Yeast Colony Survival Depends on Metabolic Adaptation and Cell Differentiation Rather Than on Stress Defense*§

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Enzymes scavenging reactive oxygen species (ROS) are important for cell protection during stress and aging. A deficiency in these enzymes leads to ROS imbalance, causing various disorders in many organisms, including yeast. In contrast to liquid cultures, where fitness of the yeast population depends on its ROS scavenging capability, the present study suggests that Saccharomyces cerevisiae cells growing in colonies capable of ammonia signaling use a broader protective strategy. Instead of maintaining high levels of antioxidant enzymes for ROS detoxification, colonies activate an alternative metabolism that prevents ROS production. Colonies of the strain deficient in cytosolic superoxide dismutase Sod1p thus developed the same way as wild type colonies. They produced comparable levels of ammonia and underwent similar developmental changes (expression of genes of alternative metabolism and center margin differentiation in ROS production, cell death occurrence, and activities of stress defense enzymes) and did not accumulate stress-resistant suppressors. An absence of cytosolic catalase Ctt1p, however, brought colonies developmental problems, which were even more prominent in the absence of mitochondrial Sod2p. sod2Δ and ctt1Δ colonies failed in ammonia production and sufficient activation of the alternative metabolism and were incapable of center margin differentiation, but they did not increase ROS levels. These new data indicate that colony disorders are not accompanied by ROS burst but could be a consequence of metabolic defects, which, however, could be elicited by imbalance in ROS produced in early developmental phases. Sod2p and homeostasis of ROS may participate in regulatory events leading to ammonia signaling.

When organisms, including yeast, grow under aerobic conditions, they produce ROS2 as a consequence of aerobic respiration. Their excess is scavenged by different stress defense enzymes. Various clinical human disorders such as porphyria, hypertension, atherosclerosis, and some neurodegenerative diseases are believed to be partly induced by unbalanced ROS levels or by a deficiency in one of the stress defense enzymes (1).

The yeast Saccharomyces cerevisiae can protect itself against various stresses (including oxidative stress) with a variety of ROS-scavenging enzymes, including superoxide dismutases, catalase, glutathione peroxidases, peroxiredoxins, and others. As do other eukaryotes, S. cerevisiae contains cytosolic CuZn-Sod1p and mitochondrial Mn-Sod2p superoxide dismutases responsible for the removal of superoxide radicals. Another S. cerevisiae enzyme that plays a role in ROS detoxification and yeast resistance to various stresses (2, 3) and that appears to be important for replicative life span (4) and programmed cell death (5) is the cytosolic catalase Ctt1p, which decomposes hydrogen peroxide.

Mutants deficient in mitochondrial Sod2p are sensitive to hyperoxia and exhibit growth inhibition on respiratory carbon sources, but during aerobic growth on glucose the absence of this enzyme has little effect (6). The loss of Sod1p leads to more pleiotropic defects, including sensitivity to various exogenous stresses and reduced growth under all aerobic conditions because of oxidative injury by internal oxygen species (7–9). A consequence of endogenous oxidative stress is damage of particular biosynthetic enzymes, resulting in lysine and methionine auxotrophy (10). Defective phenotypes associated with sod1Δ strains are usually suppressed by the accumulation of stress-resistant mutants of the two main groups. The first group includes mutations in the BSD1 and BSD2 genes involved in Mn2⁺ homeostasis (11). The sod1Δbsd mutants increase the cytosolic concentration of Mn2⁺, which possesses some intrinsic superoxide dismutase activity and partially compensates for the absence of Sod1p. The second group, designated seo (for suppressors of endogenous oxygen toxicity), includes various mutations that restore Met and Lys prototrophy, although sensitivity to environmental oxidants (e.g. Paraquat) is not reverted (10).

In contrast to liquid microbial cultures commonly studied in laboratories, microorganisms in their natural environments often organize as multicellular communities (e.g. colonies and biofilms) with unique properties (12, 13). During long term development, colonies of different yeasts can undergo developmental changes characterized by extracellular alkalization and production of volatile ammonia, which functions as a signaling molecule (14). In S. cerevisiae colonies, ammonia elicits expression changes leading to metabolic reprogramming (e.g. activation of peroxisomes and fatty acid β-oxidation, the methyl glyoxylate cycle, amino acid metabolism, and the production of

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2 The abbreviations used are: ROS, reactive oxygen species; DHE, dihydroethidium; GS, glutamine synthetase; Met+ , methionine prototrophy; PQ+, Paraquat-resistant; wt, wild type; MES, 4-morpholineethanesulfonic acid.
different plasma membrane transporters) and a parallel decrease in the expression of stress-related genes (including *CTT1* and *SOD1*) and genes of mitochondrial oxidative phosphorylation (15). Ammonia signaling is also important for constraining apoptotic-like cells to the colony center (16).

This study assesses the importance of the individual stress defense enzymes (a deficiency of which poses a serious problem for liquid yeast cultures) during the development of *S. cerevisiae* colonies. New data suggest that in colonies activation of the ammonia-induced adaptive metabolism is more important for proper development and survival than the presence of ROS scavenging enzymatic activities. Nevertheless, ROS homeostasis appears to be important for the induction of ammonia signaling and activation of metabolic changes.

**EXPERIMENTAL PROCEDURES**

Strains and Media—*S. cerevisiae* strain BY4742 (MATa, *his3Δ1, leu2Δ1, lys2Δ1, ura3Δ1*) and all isogenic mutants (*sod1Δ, sod2Δ, ctt1Δ*) were obtained from the EUROSCARF collection. Yeast giant colonies (14) were grown 6/plate at 28 °C on GMA (50 mM potassium Pi (KH2PO4/K2HPO4), pH 6.5, when approaches debris removal, the supernatant (5–15 mg/ml) was broken in 10 mM MES buffer, pH 6, with Complete, EDTA-free protease inhibitor mixture (Roche Applied Science) and 1 mM 4-(2-aminoethyl)benzenesulfon fluoride (Sigma) with glass beads in a FastPrep instrument (QBiogene). After cell debris removal, the supernatant (5–15 μg of proteins/slot determined using a Bio-Rad protein detection kit) was subjected to PAGE under nondenaturing conditions. Superoxide dismutase and catalase activity in the gels was determined (18, 19). Enzyme activities were quantified by image analyses using UltraQuant 6.0 software (Media Cybernetics).

RNA Isolation and Northern Blot Analysis—Total RNA from ~100 mg of wet colony biomass was extracted by a hot phenol method (15). For northern blots, 15 μg of total RNA was separated in 1.5% agarose gel and transferred to a positively charged nylon membrane (Amersham Biosciences). The Northern blots were densitometrically quantified using UltraQuant 6.0 software. The values presented are relative densities calculated for the maximal value of each film (set as 100%). *RDN18* was the nonregulated control gene.

**Analysis of Cell Survival and Presence of Suppresser Mutants in Liquid Media**—Survival of cells from colonies was analyzed in liquid SD, YPD, or GM (inoculated to A560 = 0.2). The number of living cells was determined after 1–7 days of cultivation by drop assay. Five-microliter drops of serial 10-fold dilutions of the cell cultures were spotted on an YPDA, and the number of colony-forming cells was determined after 3 days of cultivation. The appearance of suppressor mutants in sodΔ cultures was determined by drop assay on SDA without methionine and on YPDA supplemented with 1.5 mM Paraquat (methyl viologen; MP Biomedicals) or 5 mM MnSO4.

**Heat Shock Tolerance**—Harvested colonies were suspended in distilled water to a concentration of 7.5 mg/ml. 10-fold serial dilutions of these suspensions were incubated at 52 °C for 150 min in microtitre plates, and then 5 μl drops were spotted on YPDA. The number of colony-forming cells was determined after 3 days of cultivation. Control cells were spotted at the same dilutions before the heat treatment.

**Detection of Dying Cells**—For 4′,6′-diamidino-2-phenylindole staining of the DNA, the cells were permeabilized with 60% ethanol. 4′,6′-Diamidino-2-phenylindole was added to a final concentration of 2.5 μg/ml, and the cells were observed under a fluorescence microscope using an A filter (Leica). Nuclear morphology was determined from a minimum of 500 cells/sample. Cell morphology and the presence of shrunken cells were visualized with Nomarski contrast (supplemental Fig. S3A).

**ROS Quantification**—For DHE staining, 50 μl of 15 mg/ml cell suspension in 25 mM MES, pH 6, was incubated with 5 μl of 25 μg/ml DHE (Sigma; 1 mg/ml stock solution in Me2SO) for 25 min. The suspension was then transferred to a cuvette with 1.95 ml of distilled water. Mitochondrial superoxide production was monitored by MitoSOX Red staining. Twenty-five μl of 0.5 mM MitoSOX (Molecular Probes; 5 μM stock solution in Me2SO) was added to a cuvette with 2 ml of cell suspension (containing 0.75 mg/ml of wet biomass) and incubated for 5 min. The release of hydrogen peroxide from cells was measured with an Amplex Red horseradish peroxidase kit (Molecular Probes). Fifty μl of cell suspension (15 mg/ml in 25 mM MES buffer, pH 6.0) was incubated for 30 min in the presence of 50 μM Amplex Red, and 5 units/ml of horseradish peroxidase was diluted with 1.95 ml of distilled water. Fluorescence was measured using a FluoroMax 3 spectrofluorometer (Jobin Yvon) with the following excitation/emission wavelengths: 480/604 nm for DHE, 510/580 nm for MitoSOX Red, and 525/585 nm for Amplex Red. Readings from unstained cells were used as an autofluorescence control and subtracted from the stained cell fluorescence.

**Glutathione Peroxidase and Glutamine Synthetase Protection Assay**—The enzymatic activities of glutathione peroxidase and peroxiredoxins were measured in cell extracts prepared as described above, with the exception of using 50 mM potassium Pi, pH 7.8, for the extraction. The samples of 100 μg of protein were assayed with a glutathione peroxidase cellular activity assay kit (Sigma) detecting changes in NADPH absorbance after the addition of tert-butyl hydroperoxide. Peroxiredoxin activity was assayed by glutamine synthetase (GS) protection assay (20, 21), which is based on the sensitivity of GS to oxidative damage caused by a thiol/Fe3+/O2 mixed function oxidase system. The ability of a cell extract to protect GS from oxidative damage was determined.
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damage is mostly ascribed to the activity of peroxiredoxins (21). In the assay, 1 unit of glutamine synthetase (Sigma) in 50 μl was incubated with a mixture of 3 μM FeCl₃ and 10 mM dithiothreitol in the presence of the cell extract (containing 50 μg of protein). After 10 min, the remaining GS activity was determined by adding 950 μl of a GS activity assay mixture (0.4 mM ADP, 150 mM glutamine, 10 mM KH₂AsO₄, 20 mM NH₄OH, 0.4 mM MnCl₂, 100 mM HEPES, pH 7.4). After 15 min, the reaction was stopped with 200 μl of a mixture of 5.5% FeCl₃, 2% trichloroacetic acid, and 2% HCl. The γ-glutamylhydroxamate-Fe³⁺ complex was determined at 420 nm. The intrinsic GS activity of the cell extract was subtracted. The activity of GS prior to treatment with FeCl₃/dithiothreitol was taken as 100%. Without the protection by cell extract, the GS completely lost its activity.

Statistics—The data points representing the means ± S.D. were calculated from at least four independent experiments. Where appropriate, data significance was determined using the two-tailed t test. p values of 0.05 or less were considered statistically significant: *, p < 0.05; **, p < 0.01 or commented in the figure legends.

RESULTS

Deficiency in Sod2p but Not in Cytosolic Sod1p Causes Defects in Colony Development and Ammonia Signaling—The growth of colonies of strains deficient in Sod1p, Sod2p, or cytosolic Ctt1p on GMA was comparable with the growth of wild type (wt) BY4742 colonies (supplemental Fig. S1), exhibiting a linear increase in cell biomass that is typical for yeast colonies growing on solid media (16, 22). The colony development, however, substantially differed among strains, some being impaired in ammonia signaling. Whereas sod1Δ colonies exhibited a morphology, alkalization, and ammonia production comparable with the wt phenotype, sod2Δ colonies displayed altered morphology and failed to produce ammonia and accomplish transition to the alkali phase (Fig. 1, A and B). ctt1Δ colonies produced a reduced level of ammonia.

To find out whether sod1Δ, sod2Δ, and ctt1Δ colonies are able to activate adaptive metabolic changes ascribed to ammonia signaling (15), we monitored expression of the most relevant genes involved in these changes. Expression of carbon metabolism and transporter genes (CIT3, POX1, ATO1, ATO3, and JEN1) was activated in ammonia-producing sod1Δ colonies as well as in wt colonies. The expression of these genes reached significantly lower levels in sod2Δ colonies and was slightly diminished in ctt1Δ colonies (Fig. 1C and supplemental Fig. S2A). On the other hand, sod2Δ colonies maintained a partially higher level of stress-related gene expression (MSN4, HSP30, and CTT1), contrary to sod1Δ and wt colonies (Fig. 1D and supplemental Fig. S2B). These data showed that colonies of all three mutants initiated adaptive metabolic changes in approximately the same developmental period as did wt strain colonies, but in sod2Δ (and to some extent also in ctt1Δ) colonies the changes did not reach a level comparable with that of the wt and sod1Δ colonies.

Deficiency in Ammonia Signaling Correlates with the Absence of Cell Differentiation within sod2Δ and ctt1Δ Colonies—Because sor2Δ colonies lacking Sok2p transcription factor and defective in ammonia signaling also exhibit an altered distribution of dying apoptotic-like cells (16), we examined center margin colony differentiation in sod1Δ, sod2Δ, and ctt1Δ colonies. First, we analyzed the occurrence of cells exhibiting late dying features (chromatin condensation and fragmentation as well as the presence of “shrunken,” i.e. partially digested, cells) (Fig. 2A and supplemental Fig. S3A) in outer margin and central colony areas. As in wt colonies, the cells exhibiting modified nuclei and shrunken cells were preferentially localized to central regions in ammonia-producing sod1Δ colonies, whereas cells located at the margin were mostly normal and healthy. In contrast, the cells with both dying features were spread throughout the whole of sod2Δ and ctt1Δ colonies (impaired in ammonia production) (Fig. 2A) similarly as in sok2Δ colonies (16).

Furthermore, we determined stress-related characteristics of cells harvested from the central and margin regions of colonies from each strain. We determined ROS levels, ROS-scavenging enzyme activity, and cell tolerance to heat shock (Fig. 2, B–F). Cellular ROS production is characteristic of oxidative stress and has also been linked to ongoing apoptosis (23). The occurrence of the superoxide was detected as superoxide-mediated oxidation of either DHE (detecting total cellular superoxide) or MitoSOX Red, an indicator of mitochondrial superoxide. After entering the alkali phase (at day 11), central and outer cells in colonies of both ammonia-producing strains (wt and sod1Δ) exhibited divergent trends in both mitochondrial and cellular superoxide levels. Whereas superoxide concentration gradually increased in the colony center, we observed a simultaneous decrease in the colony margin. A significantly smaller center margin difference was observed in ammonia production-impaired sod2Δ and ctt1Δ colonies. Interestingly, however, despite the similar profiles in central and outer cells, relative levels of mitochondrial (Fig. 2C) and cellular (Fig. 2B) superoxide differed. Total cellular superoxide levels in central cells of sod2Δ and ctt1Δ colonies did not increase as in wt central cells, whereas outer cell levels were comparable with wt (Fig. 2B). Conversely, mitochondrial superoxide levels in outer cells of sod2Δ and ctt1Δ did not decrease as in their wt counterparts, whereas central cell levels remained equivalent (Fig. 2C). These data showed that the overall superoxide level did not increase in sod1Δ, sod2Δ, and ctt1Δ colonies in general, although homeostasis of mitochondrial and cellular superoxide was differentially misbalanced.

Cell production of hydrogen peroxide was determined through the Amplex Red assay (supplemental Fig. S4). The results confirmed that center margin H₂O₂ differences were nearly 40% more pronounced in wt and sod1Δ colonies as compared with sod2Δ and ctt1Δ colonies. However, the center margin difference in ammonia-producing colonies as compared with colonies defective in ammonia production was less prominent than in the case of the superoxide. This could be caused by more efficient diffusion of H₂O₂ across a colony. Overall amounts of H₂O₂ released by colonial cells were just 6–17% of the amounts released by 24-h-old wt cells cultivated in YPD liquid medium (data not shown).

Because the cellular ROS homeostasis is determined by both ROS production and ROS-scavenging enzymes, we examined catalase and superoxide dismutase activity in colonies. Within wt and sod1Δ colonies, Ctt1p activity was sig-
significantly higher in central than in outer cells, whereas it remained uniform within sod2Δ colonies (Fig. 2D). Similarly, Sod1p activity was significantly higher in central than outer wt cells but homogeneous among both sod2Δ colony regions (Fig. 2E). Sod1p activity transiently increased in the central region of ctt1Δ colonies during their transition to the abortive alkali phase (Fig. 2E). Interestingly, both enzyme activities of central sod2Δ cells were lower than those of central wt cells.

Variations in scavenging enzymatic activities can influence cell sensitivity to different stresses inducing ROS production. It was shown, for example, that sod1Δ is more sensitive to heat shock, presumably because of increased ROS production at elevated temperature (7). Heat shock tolerance of central and outer cells from both wt and sod1Δ colonies differed significantly (Fig. 2F). The survival rate of central cells was 10 times higher than that of outer cells from alkali phase and second acidic phase colonies after exposure to 52 °C for 150 min. Despite the similar trend, both central and outer cells from sod1Δ colonies were ~5–10 times more sensitive to the heat shock than wt cells. In contrast, there was no difference in heat shock sensitivity between central

FIGURE 1. Colonies formed by strains deficient in individual stress defense enzymes differ in ammonia signaling and in related metabolic changes. A, ability of wt, sod1Δ, sod2Δ, and ctt1Δ colonies to produce ammonia over 21 days ($x$ axis). *, $p < 0.05$; **, $p < 0.01$ when mutant and wt values were compared. B, morphology of colonies growing on GMA-bromcresol purple. Intensity of violet coloring of pH indicator bromcresol purple correlates with the extent of alkalinization. C, colony ability to activate adaptive changes in metabolic and transporter genes demonstrated as average change in expression of CIT3, POX1, ATO1, ATO3, and JEN1 genes. D, expression profile of stress-related genes demonstrated as average change in expression of MSN4, HSP30, and CTT1 genes. For each gene in C and D, the time point exhibiting the highest expression value was set as 100%. *, $p < 0.05$; **, $p < 0.01$; changes of particular genes are shown in supplemental Fig. S2.
and outer cells from sod2Δ and ctt1Δ colonies (Fig. 2F). Surprisingly, cells from sod2Δ and ctt1Δ colonies were in general relatively resistant. The above data show that the distribution of cells harboring dying and stress-related characteristics differed in the central and margin regions of ammonia-producing sod1Δ and wt col-
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In contrast, there was little or even no difference in their distribution in sod2Δ and ctt1Δ colonies, which are incapable of ammonia signaling. In addition, contrary to liquid cultures, there was no increase in ROS and/or ROS-scavenging enzymes in colonies deficient in these enzymes. In fact, levels of ROS and of scavenging enzymes were even decreased in some cases. To determine whether the activation of some other stress defense mechanisms could be responsible for compensating the lack of superoxide dismutases or catalase, we measured glutathione peroxidase and peroxidorexidin activities. As shown in supplemental Fig. S5, these activities in cells harvested from 23- and 30-day-old colonies were approximately the same among all strains.

Sod1p-deficient Colonies Do Not Accumulate Suppressor Mutants Resistant to Extracellular or Intracellular Oxidative Stress—Surprisingly, in contrast to the absence of mitochondrial Sod2p, the absence of cytosolic Sod1p was accompanied by no visible defect in colony development and long term survival, despite the fact that sod1Δ colonies were grown aerobically on respiratory glycerol medium GMA. In sharp contrast, it has been shown previously that the absence of Sod1p leads to pleiotropic defects in liquid yeast cultures (e.g. increased sensitivity to oxygen and various stresses, slow growth, and rapid cell dying) (7–9) (supplemental Fig. S1A) and to high rate appearance of suppressor mutants resistant to oxidants and exhibiting reversal of methionine auxotrophy (10, 11). To determine whether the wt-like behavior of GMA-grown sod1Δ colonies could be caused by the high accumulation rate of stress-resistant suppressor mutants, we tested the sensitivity of cells harvested from sod1Δ colonies to the superoxide-producing agent Paraquat (an extracellular stressor). We also monitored whether these cells still exhibit Met auxotrophy (a result of intracellular oxygen stress (10)) under aerobic conditions. As shown in Fig. 3A, even sod1Δ cells from 30-day-old GMA-grown colonies (GMA-sod1Δ) still exhibited high sensitivity to Paraquat and Met auxotrophy at normoxic O2. They were not hypersensitive to extracellular Mn2+. In contrast, a 10-day-old sod1Δ colony grown on YPDA glucose agar (YPDA-sod1Δ) contained ~1% Paraquat-resistant cells (PQr) and 5% methionine prototrophic (Met+) cells (0.1% of Met+ cells was observed even in a fresh 2-day-old YPDA-sod1Δ colony). These data demonstrate that sod1Δ cells from aged GMA-grown colonies exhibited neither of the characteristics typical of either of the two previously described groups of suppressor mutations (bsd and seo) (10, 11) nor any other suppressor mutation that would increase their resistance to either extracellular or intracellular oxidative stress.

Physiology of sod1Δ Cells from GMA-grown Colonies Differs from That of the Original sod1Δ Strain—The data described above excluded the accumulation of oxidative stress-resistant mutants in GMA-grown sod1Δ colonies. An interesting possibility remained, however, that cells within these colonies either transiently adapt or acquire mutation(s), resulting in properties optimal for their existence within this multicellular structure.
To investigate this possibility, we compared sod1Δ cells forming colonies capable of ammonia signaling with sod1Δ cells forming colonies under conditions that do not lead to ammonia signaling. In these experiments, we harvested cells either from sod1Δ colonies grown on solid GMA as described above (GMA-sod1Δ cells, i.e. those cells undergoing ammonia-guided development) or from sod1Δ colonies grown on solid glucose-based YPDA (YPDA-sod1Δ; i.e. those cells incapable of development leading to ammonia signaling) of the same ages. We subsequently inoculated these cells into various liquid media, and we monitored their growth rates, the pH levels of the cultivation media, and cell survival (i.e. number of colony-forming cells). In parallel, we analyzed the appearance of PQ' and Met⁺ suppressants in these liquid cultures.

After 24-h cultivation in the particular liquid medium (SD, YPD, or GM), the cultures inoculated with either GMA- or YPDA-sod1Δ reached similar densities (data not shown). However, prolonged cultivation revealed different viability for GMA-sod1Δ cultures as compared with the viability of YPDA-sod1Δ cultures. The survival of GMA-sod1Δ cells was only 1–10% after 4 days in liquid SD when compared with YPDA-sod1Δ cells (Fig. 4A and supplemental Fig. S6). In contrast to SD, GMA-sod1Δ and YPDA-sod1Δ cells exhibited similar viability in complex glucose YPD. The glycerol GM even favored GMA-sod1Δ over YPDA-sod1Δ (Fig. 4A and supplemental Fig. S6). Furthermore, in YPD, cells taken from GMA- and YPDA-grown colonies differed in their ability to change the pH of the cultivation medium. YPDA-sod1Δ-derived liquid cultures (similarly to original sod1Δ) acidified the medium from pH 5.5 to 4.2, whereas cultures inoculated with GMA-sod1Δ increased pH up to 6.2 (Fig. 4B). Hence, we tested whether higher pH improves poor survival of GMA-sod1Δ cells in liquid SD. Indeed, stabilizing pH of SD at about 5 increased GMA-sod1Δ-derived culture viability by 1–2 orders of magnitude (Fig. 4C).

Another striking difference between GMA-sod1Δ and YPDA-sod1Δ is connected to their abilities to form Met⁺ and PQ' suppressants. Although both SD and YPD cultures inoculated with YPDA-sod1Δ accumulated relatively high numbers of Met⁺ cells, the occurrence of these cells in GMA-sod1Δ-derived cultures was significantly diminished (Fig. 3B). When growing in YPD, GMA-sod1Δ-derived cultures (from 20–25-day-old colonies) produced less than 1% of the Met⁺ suppressants of those found in YPDA-sod1Δ cultures. No Met⁺ suppressants were detected in GMA-sod1Δ cultures growing in SD. Similarly, in contrast to YPDA-sod1Δ cultures accumulating significant levels of PQ' suppressants, no PQ' cells were detected in GMA-sod1Δ cultures growing in either YPD or SD media (Fig. 3B).

Because the GMA and YPD differ in their carbon source (glycerol and glucose, respectively), we tested whether the novel behavior of GMA-sod1Δ cells was a mere consequence of using glycerol medium. To explore this possibility, we compared the properties of sod1Δ cells from colonies grown on GMA (GMA-sod1Δ) with those of sod1Δ cells grown in liquid glycerol GM. We inoculated both cell types into liquid SD and determined survivors and Met⁺ suppressants after 4 days of cultivation. As shown in Fig. 3C, sod1Δ pregrown in liquid GM more closely resembled YPDA-sod1Δ cells and significantly differed from GMA-sod1Δ cells pregrown on GMA. sod1Δ cultures pregrown 5 days in liquid GM accumulated 100 to 1,000 times more Met⁺ suppressants during subsequent cultivation in SD than GMA-sod1Δ culture derived from 20-day-old colonies. In addition, in contrast to GMA-sod1Δ cultures, liquid GM-pregrown sod1Δ cultures were able to accumulate PQ' suppressants and did not exhibit a decreased survival rate after prolonged cultivation (even after 9 days) in SD (data not shown). These experiments show that new GMA-sod1Δ traits are not merely induced by either a carbon source or a solid medium but are rather specific for GMA-grown colonies.

An Epigenetic Change or a Mutation?—In independent experiments, GMA-sod1Δ cells always developed the specific physiological properties described above, setting themselves apart from all other sod1Δ cultures. As a rule, GMA-sod1Δ-specific traits were gradually intensified as GMA-sod1Δ colonies aged. Moreover, at least some of the properties were
preserved even after the cells were removed from the colony context and cultivated in liquid media (e.g. the inability to form oxidative stress-resistant suppressants was maintained over 7 days of cultivation in different liquid media). This suggested that the physiological change in GMA-sod1Δ cells is relatively stable, being thus either a mutation or a stable epigenetic change. To determine how the GMA-sod1Δ specific traits are acquired, we characterized the phenotypes of individual cell clones obtained from either central or margin regions during colony development. We monitored cell viability in SD as the main indicator of changed GMA-sod1Δ physiology. The procedure was as follows (Fig. 5). We harvested cells from the central or margin regions of GMA-sod1Δ colonies, plated them on YPDA, and picked up individual clones. We then inoculated the clones into SD and analyzed cell viability after 7 days of cultivation. As shown in Fig. 5A, the average survival of individual cell clones decreased as the colony aged, being only slightly higher for clones obtained from the central colony region than for those from the colony margin. When assessing survival of individual clones in SD, however, it became evident that the population within the colony exists in many intermediate stages between original sod1Δ cells and fully changed GMA-sod1Δ cells (Fig. 5B and supplemental Fig. S7). Thus, in young sod1Δ GMA-grown colonies, most of the cells resembled original sod1Δ, but there were also some cells exhibiting partially decreased viability when growing in SD (the difference in survival was 1–2 orders of magnitude). The number of these cells increased during colony aging, and new cells with an even lower viability in SD (the difference in survival being 3–5 orders of magnitude) started to appear. Old GMA-sod1Δ colonies were mostly comprised of cells with very low viabilities in SD, but they also contained cells with almost the original viability in SD.

The profile of this gradual change was similar in central (Fig. 5B) and outer regions (supplemental Fig. S7) of the GMA-sod1Δ colony.

**DISCUSSION**

Regarding liquid yeast cultures, it is taken as a rule that the life span is proportional to the oxidant scavenging capability of yeast cells. Accordingly, all three stress defense mutants sod1Δ, sod2Δ, and ctt1Δ exhibit a reduced life span when growing in liquid YPD or ethanol cultures (4, 24). Our data imply, however, that in colonies passing through ammonia-guided development, the fate and survival of the population is governed more by its ability to undergo proper development than by its capacity to defend against oxidative stress (Fig. 6). sod1Δ, sod2Δ, and ctt1Δ strains should be expected to contain more ROS (O2·− and/or H2O2) than the wt, particularly when growing on a respiratory medium. In fact, however, the cells of sod2Δ and ctt1Δ colonies growing on glycerol GMA agar do not markedly increase their cellular or mitochondrial superoxide levels as compared with wt colonies. Moreover, the absence of any Sod1p, Sod2p, or Ctt1p enzymes in colonies is not compensated by an increased level of either glutathione peroxidase or peroxiredoxin activity.

The absence of Ctt1p, Sod1p, or Sod2p has diverse impacts on the development and survival of yeast colonies. A defect in mitochondrial Sod2p (and to some extent also of cytosolic Ctt1p) significantly impairs ammonia signaling, as well as induction of adaptive metabolism and cell differentiation in aged colonies. The latter is documented by the finding that sod2Δ (and partially also ctt1Δ) colonies fail to localize dying cells and cells harboring stress-related features specifically to the colony center. On the contrary, these cells are spread throughout the whole colony. sod2Δ colonies defective in a single metabolic enzyme thus resemble to some extent colonies formed by the strain deficient in the pleiotropic transcription regulator Sok2p (16, 17) (Fig. 6). In addition to all of the defects mentioned above for sod2Δ colonies, sok2Δ colonies exhibit misbalanced levels of different stress-related factors (e.g. activities of superoxide dismutase and catalase) (17). Hence, one can speculate that an imbalance in ROS homeostasis (e.g. in mitochondria) of cells in acidic phase colonies may disrupt the initiation and/or continuation of physiological changes leading to ammonia signaling. Moreover, a stress defense enzyme itself could also play a regulatory role, because it was recently proposed that Sod2p regulates the proteins involved in chromatin remodeling and plays a role in sensing redox imbalance (25). Alternatively, superoxide radicals arising in sod2Δ mitochondria could cause oxidative damage of mitochondrial compo-
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FIGURE 6. Model of involvement of stress defense and adaptive metabolism in development and survival of different yeast populations. The cultivation of all population types starts with “chronologically young” cells. Stress defense is essential in liquid cultures to scavenge ROS. Its absence can be overcome by accumulating stress-resistant suppressants. In colonies, independently of stress defense enzymes, cells capable of ammonia signaling undergo a development leading to their differentiation, activation of adaptive metabolism (AM) and long term survival of healthy cells located at margin colony regions. This is the case of wt and sod1 Δ colonies. Lack of ammonia signaling causes developmental problems, i.e. no differentiation and reduced fitness of the whole colony population both in the presence and absence of stress defense mechanisms. This occurs in colonies of stress defense mutants sod2 Δ and ctt1 Δ as well as in colonies of the sok2 Δ mutant defective in pleiotropic regulator Sok2p (17).

In contrast to a deficiency in mitochondrial Sod2p, the absence of cytosolic Sod1p does not cause the GMA-grown colonies any significant developmental or survival problem despite the fact that this enzyme appears to be the most essential of the three for yeast cells growing in liquid cultures. Despite growing on a complex glycerol respiratory medium, the sod1 Δ population within the colony does not suffer, and the outer cells are fully viable. sod1 Δ colonies produce an amount of ammonia comparable with that of the wt colonies, induce the adaptive metabolic changes normally, and exhibit clear center margin differentiation. In contrast to sod1 Δ liquid cultures accumulating stress-resistant suppressant mutants, GMA-grown sod1 Δ cells retain their sensitivity to oxidative stress and heat shock. Nevertheless, GMA-grown sod1 Δ cells acquire new traits, which are preserved even when they are transferred to new liquid growth conditions. These include an altered physiology and survival in some liquid media and preserved loss of ability to form stress-resistant suppressants. Neither sod1 Δ cultures growing in glycerol GM or glucose YPD liquid media nor sod1 Δ colonies growing on glucose solid YPDA acquire similar properties. All of these sod1 Δ populations resemble original sod1 Δ cells. These data imply that the fitness of GMA-sod1 Δ cells results from their life history within the GMA-grown colony undergoing ammonia-guided development.

In contrast to developmental differences between colonies formed by individual stress defense mutants, which appear to be connected to the capability of a colony for ammonia signaling, the development of new traits of GMA-grown sod1 Δ cells start already in young first acidic phase colonies. These traits reproducibly intensify with colony age in both chronologically aged central and in newly grown outer cells, suggesting that the changes are not necessarily connected with cell division. The analysis of individual clones obtained from GMA-sod1 Δ colonies of various ages implies that colonial sod1 Δ cells occur in several intermediate stages before they reach what may be termed the “colony-adapted” form. This indicates that a single mutation is unlikely to be responsible for the change and suggests that the change could be related to a successive epigenetic switch and gradual cell adaptation. Such a switch in its final stage, however, must be quite stable, because it persists at least 15 generations after cell removal from the colony context (data not shown). Proteins involved in chromatin remodeling may participate in such epigenetic change (27). Some of the chromatin-remodeling genes and genes located in subtelomeric regions were found to change expression during colony development (15, 28). Of course, one cannot exclude the alternative possibility that more than one mutation accumulates in GMA-sod1 Δ cells, but these mutations should always result in the same colony-adapted phenotype. Because sod1 Δ is known to have a high rate of spontaneous mutations (8, 29), this possibility could also be feasible.

Taken together, our new findings indicate that the differentiation of the colony cell population and activation of the proper
metabolism, which allow intracellular stress to decrease naturally, is more important for the long term survival of the population than the presence and function of mechanisms dealing with an already increased stress. Moreover, the finding that mitochondrial Sod2p (and possibly Ctt1p) may be involved in the regulatory processes leading to ammonia signaling makes colonial population an excellent model for studies of the regulatory and additional metabolic functions of distinct stress defense enzymes and different kinds of ROS in particular. The absence of oxidative stress-resistant suppressants in GMA-grown sod1Δ colonies and the appearance of either mutated or switched clones show that yeast populations in colonies passing through specific developmental phases and in liquid shaken cultures are exposed to different selection pressures and need to acquire different capabilities (Fig. 6).

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