Decreased antioxidant defense during replicative aging of the yeast *Saccharomyces cerevisiae* studied using the 'baby machine' method

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Abstract Replicatively senescent cells of *Saccharomyces cerevisiae* were obtained using the 'baby machine' method by immobilizing cells on CovaLink™ NH_2 plates and allowing them to divide while exchanging medium and removing daughter cells. Centrifugation in a Percoll density gradient was employed for further purification of replicatively old yeast cells. Comparison of senescent cells showing more than 20 bud scars with cells from early stationary culture demonstrated a significant reduction of total and reduced glutathione and decrease of superoxide dismutase activity during replicative aging of yeast cells.

Key words: Aging; Yeast; Superoxide dismutase; Glutathione; *Saccharomyces cerevisiae*

1. Introduction

Replicative aging of the yeast *Saccharomyces cerevisiae* has recently become the object of increasing interest [1,2]. The demonstration that cells of *S. cerevisiae* have a limited life span (in the sense of the number of buds one cell can generate) [3] stimulated interest in studies of the effects of various factors on the replicative life span of the cells [4] and opened possibilities of identification of genes contributing to replicative longevity of the yeast [5–7]. All these studies were based on determination of the replicative life span of individual yeast cells by means of micromanipulation [8]. This method, although very useful in studies of determinants of the life span of the yeast, cannot provide sufficient amounts of material for biochemical studies of senescent yeast cells.

Due to the rapid dilution of old cells in exponentially growing yeast cultures, isolation of replicatively senescent cells (i.e. cells which have gone through large numbers of divisions) is a difficult task. An ingenious method consisted in biotinylation of cells and their recovery on streptavidin-coated magnetic beads [9]. We made use of a different idea, denoted 'baby machine' [10,11]. In this technique, cells are immobilized on a solid support and allowed to divide, thus allowing for continuous or synchronized production of daughter cells. However, another application of this technique can consist in recovering the mother cells after sufficient budding and using them for studies of biochemical changes occurring during their replicative aging. We have found previously that yeast cell during prolonged 'aging' in a stationary culture show accumulation of oxidative damage and decreased antioxidative defense [12]. These results are in line with the effect of life span shortening in superoxide dismutase-deficient mutants of the yeast. This study was aimed at determining the effect of replicative aging on the antioxidant defense of *S. cerevisiae* using the 'baby machine' method for obtaining replicatively senescent yeast cells.

2. Materials and methods

The SP-4 (wild-type) yeast strain (phenotype 4 (MATα leu1 arg4) [13] was obtained from Prof. T. Biliński (Pedagogical University of Rzeszów) and cultivated in yeast extract (1%)/bactopeptone (1%)/glucose (2%) (YPD) medium. CovaLink™ NH_2 primary amine surface plates were obtained from Nunc thanks to the kindness of Mrs. Helle Knudsen who provided us with plates with wells of surface 9.6 cm^2, more suitable for our experiments.

Replicatively senescent yeast cells were obtained using the 'baby machine' method. Cells were bound to the plates as follows: to a well, diameter 35 mm, was added: 1 ml of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide solution (6.96 mg/ml), 1 ml of a solution of the sodium salt of *N*-hydroxysulfosuccinimide (6.14 mg/ml), and 2 ml of cold (4°C) logarithmic yeast culture in YPD medium; the plates were then left at 28°C for 8 h. Then the plates were washed with YPD medium and incubated at 28°C, the medium being exchanged every 12 h for 7 days. By this time the bottom of the well was completely covered with cells. The plates were placed on a rotary shaker for 11 days, the medium was exchanged every 2 h, 12 h/day. Then the cells were scraped off, sonicated to disperse adherent daughter cells and centrifuged through a self-formed Percoll density gradient. The gradient was formed by centrifugation of 90% Percoll in 135 mM NaCl/0.5 mM KCl/5 mM glucose/10 mM HEPES, pH 7.4, in the 12110 angle rotor of a Sigma 3K30 centrifuge at $15\,000\times g$ for 1 h. 100 μl of cell suspension in 10 mM phosphate buffer, pH 6.6, containing 2% glucose (2×10^7 cells/ml) was applied at the top of 1.9 ml of the gradient in a 2 ml Eppendorf tube and centrifuged for 40 min at $10\,000\times g$, at 4°C, yielding a top layer of cells not entering Percoll, a diffuse middle layer, and a bottom layer of density higher than 1.143 g/ml, as determined with density beads. The bottom layer contained old cells showing bud scars when stained with Calcofluor and showed much higher fluorescence when analyzed in a Becton-Dickinson cell sorter after Calcofluor staining (Fig. 1). This fraction contained about 30% of total cells collected from the plates. Therefore, culture of yeast on the bottom of a 35 mm well of a CovaLink™ NH_2 plate makes it possible to obtain about 4×10^6 replicatively senescent cells of *S. cerevisiae*.

The cells were broken with glass beads (diameter 0.46 mm) on ice for 15 min. For determination of glutathione, perchloric acid was added to the cell suspension and the suspension broken with glass beads. Superoxide dismutase activity was determined with xanthine+xanthine oxidase and nitroblue tetrazolium [14]. Catalase activity

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was estimated by spectrophotometric measurement of decomposition of hydrogen peroxide [12]. Reduced glutathione was assayed fluorimetrically with *o*-phthalaldehyde [15]. Total glutathione was estimated by the recycling assay with Ellman's reagent and glutathione reductase [16]. Protein was determined according to Lowry et al. [17]. Cell diameter and number of bud scars after Calcofluor staining were determined microscopically.

3. Results and discussion

3.1. Isolation of replicatively senescent yeast cells

The 'baby machine' method makes it possible to obtain significant amounts of senescent cells of *S. cerevisiae* which after an appropriate number of washing cycles can be recovered by simple scraping of the plates. During the 18 day culture the size of the cells and the number of bud scars on the cells increased as demonstrated by flow cytometric analysis of light scattering and fluorescence of cells stained with Calcofluor which visualizes bud scars [8]. However, the fraction of cells collected from CovaLink™ NH_2 plates showed considerable heterogeneity (Fig. 1).

As some of the descendant cells are not detached from the mother cells during the washings, it was necessary to purify the old cells by density gradient centrifugation. In this study, the fraction of cells collected at the bottom of discontinuous Percoll density gradients is referred to as replicatively old cells. The main drawback in preparation of replicatively old yeast cells using this method is the difficulty in separating clumped cells, especially the oldest cells and their first descendants. Low-intensity ultrasonication or shaking with large-diameter (5 mm) glass beads was helpful but not fully successful. Therefore, cells scraped off the CovaLink plates and subjected to sonication or shaking were centrifuged through a Percoll density gradient. Even this procedure did not separate the cells completely. However, the bottom fraction contained mostly ($>84\%$) cells of diameter >6.4 μm, contamination of this fraction with younger cells being pretty low (Table 1). In agreement with a previous report [18], replicatively aged cells had a much larger diameter and numerous bud scars as demonstrated by microscopic examination (Table 2) and flow cytometry measurements (Fig. 2).

Therefore, the procedure applied makes it possible to obtain populations of yeast cells containing mostly senescent cells which can be used for studies of changes occurring during replicative aging of yeast cells.

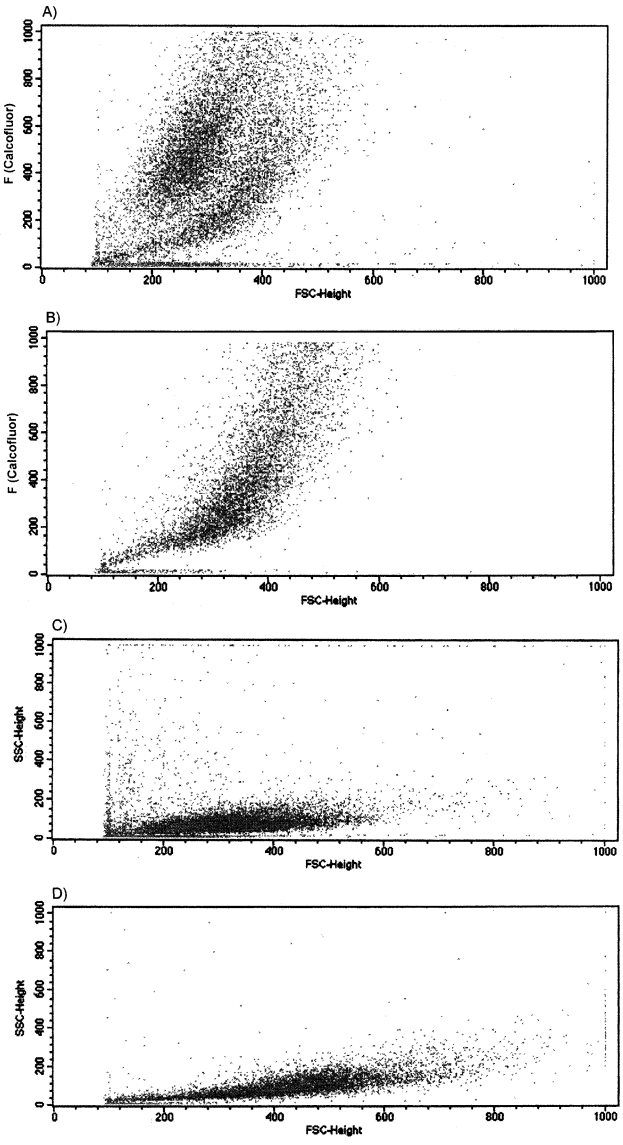

Fig. 1. Calcofluor fluorescence vs. forward scattering (A, B) and side scattering vs. forward scattering plots (C, D) for cells of early stationary culture (2 days old) (A, C) and cells collected from CovaLink™ NH_2 plates after 18 days (B, D). Mean values of Calcofluor fluorescence: 456 AU for young cells, 635 AU for old cells.

3.2. Decreased antioxidant defense in replicatively senescent yeast

Superoxide dismutase activity was considerably decreased in senescent yeast cells, more than four-fold with respect to control cells. In contrast, catalase activity was only slightly diminished in the oldest cell fraction, and increased in the top and, especially, middle fractions of the density gradient indicating that expression of this enzyme is transiently activated during cellular aging which may represent a stress response [12]. Reduced glutathione showed a dramatic (more than 15-fold) decrease in senescent cells. Total glutathione

Table 1
Percent virgin cells (with no bud scars) and percent old cells (diameter >7.1 μm) in various fractions collected from Percoll density gradient after separation of cells grown on CovaLink™ NH_2 plates for 18 days

Cell fraction	Virgin cells (%)	Old cells (%)
Top	1.7	0.8
Middle	10.8	10
Bottom	1.7	84

Mean values from a typical experiment.

Table 2
Diameter of control yeast cells and number of bud scars in control and in cell fractions collected from CovaLink™ NH_2 plates and separated in a Percoll density gradient

Cell fraction	Diameter (μm) ($n=480$)	Number of bud scars ($n=60$)
Control	2.7 ± 0.7	1.4 ± 2.0
Top	3.9 ± 1.3	1.6 ± 2.1
Middle	5.4 ± 1.1	11.7 ± 5.9
Bottom	7.1 ± 0.7	>20[a]

Control: cells from 2 day old early stationary culture.
[a]Difficult to count precisely.

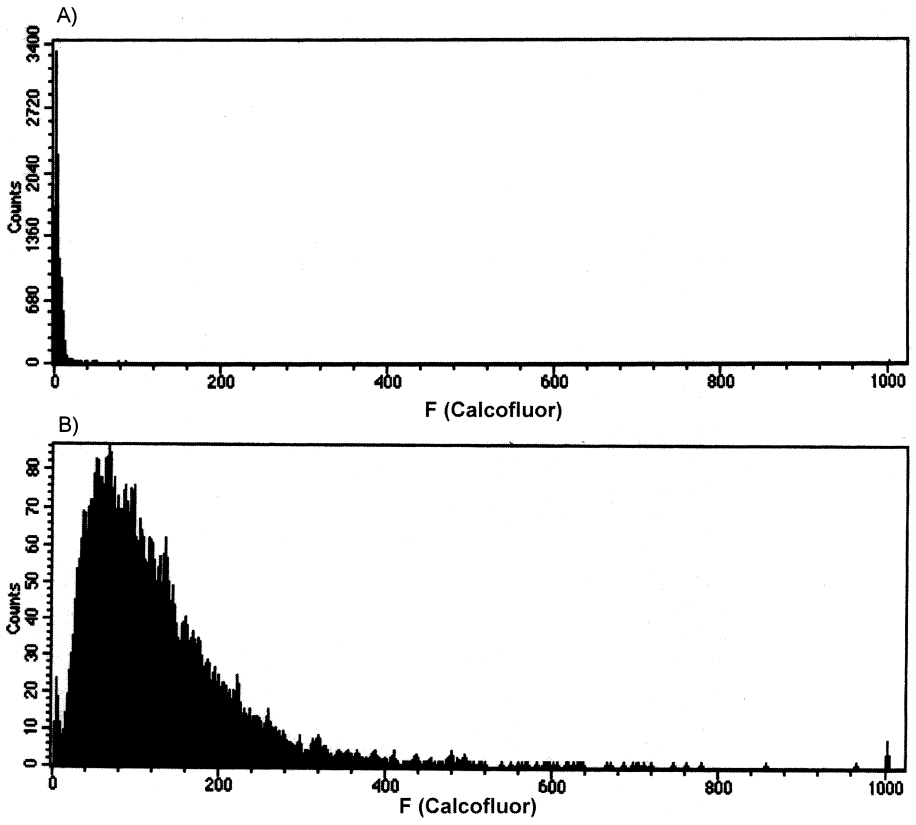

Fig. 2. Distribution of Calcofluor fluorescence in cells of early stationary culture (A) and the Percoll fraction of densest cells (B). Mean values of Calcofluor fluorescence: 8.2 for control cells and 118.7 for old cells.

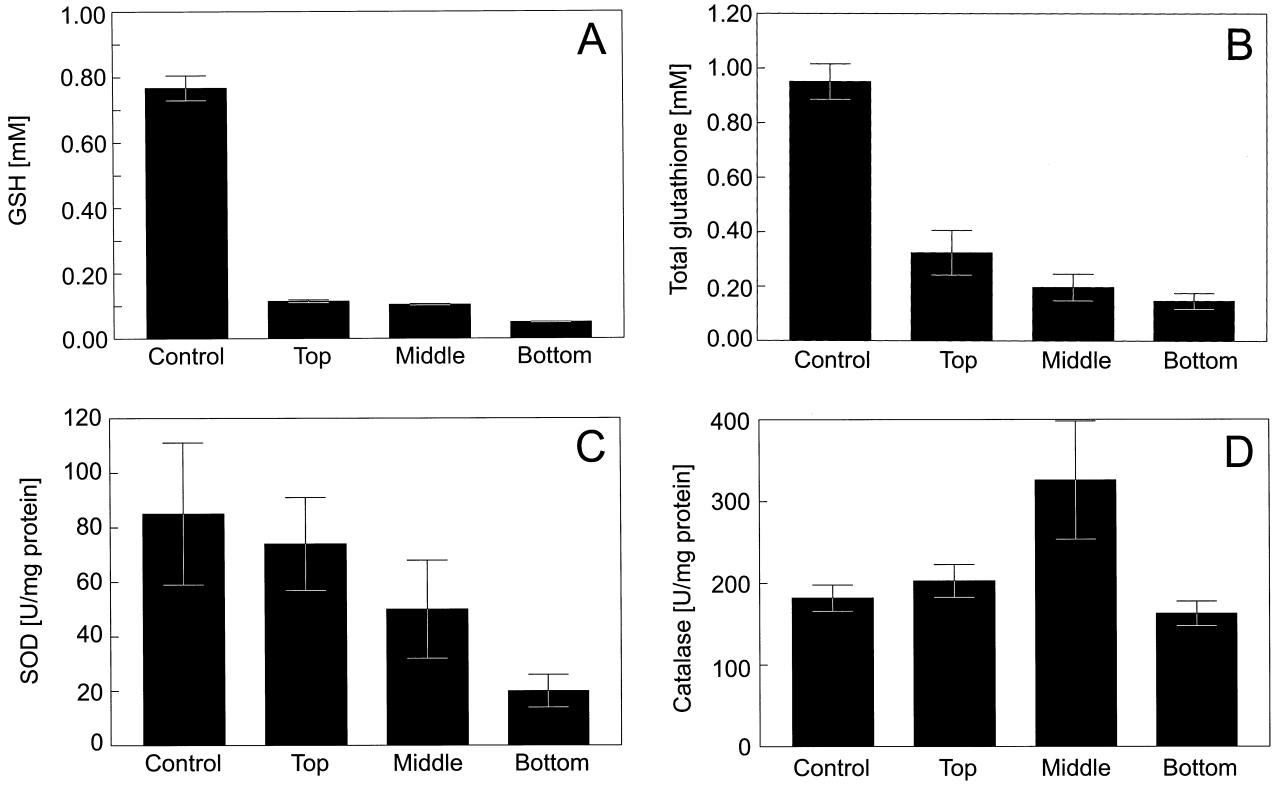

Fig. 3. Level of reduced glutathione (A) and of total glutathione (B), superoxide dismutase activity (C), and glutathione peroxidase activity (D) in cells of early stationary culture and in various fractions of Percoll-fractionated cells collected from CovaLink™ NH_2 plates ($n = 4$).

also decreased considerably (more than six-fold) (Fig. 3). Though reduced and total glutathione were estimated by different methods and direct comparison between the results is not possible, these data suggest that the ratio of oxidized to reduced glutathione increases during replicative aging of the yeast.

The obtained data demonstrate that replicative aging of yeast cells is associated with a considerable attenuation of the antioxidant barrier of the cells. This phenomenon corresponds to that found during aging of stationary cultures of *S. cerevisiae* [12] and is in line with the findings of decreased replicative life span of yeast mutants lacking superoxide dismutases [19,20], prolongation of replicative span under oxygen-rich atmosphere by exogenous glutathione [21], and the importance of superoxide dismutase for the stationary phase survival of *S. cerevisiae* [22]. These results further support the important role played by reactive oxygen species in the process of aging.

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