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Dependence of the yeast *Saccharomyces cerevisiae* post-reproductive lifespan on the reproductive potential

Renata Zadrag-Tecza[1]✉, Mateusz Molon[1], Jan Mamczur[2] and Tomasz Bilinski[1]

[1]Department of Biochemistry and Cell Biology, University of Rzeszow, Rzeszow, Poland; [2]Faculty of Mathematics and Applied Physics, Rzeszow University of Technology, Rzeszow, Poland

The lifespan of budding yeast cells is divided into two stages: reproductive and post-reproductive. The post-reproductive stage of the yeast's lifespan has never been characterized before. We have analyzed the influence of various mutations on the post-reproductive (PRLS) and replicative (RLS) lifespans. The results indicate that PRLS demonstrates an inverse relationship with RLS. The observed lack of differences in the total lifespan (TLS) (expressed in units of time) of strains differing up to five times in RLS (expressed in the number of daughters formed) suggests the necessity of revision of opinions concerning the use of yeast as a model organism of gerontology.

Key words: yeast, lifespan, oxidative stress, aging

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INTRODUCTION

The yeast *Saccharomyces cerevisiae* has been widely accepted as a simple eukaryotic model organism for the studies of aging processes. A single yeast cell can undergo only a limited number of cell cycles, which is referred to as the replicative lifespan (RLS). Studies on the replicative aging of the budding yeast, carried on since 1989 have been based on several *ad hoc* assumptions. The basic assumption was that yeast cells cannot reproduce infinitely due to the fact that yeast mother cells actively accumulate hypothetical "senescence factor" which is soluble, diffusible and partly degradable (Egilmez & Jazwinski, 1989). The most commonly postulated "senescence factor" are rDNA circles (ERCs) (Sinclair & Guarente, 1997), oxidatively damaged proteins (Aguilaniu, *et al.*, 2003) or other molecular damages. This factor was supposed to have a universal character, and therefore it was believed that by studying yeast one could discover the basic mechanisms of human aging (Ganley, *et al.*, 2012; Kaeberlein, 2012). This opinion, remaining at the core of the accepted paradigm is not shared by two groups of researchers (Bilinski, 2012; Bilinski, *et al.*, 2012; Wright, *et al.*, 2012; Yang, *et al.*, 2011; Zadrag-Tecza, *et al.*, 2009). We propose that limitation of the reproductive capacity may be due to reaching excessive volume by yeast cells (Bilinski, *et al.*, 2012).

The second assumption of the experimental yeast aging research based on tracing survival curves was that yeast cells, which are unable to reproduce further, are dead. This assumption appears to be false (Minois, *et al.*, 2005; Zadrag, *et al.*, 2008). In other words, the ordinate axis of yeast survival curves should be rather marked as percentage of reproducing cells, rather than as percentage of survival. It is worth noting that replicative lifespan of yeast is not expressed in time units, but as the number of daughter cells produced during the life of the mother cell (Sinclair, *et al.*, 1998). Yeast is the only model organism of gerontology, for which the time of life has never been analyzed in mainstream publications. Even the length of life of "longevity mutants" and their parental strains is not expressed in units of time. Very early (Gershon & Gershon, 2000) it was suggested that such presentation of the results corresponds rather to fecundity than to longevity. Thus, both axes of yeast „survival curves" are inappropriately marked. Consequently, calling the mutants that produce more daughters "longevity mutants" and describing them as "long lived", is unauthorized. However, many authors compare the "longevity" of these mutants to longevity of animal mutants, despite the fact that for animals longevity is always expressed in units of time. These two values are in fact incomparable.

The yeast lifespan is expressed in units of time, but only in case of studies of "chronologic" lifespan. This experimental approach was proposed to reveal the mechanisms of aging of non-dividing human somatic cells (Longo, *et al.*, 1996). During these studies the survival of stationary phase yeast cells was measured. This approach describes the mechanisms of starvation stress tolerance, accompanied by other kinds of stress resulting from acetic acid accumulation (Longo, *et al.*, 2012; Mirisola & Longo, 2012), and possibly some other products of metabolism and autolysis of dead cells. Our somatic cells never encounter such severe conditions during the normal process of aging, because they live under the conditions of homeostasis, even if the organism is transiently starving.

In contrast, the procedure of measuring RLS assures optimal conditions for the studied cells during their life. Thus, measuring the time parameters during reproductive and post-reproductive phases of life, allows for the analysis of the behavior of yeast cells under the conditions better corresponding to those of our human somatic cells.

The aim of our research was to test the influence of the reproductive potential (replicative lifespan) of cells in relation to the reproductive and post-reproductive lifespan (PRLS) expressed in units of time.

✉e-mail: retecza@univ.rzeszow.pl

Abbreviations: YPD, yeast extract-peptone-dextrose; RLS, replicative lifespan; PRLS, post-reproductive lifespan, TLS, total lifespan

MATERIALS AND METHODS

Yeast strains, media and growth conditions. The genetic background has a very strong influence on various aspects of the yeast aging studies. Therefore, we have commenced our studies with constructing mutants in a selected background. The following yeast strains were used: wild-type SP4 *MATα leu1 arg4* (Bilinski*, et al.*, 1978), Δ*sod1* mutant, isogenic to SP4, *MATα leu1 arg4* Δ*sod1* (Bilinski*, et al.*, 1985), Δ*fob1* mutant, isogenic to SP4, *MATα leu1 arg4* Δ*fob1* (*in this study*). The *FOB1* gene in a wild-type — SP4 strain was disrupted by excision (from Δ*fob1* mutant — EUROSCARF), and then amplification of the disruption cassette kanMX4 in PCR reaction and transformation wild-type — SP4 cells with the PCR product. The G418-resistant transformants were confirmed by PCR, using gene-specific and kanMX4 primers. Yeast cells used in this study are only haploid cells. Yeast was grown in the standard liquid YPD medium (1% Yeast Extract, 1% Yeast Bacto-Peptone, 2% glucose) on a rotary shaker at 150 rpm, or on the solid YPD medium containing 2% agar, at the temperature of 28°C.

Determining the reproductive potential, reproductive and post-reproductive lifespan. Yeast *Saccharomyces cerevisiae* lifespan (reproductive and post-reproductive) was determined as described previously (Minois*, et al.*, 2005) with a small modification described in Zadrag *et al.* (2008). Yeast cultures were grown in YPD liquid medium overnight. One-microliter aliquots of culture were dropped on YPD plates with solid medium containing Phloxine B at the concentration 10 μg/ml. For each experiment, forty single cells were micromanipulated to the appointed area. The first daughters were chosen as starting cells and their successive buddings were followed to determine the reproductive potential and the reproductive lifespan expressed in time units. After the completion of buddings, the yeast cells were inspected in one-hour intervals to determine their post-reproductive lifespan. The total lifespan was calculated as the sum of the reproductive and post-reproductive lifespans. During the manipulation the plates were kept at 28°C for 16 h and at 4°C during the night (8 h). Data represent mean values from two separate experiments.

Calculating the parameters of cell life

Average life time and standard deviation. Each of the lifespan plots concerning the percentage of either dividing or living cells can be treated as the plot of cumulative distribution function (CDF) with percentage probability, $F(t)$, related to time. Then, the probability density function (PDF), $p(t)$, which describes the likelihood for the cell life time to take on a given value t, is a derivative of CDF with a minus sign. The minus sign comes from the CDF definition accepted in this paper: $F(t)$ describes the probability that the life time of a given cell will be found at a value t' greater than, or equal to t. Such CDF is also called the *survival function,* while the abovementioned PDF is called the *unconditional density of failure at age t* (Cunningham R*, et al.*, 2008). The average life time of a cell can be calculated from the common definition of the expected value:

$$t_{av} = E(\mathbf{t}) = \int_0^\infty t \cdot p(t)\,\mathrm{d}t = -\int_0^\infty t \cdot \frac{\mathrm{d}F(t)}{\mathrm{d}t}\,\mathrm{d}t = \int_0^1 t(F)\,\mathrm{d}F \tag{1}$$

The last integral is equal to the area of the region bounded by the $F(t)$ graph and the ordinate F axis. Since the function F is monotonic and defined in the interval $[0,\infty)$, the above region is identical to the region constrained by the $F(t)$ graph and the abscissa t axis, which yields

$$t_{av} = \int_0^\infty F(t)\,\mathrm{d}t \tag{2}$$

The variance of the life time can be calculated from the common definition:

$$D^2(\mathbf{t}) = \int_0^\infty t^2 \cdot \frac{\mathrm{d}F(t)}{\mathrm{d}t}\,\mathrm{d}t - [E(\mathbf{t})]^2 = \int_0^1 [t(F)]^2\,\mathrm{d}F - [E(\mathbf{t})]^2 \tag{3}$$

The last integral can be transformed by manipulating in a similar manner the area of analogous region bounded by the graph and the axes, which yields:

$$D^2(\boldsymbol{t}) = \int_0^\infty F(t^2)\,\mathrm{d}t^2 - [E(\boldsymbol{t})]^2 = \int_0^\infty F(t)\cdot 2t\,\mathrm{d}t - [E(\mathbf{t})]^2 \tag{4}$$

Since we know the CDF by its statistical realization $F(t)$ defined in the discrete domain, the above integrals can be calculated by a finite sum according to the method of trapezoids, which gives the following formulae for the average life time and the standard deviation

$$t_{av} = \tfrac{1}{2} F(t_0)\cdot\Delta t + \sum_{i=1}^{n-1} F(t_i)\cdot\Delta t \tag{5}$$

$$\sigma(\boldsymbol{t}) = \sqrt{D^2(\boldsymbol{t})} = \sqrt{F(t_0)\cdot t_0\cdot\Delta t + 2\sum_{i=1}^{n-1} F(t_i)\cdot t_i\cdot\Delta t - {t_{av}}^2} \tag{6}$$

It should be emphasized that the standard deviation given by Eqn. (4) or (6) does not result from any measuring error made during counting dividing or alive cells (Fig. 1). On the contrary, the standard deviation follows from the natural discrepancy between life times of different cells.

Total life time. The total life time could be defined as the time until the death of the last cell. Unfortunately, the last cell may live unexpectedly long and little can be concluded from that isolated incident. Instead, we should rather try to approximate the real lifespan by a suitable function $F(t)$ (survival function) that plays the role of CDF, and then find the time at which $F(t)$ takes on the value 0.01, i.e. find the last-percentile of time $t_{1\%}$, above which only 1% of cells survives theoretically.

Two mathematical forms of CDF have been considered, with power or exponential force of mortality, $\mu(t)$. The function $\mu(t)$ can be interpreted as the conditional density of failure at age t (1). The two types of CDF are given by the following equations that express the Weibull and Gompertz-Makeham laws of mortality (Bowers NL*, et al.*, 1986; Gerber, 1990), correspondingly

$$F_1(t) = \exp\left(-\frac{c}{n+1}t^{n+1}\right) \tag{7a}$$

$$F_2(t) = \exp\left\{c\left[t - \tau\left(e^{t/\tau} - 1\right)\right]\right\} \tag{7b}$$

The selection is justified by the fact that all total lifespans tested, $F(t)$, seem to have zero slope at $t=0$, that is the time derivative of $F(t)$ equals zero for $t=0$.

We have fitted the two abovementioned functions to all total lifespans obtained during yeast cell investigation. This has been done using the Least Squares Approximation method. Additionally, the goodness-of-fit parameter, least squares sum (LSS), has been evaluated in every fit by the following formula

$$LSS = \sum_i [F(t_i) - L_i]^2 \quad (8)$$

where L_i is the value of the lifespan for time t_i. It has turned out that the LSS value is less for the fitting function $F_1(t)$ than for the function $F_2(t)$ in case of every lifespan, thus indicating that the function $F_1(t)$ describes the real lifespan better than the other one. Therefore, we have accepted the CDF $F_1(t)$, given by Eqn. (7a), as an approximating function for all the lifespans considered. As it has been mentioned at the beginning of the current section, the last percentile time $t_{1\%}$, above which only 1% of cells survives theoretically, has been calculated for each lifespan from the condition $F_1(t_{1\%})=0.01$ in the following way

$$t_{1\%} = \sqrt[n+1]{-\frac{n+1}{c}\ln(0.01)} \quad (9)$$

where n and c are the coefficients found in the fitting.

Statistical analysis. The results represent the mean ±S.D. from all cells tested in two independent experiments (80 cells). The statistical significance of differences between means of yeast strains tested was estimated using one-way ANOVA and the Tukey's *post-hoc* test. The differences between the mutant strain compared to the wild-type strain were estimated using one-way ANOVA and the Dunnett's *post-hoc* test. The values were considered significant if $P<0.05$. Statistical analysis was performed using the Statistica 10 software.

RESULTS

Genetic studies have identified many genes having significant influence on the reproductive capacity of yeast cells. We have selected only those genes, the knockout of which results in big changes in the reproductive potential, Δ*sod1* decrease and Δ*fob1* increase, when compared to that of the parental strain (Kaeberlein, *et al.*, 2005; Wawryn, *et al.*, 2002). We have chosen the Δ*fob1* mutant as the representative of "longevity" mutants also because only this type of mutation is strongly expressed in the genetic background of our strains.

Our results are described as the budding lifespan, which corresponds to the replicative lifespan (RLS) presented in the mainstream publications, the reproductive lifespan expressed in units of time, which covers the replicative lifespan, and the post-reproductive lifespan (PRLS), from the moment of the last cell cycle completion till the death of the cell. Description of these parameters is taken from the publication of Minois et al. (Minois, et al., 2005). Additionally, we have also introduced (Zadrag, et al., 2008) the term of total lifespan (TLS), which is the sum of the reproductive and post-reproductive lifespans. It makes the yeast lifespan comparable to the lifespan of humans and animals.

In the previous paper (Zadrag, et al., 2008) we have shown that various mutations lowering the level of anti-oxidative potential do not influence the "total" lifespan. These studies were however carried on pairs of strains of different backgrounds.

Figure 1 presents data concerning two mutants obtained in the strain, having the same genetic background. The fivefold difference in the budding lifespans of these strains (1A) became much less spectacular when we expressed the reproductive lifespan in units of time, instead of in the number of daughters (1B). Figure 1D shows that all differences between presented yeast strains disappeared when we additionally took into account the post-reproductive lifespan of each cell. Table 1 presents mean values of the studied parameters. It is quite surprising that extremely "short lived" mutant Δ*sod1* lives just as long as one of the best "longevity mutants". This astonishing fact can be explained by the results presented in Figure 1C, showing that the post-reproductive lifespan seems to be negatively correlated with the budding lifespan i.e. the reproductive capacity of the cell. In other words, high fecundity shortens the post-reproductive lifespan (PRLS). The differences between mean values for yeast strains tested observed on the Figure 1 and Table 1 are statistically significant for budding and reproductive lifespans in all tested cases (*the comparison of all tested strains with each other and the comparison of mutant strain with the wild-type strain*). While no statistical significance of differences between means was found

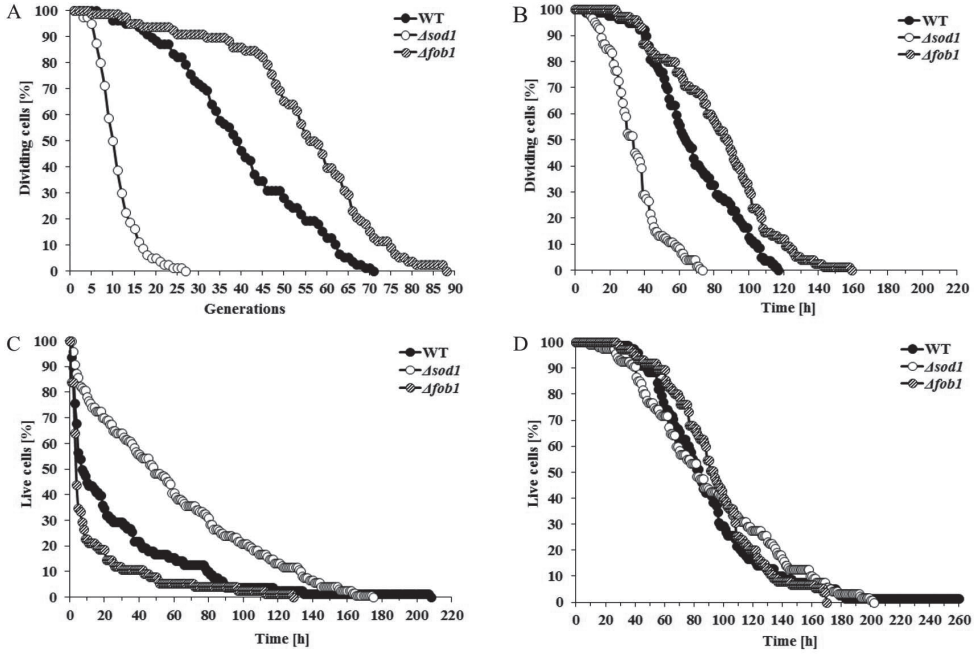

Figure 1. Budding lifespan (A), reproductive lifespan (B), post-reproductive lifespan (C), and total lifespan (D) of a wild-type strain SP4 (WT) and mutants: Δ*sod1* lacking CuZn-superoxide dismutase and Δ*fob1*. Data represent mean of two independent experiments.

Table 1. The budding lifespan (number of generations), reproductive lifespan, post-reproductive lifespan, and total lifespan of the yeast strains used.
Data are presented as mean ± S.D. from all cells tested during two independent experiments (80 cells). ***$P < 0.001$ as compared to wild-type cells (one-way ANOVA and the Dunnett's test).

Strain	Budding lifespan	Reproductive lifespan	Post-reproductive lifespan	Total lifespan
	Generations	Time [h]	Time [h]	Time [h]
Δsod1	10.6 ± 4.5***	33.9 ± 14.9***	57.6 ± 46.6***	91.4 ± 45.1
wild-type	39.6 ± 15.5	67.6 ± 24.7	26.0 ± 36.2	90.1 ± 38.5
Δfob1	54.8 ± 16.9***	82.8 ± 30.1***	13.5 ± 24.2	95.1 ± 30.8

for the total lifespan, the post-reproductive lifespan shows no statistical significance of differences between means only for the wild-type strain and Δ*fob1* strain.

Final conclusions should not be drawn on the basis of the results concerning only three strains. Therefore, we collected the results of our other experiments, concerning PRLS of all tested strains so far, irrespective of their genetic background.

Figure 2 presents the relationship between the mean number of generations (number of daughter cells produced) and the mean length of the post-reproductive lifespan. The post-reproductive lifespan is expressed not in units of time, but as the contribution of PRLS to the total lifespan (TLS) (both expressed in units of time). This recalculation of the original data allows for the comparison of strains differing in the length of life due to various genetic backgrounds. Similar approach is used to compare PRLS of various species of mammals. The value of $R^2 = 0.97$ presented in Fig. 2 is very high. This relationship still remains very strong ($R^2 = 0.72$), even though we add the data from other yeast strains with mutation in genes coding the ribosomal proteins or defective in DNA repair system (data not published). These results confirmed the opinion drawn from Fig. 1.

DISCUSSION

The yeast *Saccharomyces cerevisiae* was accepted as a model organism for the studies of aging, based on the assumption that basic mechanisms of aging are conserved among eukaryotic organisms. Several factors have been suggested to determine the yeast replicative lifespan, including the accumulation of extrachromosomal rDNA circles (ERCs), transcriptional silencing at the rDNA mediated by the Sir2 histone deacetylases, genomic instability, oxidative stress, and dietary restriction (Barros, *et al.*, 2004; Bitterman, *et al.*, 2002; Sinclair, *et al.*, 1998). All tested factors that modulate the yeast lifespan alter the reproductive capacity (number of generations). Therefore, the main problem of the usefulness of this organism for gerontology research is the way of expressing lifespan only by the number of daughter cells produced. This method of expressing life expectancy is associated with the assumption that the yeast cell dies after forming the last bud. This assumption has been verified and it is known that the cell can live a long time after the end of its reproduction period (Minois, *et al.*, 2005; Zadrag, *et al.*, 2008). Thus, the lifespan of yeast cells can be divided into two stages: reproductive and post-reproductive, and each of them may be regulated by common or different factors.

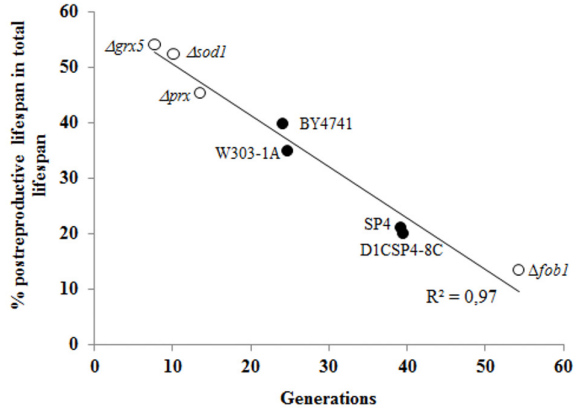

Figure 2. The relationship between the mean number of generations and the mean length of the post-reproductive lifespan expressed as the percentage of the total lifespan.
Yeast mutants (**open circles**) with defective antioxidant systems, like Δ*prx*, Δ*grx5*, Δ*sod1*, "longevity" mutant Δ*fob1* and their wild-type counterparts BY4741, W303-1A, D1CSP4-8C (**solid circles**) were described in the paper (Zadrag *et al.*, 2008).

The obtained results indicate a negative correlation between the duration of PRLS and RLS. The negative correlation of fecundity (number of generations), the known from the mainstream publications as the replicative lifespan (RLS), and PRLS, suggest that the nature of the tested mutations is less important. The mutations reducing reproductive capacity disturb the lifespan of these strains by decreasing the level of antioxidant barrier (Δ*sod1* — superoxide dismutase; Δ*grx5* — glutaredoxin 5; Δ*prx* — peroxiredoxins) (Rodriguez-Manzaneque, *et al.*, 1999; Wawryn, *et al.*, 2002; Wong, *et al.*, 2004). On the other hand, the Δ*fob1* (nucleolar protein that binds the rDNA replication fork barrier site) mutation increases the reproductive capacity (Kaeberlein, *et al.*, 2005). Therefore, PRLS does not depend on the nature of the mutations as much as on the reproductive capacity of the studied cells. This opinion is strongly supported by the results of studies carried out on the mutants with disturbed DNA repair mechanisms, the RLS of which is strongly reduced, but PRLS is high (data not published). One can postulate that increasing the number of progeny lowers the vitality of the cell. It is likely that mother cells exhaust during each cell cycle some of the resources necessary for further survival.

Very high value of PRLS in mutants with defective antioxidant barrier and comparable values of the total lifespan suggest that oxidative damage does not play an important role in the process of yeast aging. This finding confirms the opinion of Blagosklonny (Blagosklonny, 2008) that the role of free radical damage in aging of humans or animals is undetectable. Increased fecundity in humans or animals negatively correlates with the total lifespan (Kirkwood & Holliday, 1979; Shanley, *et al.*, 2007). In budding yeast, despite significant differences in the number of generations (equivalent of the animal fe-

cundity), the total lifespan is very similar. These results suggest the necessity of reconsidering the opinion that the "budding yeast is a preeminent model organism in studies of cellular aging pathways that are conserved in eukaryotes, including humans" (Polymenis & Kennedy, 2012).

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