responsible for lifespan extension. The methylamine oxidation product formaldehyde is further oxidized resulting in NADH generation, which contributes to increased ATP generation and reduction of ROS levels in the stationary phase.

Conclusion/Significance: We conclude that primary peroxisome metabolism enhanced chronological lifespan of H. polymorpha. Moreover, the possibility to generate NADH at carbon starvation conditions by an organic nitrogen source supports further extension of the lifespan of the cell. Consequently, the interpretation of CLS analyses in yeast should include possible effects on the energy status of the cell.

Introduction

Aging is a degenerative process characterized by a progressive deterioration of cellular components resulting in enhanced mortality. Short lived model organisms, such as yeast, have strongly contributed to our current understanding of the molecular determinants of aging [1]. In yeast, two types of lifespan can be discriminated, referred to as replicative lifespan (RLS) and chronological lifespan (CLS). RLS is defined by the number of daughter cells a mother cell can produce before cell division ceases [2], whereas CLS is the time cells survive in the stationary phase [3]. These two types of lifespan can serve as models for proliferating (mitotic) and non-proliferating (post-mitotic) tissues in higher eukaryotes, respectively [4]. Research on CLS of glucose-grown Saccharomyces cerevisiae cells have strongly contributed to the identification of factors that contribute to aging. Using deletion or overexpression strains, several proteins have been identified that either negatively or positively influence CLS. For instance, deletion of certain genes involved in nutrient adaptation response, like TOR1, lengthens the lifespan of S. cerevisiae [5,6], whereas the deletion of genes required for autophagy results in a reduced lifespan. In addition to genetic factors, also growth conditions have been shown to have major impact on the CLS of S. cerevisiae [7,8]. Important parameters include the composition of the growth medium as well as the pH [9]. Finally, the addition of various compounds such as spermidine, resveratrol and rapamycin were demonstrated to have a positive effect on the CLS of S. cerevisiae [10,11]. Both spermidine and rapamycin stimulate autophagy, underlining the importance of this process for longevity.

For several factors that affect aging in S. cerevisiae the molecular mechanisms have been elucidated in detail and shown to be conserved in higher eukaryotes. However, for many others the mechanisms are unclear or even highly debated.

Although S. cerevisiae is a highly attractive model organism for aging research, because of the unprecedented availability of tools and knowledge, this organism and the generally used growth substrate glucose, have also distinct disadvantages. First, S. cerevisiae is a Crabtree positive yeast, implying that mitochondrial respiration is turned down at high glucose conditions. As a result the
organism shows diauxic growth on glucose: first glucose is consumed and converted into ethanol, followed by growth on ethanol. However, the metabolic intermediate ethanol (but also acetate that is formed as well) is an important determinant in CLS. Hence, a substrate that does not result in ethanol and acetate formation can have advantages in certain aging studies. Second, S. cerevisiae has lost several properties/gene during evolution that are still conserved from their common ancestor in other yeast species and animals. Finally, different from most yeast species, extensive gene duplication has occurred in S. cerevisiae, which requires the construction of double mutants in order to detect specific phenotypes. Indeed, the analysis of CLS in alternative yeast species, such as Candida albicans, Kluyveromyces lactis and Schizosaccharomyces pombe, has already been shown to contribute to the identification of universal molecular factors acting on aging [12].

In the present work, we have used the methylotrophic yeast species Hansenula polymorpha to study chronological aging. In contrast to S. cerevisiae, this yeast is Crabtree negative. This yeast also is capable to metabolize a range of carbon (e.g. methanol and ethanol) and organic nitrogen sources (primary amines, D-amino acids) that all require peroxisome function for growth which have not been analyzed in CLS studies before. Our data indicate that utilization of these compounds (ethanol, methanol, D-alanine, methylamine) results in enhanced CLS relative to glucose/ammonium sulphate.

**Materials and Methods**

**Strain and growth conditions**

*H. polymorpha* NCYC495 *leu1.1*, an amine oxidase deletion strain derived from this wild-type strain and *atg* [13] were used throughout this study. Yeast cells were grown at 37°C on mineral medium (MM) [14] supplemented with different carbon sources (0.5% glucose, 0.5% methanol and 0.35% ethanol) and nitrogen sources (0.0025%, 0.25% MA and 0.33% D-alanine), unless stated otherwise. Leucine was added to a final concentration of 30 μg/ml. For viability determination cells were plated on YPD agar plates containing 1% yeast extract, 1% peptone, 1% glucose and 1.0% agar. For cloning purposes, *E. coli* DH5α was used; cells were grown at 37°C on LB media supplemented with 100 μg/ml ampicillin or 50 μg/ml kanamycin when required.

**Chronological lifespan measurements**

Yeast cells from fresh YPD plates were inoculated into MM supplemented with 0.5% glucose and 0.25% ammonium sulphate and grown overnight. Overnight cultures were diluted to an OD<sub>600</sub> of 0.1 in the same medium and grown till OD<sub>600</sub> of 1.0 and again diluted 1/10 to the same medium. When the cultures reached an OD<sub>600</sub> of 1.5, cells were transferred to MM supplemented with different carbon and nitrogen sources at a start OD of 0.1. CLS measurements were started when the culture reached the stationary phase (16 h on glucose and 40 h on methanol and ethanol containing media) and was referred as day 1. Cells were kept in the spent medium except for the experiments shown in Fig. 1B, where stationary phase cells were collected by centrifugation and resuspended in 25 mM phosphate buffer pH 6.0. For viability assays, the number of cells per ml was determined using CASY® Model TT (Roche Applied Science). 500 cells were plated on YPD agar plates and incubated at 37°C until colonies appeared. The lifespan curves show represent the average of 4–6 experiments.

**ROS measurements**

ROS accumulation, mainly peroxides and peroxinitrites, was measured with dihydrodihydrofluorescein 125 (DHR, Invitrogen). 10<sup>7</sup> cells were stained with 20 μg/ml DHR for 30 minutes. Mean of fluorescence was measured using a FACSc Aria II Cell sorter (BD Biosciences) equipped with a 488 nm laser and 330/30 nm band-pass filter. FACSDiva software version 6.1.2 was used for data acquisition and analysis.

**Construction of Hansenula polymorpha Δamo mutant**

The Δamo strain was constructed by replacing the genomic region of *AMO* comprising nucleotides +103 to +1925 by the hygromycin B resistance gene, *HphMX4* in the wild-type cells. Two DNA fragments from −515 to +102 and +1926 to +2412 of the *AMO* genomic region were amplified by PCR using primers 5’*Amo*-FP (5’-GGGGGACACTTTTGTCAGGATGTGCCTCTTCT-3’) /5’*Amo*-RP (5’-GGGGA-CTCTTGGTTGACACTCTTCTTCT-3’). For viability assays, the number of cells per ml was determined using CASY® Model TT (Roche Applied Science). 500 cells were plated on YPD agar plates and incubated at 37°C until OD<sub>600</sub> of 0.1 in the same medium and grown till OD<sub>600</sub> of 1.0 and again diluted 1/10 to the same medium. When the cultures reached an OD<sub>600</sub> of 1.5, cells were transferred to MM supplemented with different carbon and nitrogen sources at a start OD of 0.1. CLS measurements were started when the culture reached the stationary phase (16 h on glucose and 40 h on methanol and ethanol containing media) and was referred as day 1. Cells were kept in the spent medium except for the experiments shown in Fig. 1B, where stationary phase cells were collected by centrifugation and resuspended in 25 mM phosphate buffer pH 6.0. For viability assays, the number of cells per ml was determined using CASY® Model TT (Roche Applied Science). 500 cells were plated on YPD agar plates and incubated at 37°C until colonies appeared. The lifespan curves show represent the average of 4–6 experiments.

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Results

Growth on ethanol or methanol extend the CLS relative to glucose

To investigate the effect of different carbon sources which are metabolized by peroxisome-borne enzymes on the CLS of *H. polymorpha*, we cultivated wild-type cells on mineral media containing ethanol or methanol as sole carbon source in the presence of ammonium sulphate (AS) as sole nitrogen source, using glucose/AS as control. The survival measurements started when the cells reached the stationary phase (day 1) and the cells were kept in their original medium. The data presented in Fig. 1A show that the CLS (both medium and maximum lifespan; Table 1) of the ethanol and methanol cultures was strongly extended relative to that of the glucose culture.

In *S. cerevisiae* acidification of the medium is an important factor in the CLS of glucose-grown cells. We therefore monitored whether differences occurred in the pH of the cultures during the CLS experiments. The pH of the glucose culture rapidly dropped from 6.2 to 3.8 during the first day of the experiment, whereas in the ethanol and methanol cultures the pH never dropped below 4.5.

To analyse whether medium acidification explained the reduced lifespan of the glucose cultures, CLS experiments were repeated using cells which were precultivated on the three different carbon sources and, upon reaching the stationary phase, collected by centrifugation and resuspended into phosphate buffer (pH 6). As
shown in Fig. 1B and Table 1, the mean and maximum lifespan of the glucose-grown cells was strongly enhanced in phosphate buffer relative to the cultures that remained in the original medium. For the methanol and ethanol cultures the median and maximum lifespan only slightly increased. Hence, medium acidification is an important factor in the relatively short CLS of glucose-grown H. polymorpha cells, but plays a minor role in survival of methanol or ethanol cultures.

In addition to medium acidification, intracellular ROS levels are important in determining yeast CLS. We therefore analysed the levels of these reactive compounds in the three cultures using the dye DHR and FACS. As shown in Fig. 1C, glucose-grown cells accumulate similar, relatively low ROS levels like ethanol or methanol grown cells during the first 3 days after reaching the stationary phase, when large differences in survival were already apparent (compare Fig. 1A). At later time points (day 4 and 5) we only measured ROS in methanol and ethanol cultures as most cells of the glucose culture already had died after day 3. On days 4 and 5 ROS levels increased in both cultures. The highest levels were observed in methanol-grown cells even though the survival of these cells was similar to ethanol-grown cells (Fig. 1A). These findings suggest that growth of cells on media that require peroxisome function has a positive effect on their despite the enhanced ROS levels.

**Methylamine extends the lifespan**

Different from S. cerevisiae, H. polymorpha is capable to utilize a large range of organic nitrogen sources. To test the effect of an organic nitrogen source on chronological aging, cells were grown on methanol in the presence of AS or methylamine (MA). As shown in Figure 2A, MA significantly extends the median and maximum lifespan relative to AS. In both the methanol/AS and methanol/MA cultures the pH had decreased only slightly (to 5.2) at day 1. After this time point the pH of both cultures remained constant suggesting that differences in acidification do not explain the observed CLS extension by MA.

We also measured ROS levels in both cultures. The data (Fig. 2B) revealed that during the first 3 days, when differences in survival were evident, ROS levels were at a similar, low level in both cultures. At days 4 and 5 the ROS levels remained low in the MA cultures whereas they increased in the AS cultures. These data indicate that during the first days of the CLS experiment neither reduced acidification nor altered ROS levels can explain the positive effect of the utilization of MA as nitrogen source on the chronological lifespan. Hence, it is likely that additional processes play an important factor in the lifespan extension by MA.

### Table 1. The effect of buffer on median and maximum lifespan of H. polymorpha.

<table>
<thead>
<tr>
<th>Carbon source*</th>
<th>Condition**</th>
<th>Median lifespan (days)</th>
<th>Maximum lifespan (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Medium</td>
<td>2.2</td>
<td>3.8</td>
</tr>
<tr>
<td>Glucose</td>
<td>Buffer</td>
<td>4.2</td>
<td>5.9</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Medium</td>
<td>4.6</td>
<td>7.3</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Buffer</td>
<td>6.8</td>
<td>7.1</td>
</tr>
<tr>
<td>Methanol</td>
<td>Medium</td>
<td>5.1</td>
<td>7.9</td>
</tr>
<tr>
<td>Methanol</td>
<td>Buffer</td>
<td>6.2</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* Cells were grown in the presence of ammonium sulphate as nitrogen source.

** Upon reaching the stationary phase the cells were kept in the same medium or collected by centrifugation and resuspended in buffer.

The median lifespan is defined as time point when 50% of the cells survive, the maximum lifespan when 10% of the cells survive (Fig. 1A).

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The CLS extension by MA is not dependent on autophagy

Autophagy has been shown to be important for yeast chronological aging. The polyamine spermidine prolongs the CLS in S. cerevisiae by inducing autophagy [11]. We therefore asked whether the lifespan extension caused by MA is related to changes in autophagy. If alterations in autophagy would explain the lifespan extension by MA, this extension should not occur in cells defective in autophagy. We therefore performed a CLS experiment using H. polymorpha Δatg1 cells, which are deficient in autophagy. These experiments indicated that the CLS of H. polymorpha Δatg1 cells is strongly reduced relative to that of the wild-type control (Fig. 3, compare also Fig. 1A). However, also in Δatg1 cultures a positive effect of MA on survival was evident during the first three days. In addition a slight increase in median and maximum lifespan was observed (Fig. 3). This result suggests that the use of MA has a positive effect on cell survival in an autophagy-independent way.

The extended CLS is dependent on the MA concentration in the growth medium

Like spermidine, MA may directly trigger specific cellular processes that contribute to cell survival. Alternatively, MA metabolism may be responsible for the observed lifespan extension. In the latter case it is likely that the positive effect of MA is only observed at relatively high concentrations. To test this, we analysed the effect of reducing the concentration of MA. No change in the CLS curve was observed (relative to the methanol/AS) when the MA concentration was 100 fold reduced (Fig. 4A). This was not due to negative effects of the low MA concentration on the initial growth phase as both the doubling time (Fig. 4B) and the final yield of the culture (Fig. 4C) were unaltered at reduced MA concentrations. This suggests that the metabolism of MA during the stationary phase is important for lifespan extension. If so, the presence of MA in the medium should be essential to extend the CLS. To analyse this, we removed MA from the stationary phase cultures. To this end cells were precultivated on methanol/AS or methanol/MA until the stationary phase. Subsequently both cultures were harvested by centrifugation and resuspended in phosphate buffer (pH 6.0). As shown in Figure 5A, methanol/AS and methanol/MA grown cells showed a very similar CLS curve in buffer.

When MA metabolism is responsible for the CLS extension, this effect should be abolished in a strain lacking amine oxidase (AMO) activity. Indeed, MA did not result in a CLS extension in an AMO deficient strain (Δamo Fig. 5B).
Finally, if MA consumption is important the levels of free amines in the media should drop during chronological aging and the cells should display AMO activity in the stationary phase. Measurement of free amine levels (Fig. 5C) revealed a gradual decreased, indicating that MA is consumed during the initial growth but also during the stationary phase. As expected, no significant decline was observed in cultures of the D amo control strain.

Enzyme assays revealed that AMO activity was present during the CLS experiment, but somewhat reduced after 7 days relative to the values observed at day 1 and 3 (Fig. 6A). The reduction in AMO activity at day 7 was paralleled by a reduction in AMO protein as was observed by Western blot experiments (Fig. 6B, C).

Formaldehyde can cause an increase in CLS similar as MA

MA is oxidized by AMO into ammonium and formaldehyde [17]. In H. polymorpha formaldehyde is further oxidized by formaldehyde dehydrogenase and formate dehydrogenase into CO₂. This process results in the generation of 2 NADH molecules (Fig. 7). To test whether the formaldehyde oxidation product of MA was responsible for the extension of the CLS by MA, we supplemented a methanol/AS grown stationary phase culture with 37.8 mM formaldehyde, using cultures with 37.8 mM MA (corresponding to 0.25% w/v) as a control. The data revealed that the chronological lifespan of cultures supplemented with formaldehyde is extended to the same extent as MA cultures (Fig. 8). These data suggest that oxidation of formaldehyde, generated by MA oxidation during the stationary phase, is likely responsible for extra NADH supply which leads to an enhanced lifespan.

D-alanine results in lifespan extension

H. polymorpha can also use D-alanine as nitrogen source. If energy generation during the stationary phase can extend the lifespan of cells, D-alanine is expected to cause a similar effect as MA. D-alanine is oxidized by D-amino acid oxidase into ammonium and pyruvate [18]. As shown in Fig. 9, indeed D-alanine prolongs the chronological lifespan of H. polymorpha.

Discussion

Yeast chronological aging has a multifactorial nature. Many cellular processes and extrinsic factors negatively influence the CLS. Examples include oxidative stress, reduced autophagy or medium acidification. Processes which induce stress responsive genes extend yeast lifespan [19] . Hence yeast CLS is determined by the resultant of multiple positive and negative processes. Because of this complexity several factors implicated in yeast aging are still highly debated.

So far most research on yeast CLS is performed with S. cerevisiae using glucose/ammonium sulphate containing media. In this paper we analysed the chronological lifespan of the yeast H. polymorpha in relation to growth on different carbon and nitrogen sources.

Our data revealed that of the three carbon sources tested (glucose relative to two compounds that require peroxisome function for growth namely ethanol and methanol) H. polymorpha shows the shortest chronological lifespan on glucose. Compared to S. cerevisiae, H. polymorpha dies relatively fast with a short maximum lifespan of less than 4 days when grown on 0.5% glucose. At these conditions the maximum lifespan of S. cerevisiae is generally above 10 days [20].

Glucose metabolism involves glycolysis, which in S. cerevisiae leads to acetic acid formation that is associated with induction of the mitochondrial apoptosis pathway [21]. In S. cerevisiae acetic acid production is strongly reduced upon growth on glycerol instead of glucose [22] . Similar mechanisms most likely operate...
in *H. polymorpha*, because the medium of glucose cultures acidified more strongly relative to those containing ethanol- or methanol. Although placing the glucose-grown cells in fresh buffer significantly extended the lifespan, neither the median nor maximum lifespan reached values obtained for methanol or ethanol cultures (Table 1). One explanation may be that prior to placing the glucose-grown cells in buffer, they already experienced the toxic effects related to the low pH when reaching the stationary phase.

### Figure 4. CLS extension relates to MA concentrations in the cultivation media.

(A) CLS of wild-type cells grown on methanol in the presence of 0.25% ammonium sulphate (AS) or 0.25% or 0.0025% MA. Bars indicate the standard error of mean of four experiments. (B) Growth curves of wild-type *H. polymorpha* grown on methanol in the presence of different concentrations of MA (0.25%, 0.025% or 0.0025%). Optical densities are expressed as absorption at 600 nm. (C) Cell concentrations expressed as number of cells per ml in stationary cultures grown on media containing 0.25% or 0.0025% MA. doi:10.1371/journal.pone.0048982.g004

### Figure 5. MA metabolism is required for CLS extension.

(A) CLS of cells which were pregrown on methanol/AS or methanol/MA until the stationary phase and subsequently harvested and resuspended in phosphate buffer. (B) CLS of wild-type and *Δamo* cultures grown on methanol in the presence of 0.25% MA or 0.25% AS. Bars indicate the standard error of mean of two independent experiments. (C) Levels of free amines (-NH₂) in the cultivation media of cultures of wild-type and *Δamo* cells grown on methanol in the presence of 0.25% MA. The level of free amines in medium containing 0.25% MA before inoculation was set to 100%. Bars indicate the standard error of mean of two independent experiments. Statistical analysis was performed by student t-test, * = p<0.05; ** = p<0.01. doi:10.1371/journal.pone.0048982.g005

In addition to medium acidification, ROS are important factors in determining yeast CLS. ROS initially were assumed to be harmful as they caused oxidative damage. However, data have been presented indicating that ROS also can have a positive effect as signaling molecules that induce stress responsive genes
Moreover, recent findings suggest that in *S. cerevisiae* also the type of ROS (e.g. superoxide versus hydrogen peroxide) and the growth stage at which they occur are important for lifespan extension [23,24], which illustrates a complex role of ROS in yeast aging.

We observed that in *H. polymorpha* ROS levels were equally low during the first days of the CLS experiments in glucose, ethanol and methanol cultures. Because in this period differences in survival were already evident, ROS levels alone most likely are not sufficient to explain the observed differences in survival.

![Figure 6. Specific AMO activities and AMO protein levels decrease during chronological aging.](image)

Figure 6. Specific AMO activities and AMO protein levels decrease during chronological aging. (A) Detection of AMO activities in wild-type cells in cultures grown on methanol in the presence of 0.25% methylamine during chronological aging. Bars indicate the standard error of mean of two independent experiments. (B) Western blot analysis of AMO protein levels in wild-type cells grown in the presence of 0.25% MA or on 0.25% ammonium sulphate (AS). AMO, 80 kDa. FD, 40 kDa. (C) Quantification of the AMO levels using densitometric scanning of the blots. Two independent blots were quantified. The error bars indicate the standard error. The loading control was set to 100%. doi:10.1371/journal.pone.0048982.g006

![Figure 7. Schematic overview of MA metabolism in *H. polymorpha*.](image)

Figure 7. Schematic overview of MA metabolism in *H. polymorpha*. MA is oxidized by peroxisomal amine oxidase (AMO) to generate formaldehyde, ammonium and hydrogen peroxide. After binding of glutathione (GSH) to formaldehyde, the produced S-hydroxymethylglutathione is converted to S-formylglutathione by formaldehyde dehydrogenase (FLD). GSH is removed by S-formyl glutathione hydrolase (FGH) and formate is converted to CO₂ by formate dehydrogenase (FDH). Oxidation of formaldehyde generates 2 molecules of NADH that are used for ATP generation in mitochondria [30]. doi:10.1371/journal.pone.0048982.g007

![Figure 8. Formaldehyde extends the CLS.](image)

Figure 8. Formaldehyde extends the CLS. Wild-type cells were grown on methanol media containing 0.25% ammonium sulphate or 0.25% MA. Upon reaching the stationary phase, the ammonium sulphate containing culture was supplemented with 37.8 mM formaldehyde. The MA cultures were kept in the same medium as a control. Bars indicate the standard error of mean of 2 experiments. doi:10.1371/journal.pone.0048982.g008
the major determinants in the observed differences in lifespan. At later stages ROS levels increased in the methanol and ethanol cultures. Given the multifactorial nature of CLS, it is yet unclear whether this may have caused negative and/or positive effects. Also, ROS measurements performed with fluorescent dyes have to be interpreted with care when cells are grown on different carbon sources. We used DHR, which forms fluorescent rhodamine efficiently upon reaction with free \( \cdot OH \) or \( \cdot NO_2 \) radicals, but requires a catalyst for oxidation by \( O_2 \) or \( H_2O_2 \) [25]. Important catalysts are iron, heme and cytochrome c oxidase. These catalysts as well as the composition of the ROS may vary significantly upon growth of \( H. \) polymorpha cells on the different carbon sources. For instance, the peroxisomal heme containing enzyme catalase is strongly induced on methanol, to moderate levels on ethanol but repressed on glucose [26]. Hence, relative to glucose heme levels and most likely also iron (e.g. released from catalase in aged cells) are may be significantly higher in methanol and ethanol cells which may add to the observed increase in ROS levels. Together, our data lend support to the view that cultivation of cells at an instance, the peroxisomal heme containing enzyme catalase is strongly induced on methanol, to moderate levels on ethanol but repressed on glucose [26]. Hence, relative to glucose heme levels and most likely also iron (e.g. released from catalase in aged cells) are may be significantly higher in methanol and ethanol cells which may add to the observed increase in ROS levels. Together, our data lend support to the view that cultivation of cells at

## References