Superoxide is a mediator of an altruistic aging program in *Saccharomyces cerevisiae*

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Aging is believed to be a nonadaptive process that escapes the force of natural selection. Here, we challenge this dogma by showing that yeast laboratory strains and strains isolated from grapes undergo an age- and pH-dependent death with features of mammalian programmed cell death (apoptosis). After 90–99% of the population dies, a small mutant subpopulation uses the nutrients released by dead cells to grow. This adaptive regrowth is inversely correlated with protection against superoxide toxicity and life span and is associated with elevated age-dependent release of nutrients and increased mutation frequency. Computational simulations confirm that premature aging together with a relatively high mutation frequency can result in a major advantage in adaptation to changing environments. These results suggest that under conditions that model natural environments, yeast organisms undergo an altruistic and premature aging and death program, mediated in part by superoxide. The role of similar pathways in the regulation of longevity in organisms ranging from yeast to mice raises the possibility that mammals may also undergo programmed aging.

Introduction

Longevity in yeast, worms, flies, and mice can be extended by up to severalfold by mutations that increase protection against superoxide and other stresses (Longo and Finch, 2003). Although several mutations that extend longevity cause growth defects, some long-lived mutants grow and reproduce at normal rates. For example, certain yeast and worm mutants have doubled life spans but grow normally (Longo, 1997; Dillin et al., 2002; Fabrizio et al., 2003). If antioxidant defenses and life span can be increased without affecting reproduction, it is surprising that organisms do not express higher levels of antioxidant enzymes or acquire mutations that increase stress resistance and longevity. One explanation may be that the fitness cost of life span extension is not easily detectable. In fact, in *Caenorhabditis elegans*, a mutation that increases the lifespan does not cause a measurable reproductive disadvantage but impairs the ability to undergo cycles of feeding and starvation (Walker et al., 2000). It is also possible that in certain organisms the fitness cost of life span extension is at the population and not individual level (Longo, 1997; Longo et al., 1997; Skulachev, 2002). Although the rapid senescence and death of pacific salmon after spawning and the sudden death of certain organisms after reproduction (Finch, 1990) is consistent with programmed death, an altruistic program that causes accelerated aging and premature death has not been demonstrated in any organism.

Analogously to *C. elegans* and some hibernating mammals, yeast enter different phases based on the availability of nutrients. After growing to the maximum density by using the glucose and other nutrients available on rotting fruit, yeast populations can enter a low metabolism stationary phase and survive for weeks or generate dormant spores that can remain viable for years (Phaff et al., 1966). Moreover, when incubated in medium containing a high concentration of glucose (2% synthetic dextrose complete [SDC] medium) yeast can enter a third survival phase characterized by high metabolic rates and a shorter life span (Longo and Fabrizio, 2002; Fabrizio et al., 2003). We

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Abbreviations used in this paper: SDC, synthetic dextrose complete; Sod, superoxide dismutase.

termed survival in these low and high metabolism phases "chronological life span" (Longo, 1997; Fabrizio and Longo, 2003). *Saccharomyces cerevisiae* wild-type strains DBY746 and SP1 grown in SDC medium have a mean survival of 6 d (see Fig. 1 A; Longo et al., 1996, 1997). Survival in SDC medium provides a model for natural environments such as fruits that contain a high level of glucose (rotting figs), from which ancestors of the wild-type yeast strains used in our experiments were isolated (Mortimer and Johnston, 1986). Because the death of a yeast population is mediated in part by mitochondrial superoxide (Fabrizio et al., 2001, 2003) and is delayed by overexpression of superoxide dismutases (Sods) or the human antiapoptotic protein Bcl-2, we hypothesized that it may represent a form of programmed death (Longo et al., 1997). Apoptosis has been shown to occur in yeast after treatment with hydrogen peroxide or acetate (Madeo et al., 1997, 1999; Ludovico et al., 2001; Skulachev, 2002). Analogously to mammalian cells, a caspase-related protease regulates hydrogen peroxide–induced programmed cell death in yeast (Madeo et al., 2002). Although markers of apoptosis have been detected in yeast cells undergoing replicative or chronological senescence (Laun et al., 2001; Herker et al., 2004), the existence programmed or altruistic aging has not been demonstrated in any organism.

Here, we study several yeast strains isolated from grapes and three different laboratory wild-type strains under conditions that model natural environments and provide evidence for the role of superoxide in an altruistic aging and death program that kills over 90% of a yeast population to release nutrients that promote the growth of a small better-adapted mutant subpopulation.

Results

Features of apoptotic death in aging yeast

SCH9 encodes for a serine/threonine kinase that promotes aging and death in yeast (Fig. 1 A; Fabrizio et al., 2001, 2003). The deletion of *RAS2* and incubation in water also prevent age-dependent death (Fig. 1 A; Longo et al., 1997; Fabrizio et al., 2003). To determine whether or not the death of the yeast population is programmed, we looked for morphological and biochemical features of apoptosis. Chromatin condensation, which is a major characteristic of mammalian cell apoptosis, is observed in yeast undergoing hydrogen peroxide– or Bax-dependent death (Madeo et al., 1997, 2002; Jin and Reed, 2002). At day 7, during the major mortality phase, chromatin staining of wild-type yeast with DAPI shows abnormal nuclear morphology including chromatin condensation and multiple stained regions (Fig. 1 B). Similar results have been reported by Herker et al. (2004). In contrast, the 7-d-old population of *sch9*Δ mutants maintains nuclear morphology similar to that of young cells as suggested by the absence of chromatin condensation and multiple DAPI-stained nuclear regions (Fig. 1 B). Apoptotic mammalian cells lose the ability to maintain phosphatidylserine predominantly in the inner leaflet of the plasma membrane. Consequently, phosphatidylserine is detected on the outer plasma membrane. Annexin V staining of aging yeast populations indicates that at day 7, phosphatidylserine is located in the outer phase of the cytoplasmic membrane (Fig. 1 B). The small percentage of propidium iodide–stained yeast confirmed that the annexin V did not simply stain phospatidylserines exposed after cell death (Fig. 1 B). Neither annexin V nor propidium iodide stained the long-lived *sch9*Δ mutants (Fig. 1 B). Another early event associated with apoptosis in mammalian cells is the alkalinization of the mitochondrial matrix and the acidification of the cytosol (Matsuyama et al., 2000). We tested if cytosolic acidification contributes to aging and death by monitoring survival after increasing the pH (Imai and Ohno, 1995). Raising the extracellular pH from 5 to 6.5 causes an increase of intracellular pH from 5.8 to 6.2 (Imai and Ohno, 1995). Death was prevented by increasing the extracellular pH at days 3–11 from the physiologic 3.5–4 pH (days 1–13) to pH 6.5 (Fig. 1 C). The effect of low pH on aging and death may be explained by a role for intracellular acidification in the activation of the Ras pathway (Thevelein and de Winde, 1999). In fact, the deletion of *RAS2* doubles the life span of yeast cells (Fig. 1 A; Fabrizio et al., 2003).

Superoxide and Sods play major roles in the aging and death of yeast and higher eukaryotes (Honda and Honda, 1999; Sun and Tower, 1999; Fabrizio et al., 2001, 2003) and in the apoptosis of mammalian cells (Tanaka et al., 2002). Furthermore, mitochondrial Sod (Sod2) functions downstream of Sch9 and Ras2 to extend longevity in yeast (Fabrizio et al., 2003). To determine whether or not wild-type yeast may down-regulate Sod2 as part of an aging and death program, we assayed the activity of Sod2, which is expressed at low levels during growth in glucose medium but increases by 5–10-fold in carbon sources that require mitochondrial respiration (Maris et al., 2001). Although respiratory rates are high at day three (Fabrizio et al., 2003), mitochondrial Sod2 activity in wild-type extracts from day 3 and 5 was two- to threefold lower than in *sch9*Δ mutant extracts (Fig. 1 D). Furthermore, the long-lived *sch9*Δ and *ras2*Δ mutants and *SOD1CTT1* overexpressors were resistant to the superoxide-generating agent menadione and to hydrogen peroxide (Fig. 1 E and not depicted), indicating that the wild-type yeast population is underprotected against oxidative toxicity during a phase in which mitochondrial respiration and oxidant generation are high. Together, these data suggest that yeast grown in SDC medium undergo an age-dependent death with characteristics of mammalian apoptosis including chromatin condensation, exposure of phosphatidylserine, cytosolic acidification, low Sod2 activity, and sensitivity to superoxide and hydrogen peroxide. These results are consistent with recent data showing chromatin condensation and exposure to phosphatidylserine in yeast cells aging chronologically (Herker et al., 2004). The ability of a switch from SDC medium to water or of mutations in the Sch9 and Ras pathways to cause a major delay in cell death (Fig. 1 A) is also consistent with the existence of programmed aging.

Budding yeast isolated from grapes undergo age-dependent death and adaptive regrowth

To determine if "programmed aging" may be limited to laboratory yeast strains, we isolated yeast cells from or-

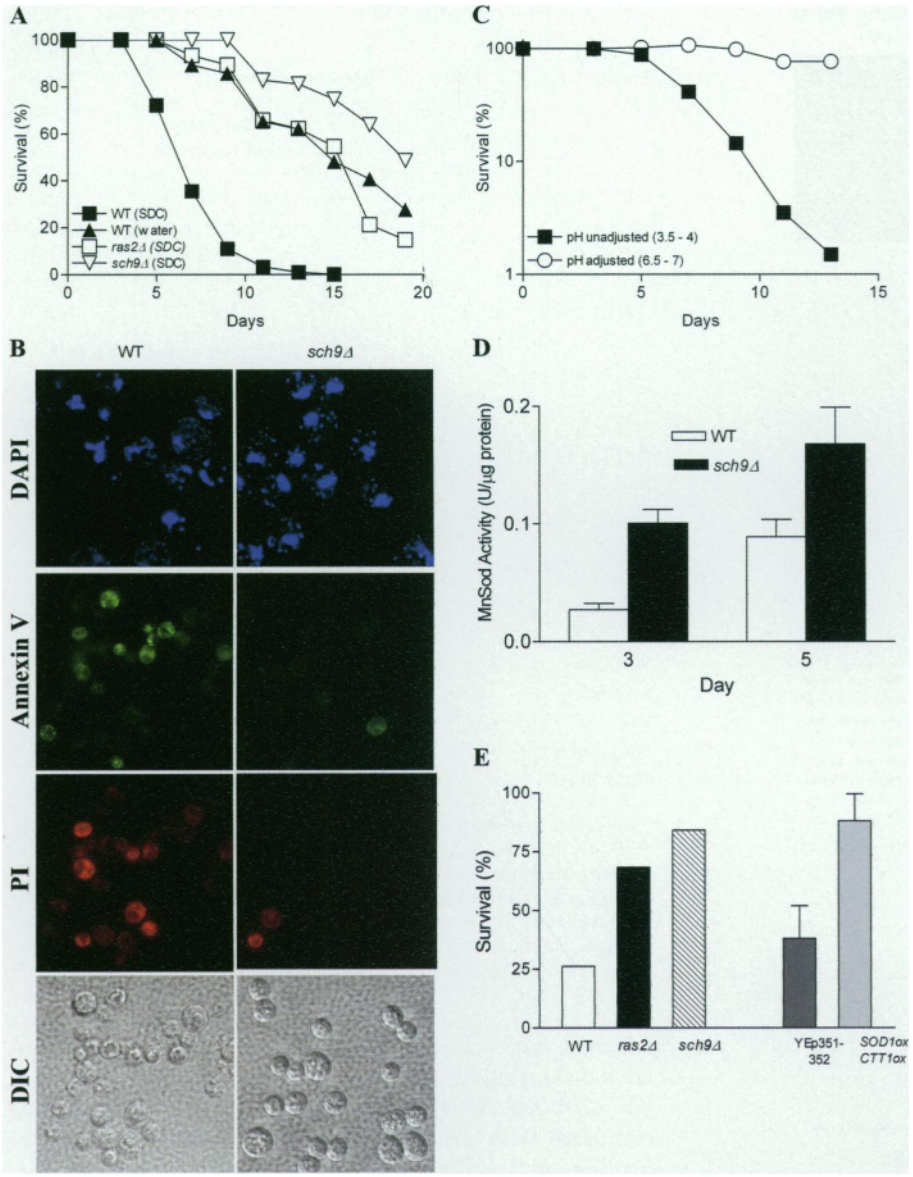

Figure 1. **Aging yeast cells show features of apoptotic death.** (A) Chronological survival of wild-type (DBY746) and long-lived mutants *ras2*Δ and *sch9*Δ populations. Life span measurement were performed by incubating wild-type (WT) cells, *ras2*Δ, and *sch9*Δ mutants either in synthetic dextrose complete (SDC) medium or in water. Viability is expressed as percent survival (100% at day 3). Results shown represent the average of 4–12 experiments. (B) Day 7 wild-type and *sch9*Δ mutants were stained with DAPI for analysis of nuclear morphology (top), GFP-labeled annexin V for detection of exposed phosphatidylserine and propidium iodide for detection of damaged/dead cells (middle panels). Approximately 25–30 cells for each strain were present on each field (bottom, DIC images) and were treated simultaneously with annexin V and propidium iodide. (C) Chronological survival of wild-type cells incubated in standard SDC medium (pH 3.5–4) and in SDC medium with pH adjusted and maintained at 6.5–7 from day 3 to 13. The experiment was repeated five times with similar results. A representative experiment is shown. (D) Mitochondrial Sod (Sod2) activity of wild-type and *sch9*Δ mutants was measured at day 3 and 5. Results shown represent the average of two experiments conducted in triplicate. Error bars show SEM. (E) Oxidative stress resistance of wild type, *ras2*Δ mutants, *sch9*Δ mutants, wild type overexpressing control vectors YEp351-YEp352, and *SOD1/SOD2* overexpressors. Cells removed from day 3 in the postdiauxic phase were incubated for 60 min in phosphate buffer containing 200 μM of the superoxide H_2O_2-generating agent menadione. Viability was measured by plating cells onto rich medium before and after treatment. A representative experiment performed in duplicate is shown for the deletion strains. The average of two experiments is shown for the overexpressors. Error bars show SEM.

ganically grown California red grapes. We selected cells that grew within 48 h in YPD medium, generated colonies similar to those formed by *S. cerevisiae*, and divided by asymmetric budding (Fig. 2 A). The survival of populations generated from cells derived from three different wild colonies was similar to that of wild-type laboratory strains (Fig. 2 B). After ~90–99% of the "wild" population died (day 3–11), the number of viable cells in the culture increased, suggesting that adaptive regrowth is occurring. In contrast, incubation in water prevented age-dependent death and regrowth (Fig. 2 C). As observed in the laboratory wild-type strains, the pH of wild yeast grown in SDC medium decreases to approximately pH 3 by day 1. Adjustment of the extracellular pH from 3 to 6.5 prevented age-dependent death (Fig. 2 D). These data confirm our results with laboratory strains and suggest that programmed aging occurs in wild strains isolated from natural environments.

Effect of superoxide and hydrogen peroxide in promoting adaptive regrowth

We observed that in many laboratory wild-type cultures, the number of viable organisms (colony-forming units) increases after 90–99% of the population dies (Fig. 3 A). This adaptive regrowth, which is reminiscent of the growth (gasping) of bacterial populations maintained in stationary phase (Zambrano and Kolter, 1996), is important for adaptation to starvation conditions. To determine whether or not early death may be adaptive, we monitored the ability of various wild-type, short-, and long-lived populations to regrow during stationary phase by using nutrients available in the expired medium (Fig. 3 A and Table I). In ~50% of

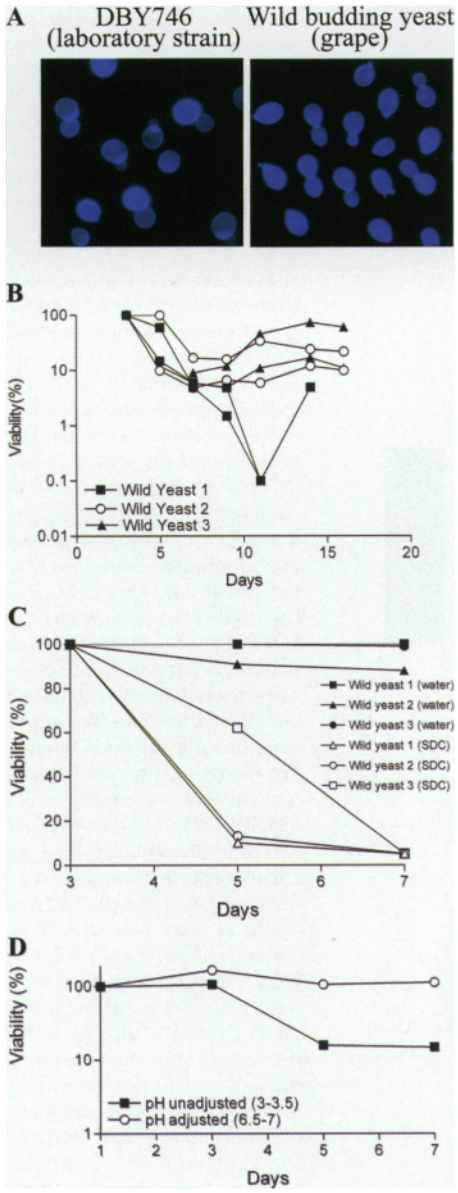

Figure 2. **Adaptive regrowth in wild yeast.** (A) Wild-type DBY746 and wild yeast isolated from grapes were stained with Calcofluor to analyze their morphology and budding pattern. Chronological survival/regrowth of wild yeast cells incubated in SDC medium (B), water (C), and standard SDC medium (pH 3–3.5) and SDC medium with pH adjusted and maintained at 6.5–7 from day 1 to 7 (D). Viability was measured every 2 d. These experiments were performed twice in duplicate with similar results.

wild-type cultures, a small subpopulation regrew after day 10, when the majority of the population had lost viability (88 flasks; Table I and Fig. 3). Increased levels of cytosolic superoxide, which we have previously shown to play a major role in the chronological aging of yeast, caused increased adaptation as evidenced by adaptive regrowth in 89% of populations of yeast lacking *SOD1* (*sod1*Δ) and 75% of those lacking both *SOD1* and catalase (*sod1*Δ *ctt1*Δ; Fig. 3, A and B; and Table I). In most studies, *sod1*Δ and *sod1*Δ *ctt1*Δ mutants died within 24 h and were able to grow back within the first 7 d (Fig. 3, A and B). The deletion of *SOD1* also caused early growth in the parental strain BY4700,

Table I. Adaptive regrowth in yeast with different life span

Strain	Adaptive regrowth			Mean Life Span
	Yes	No	Percentage of regrowth	
DBY746 (WT)	42	46	48	6.2
*sod1*Δ	17	2	89[c]	1
*sod1*Δ *ctt1*Δ	3	1	75	1
*ctt1*Δ	1	1	50	6.2
*sod1*Δ Bcl-2	4	6	40	6.2
SOD1ox SOD2ox	0	9	0[c]	7.8
SOD1ox CTT1ox	0	13	0[c]	7.5
SOD1ox	6	13	31.5[c]	7.2
SOD2ox	0	6	0[c]	6.65
CTT1ox	3	7	30[c]	6.2
Bcl-2ox	0	9	0[c]	7
*ras2*Δ	0	15	0[c]	15
*sch9*Δ	0	14	0[c]	19
*yap1*Δ	1	10	10[c]	6
*skn7*Δ	2	10	20[c]	6
SP1 (WT)	3	1	75	7.8
SOD1ox	1	3	25	9
Summary				
WT	45	47	49[c]	7
Long-lived[a]	6	82	6.8[c]	9.9
Short-lived[b]	20	3	87[c]	1

[a]Superoxide resistant: *SOD1ox SOD2ox*, *SOD1ox CTT1ox*, *SOD1ox*, *SOD2ox*, Bcl-2ox, *ras2*Δ, and *sch9*Δ.
[b]Superoxide sensitive: *sod1*Δ and *sod1*Δ *ctt1*Δ.
[c]Significantly different versus wild type (WT). When four or less samples were available, the corresponding strains were excluded from statistical analysis. Chi square test ($\chi^2 = 64$, df = 10, $P < 0.05$) followed by Scheffé's post-test for multiple comparisons ($P < 0.05$).

which is more closely related to the strains originally isolated on rotting figs (Mortimer and Johnston, 1986). Strain BY 4700/*sod1*Δ exhibited an early adaptation advantage compared with wild-type strain BY4700 (Fig. 3 C), confirming our results with strains DBY746. However, the strain lacking *SOD1* eventually died, whereas the wild-type strain reached and maintained a viability of ~1% of the initial viability for over 60 d (Fig. 3 C and not depicted). *sod1*Δ mutants in the DBY746 genetic background also died after more than 40 d in culture (unpublished data). These results suggest that the beneficial effect of greatly elevated superoxide on adaptive regrowth is only temporary and may explain in part why natural selection has prevented loss-of-function mutations in the *SOD1* gene.

To further test the hypothesis that the oxidant-dependent adaptation is related to mammalian apoptosis, we overexpressed the human antiapoptotic protein Bcl-2 in *sod1*Δ mutants and wild-type yeast. Bcl-2 overexpression, which extends survival (Longo et al., 1997), caused a major decrease in the frequency of adaptive regrowth of both *sod1*Δ mutants and wild-type cultures (Fig. 3 A and Table I). The overexpression of *SOD1* in *sod1*Δ mutants also prevented adaptive regrowth (unpublished data).

In contrast to wild-type and *sod1*Δ mutants, long-lived yeast overexpressing antioxidant enzymes (*SOD2ox*, *SOD1ox SOD2ox*, and *SOD1ox CTT1ox*), lacking *RAS2* (*ras2*Δ) or

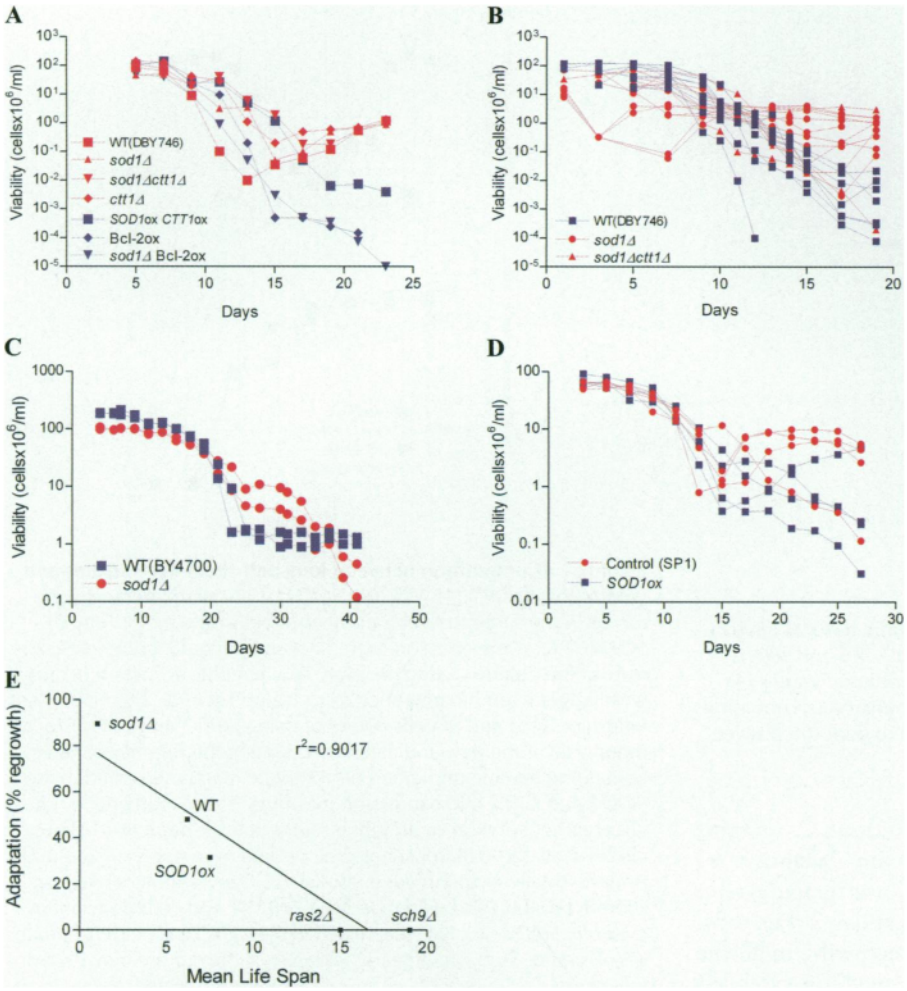

Figure 3. **Adaptive regrowth in short- and long-lived strains.** Red lines and symbols are used for strains that have a regrowth advantage relative to the other strain(s) (blue) in that experiment. (A) The ability to regrow after the majority of the population had died was monitored in various wild-type (WT DBY746 and control strain YEp351-YEp352), short-lived mutants lacking cytosolic Sod (*sod1*Δ and *sod1*Δ *ctt1*Δ), and long-lived strains overexpressing antioxidant enzymes or the antiapoptotic mammalian protein Bcl-2 (*SOD1ox CTT1ox* and Bcl-2ox). *sod1*Δ mutants overexpressing Bcl-2 and *ctt1*Δ mutants were also included in the study. A single representative experiment is shown (see Table I for all experiments). (B) Adaptive regrowth was monitored in DBY746 wild-type (blue line) and short-lived strains (*sod1*Δ and *sod1*Δ *ctt1*Δ; red line). Results from four different experiments are plotted. Each line represents a single sample. A total of 8 wild-type samples, 11 *sod1*Δ, and 3 *sod1*Δ *ctt1*Δ is shown. (C) Adaptive regrowth of wild-type strain BY4700 and of a *sod1*Δ mutant originated in the same background. The experiment was performed three times in duplicate. A representative experiment is shown. (D) Adaptive regrowth of wild-type strain SP1 and of *SOD1* overexpressors originated in the same background. A representative experiment is shown. (E) Correlation between adaptive regrowth and mean life span ($r = 0.90$).

lacking *SCH9 (sch9*Δ*),* exhibited a major reduction of regrowth frequency (Fig. 3 A and Table I). Adaptive regrowth was not observed in any of the cultures overexpressing both *SOD1* and catalase (*SOD1ox CTT1ox*; Fig. 3 A, and Table I, 13 cultures) and was reduced from a 48 to 31% frequency by the overexpression of *SOD1* (19 cultures; Table I). Overexpression of *SOD1* also caused a major adaptive regrowth disadvantage in another parental strain (SP1; Fig. 3 D and Table I).

Growth under starvation condition was not observed in any of the 29 independent cultures of the long-lived *ras2*Δ and *sch9*Δ (Table I). This effect of mutations in the *RAS2* or *SCH9* genes on stationary phase regrowth may be mediated, in part, by decreased superoxide and hydrogen peroxide levels. In fact, deletion mutations in *RAS2* or *SCH9* increase resistance to both superoxide and hydrogen peroxide (Fig. 1 E), decrease the level of superoxide, and extend longevity by inducing the expression of Sod2 and other stress resistance genes (Fabrizio et al., 2001, 2003).

In summary, regrowth was observed in 90% of the cultures of short-lived mutants deficient in *SOD1*, in 50% of wild-type cultures, but in <10% of long-lived yeast overexpressing antioxidant enzymes (Table I). The strong inverse correlation between mean life span and the ability to grow in stationary phase ($r = 0.90$; Fig. 3 E) is consistent with a role for superoxide and hydrogen peroxide in a death program that increases the chance of adapting to a nutrient-poor environment. These results are also consistent with the initiation of caspase-dependent apoptosis in yeast exposed to hydrogen peroxide (Madeo et al., 2002). However, a major increase in longevity may not be required to prevent adaptive growth. In fact, the overexpression of *SOD1CTT1* or *SOD1SOD2* prevents growth in stationary phase cultures but only causes a 10–30% increase in life span.

Adaptive regrowth in media that model natural environments

To test whether or not the age-dependent adaptive regrowth may be an artifact of the laboratory medium, we monitored viability in yeast cultures aged in medium obtained by processing organic red grapes or in medium containing 10% of the ammonium sulfate compared with the standard SDC medium. This decrease in ammonium sulfate can serve to model the low levels of nitrogen, characteristic of certain natural environments (e.g., grapes). The *S. cerevisiae* DBY746 strain grown in grape extracts died at rates similar to those observed in SDC medium (Fig. 4 A). A leveling off in mortality and then adaptive regrowth was observed after day 15 (Fig. 4 A and not depicted). In contrast, adaptive regrowth in *sod1*Δ cultures maintained in "grape extracts" was observed much earlier (day 3; Fig. 4 A).