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Defects Arising From Whole-Genome Duplications in *Saccharomyces cerevisiae*

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ABSTRACT

Comparisons among closely related species have led to the proposal that the duplications found in many extant genomes are the remnants of an ancient polyploidization event, rather than a result of successive duplications of individual chromosomal segments. If this interpretation is correct, it would support Ohno's proposal that polyploidization drives evolution by generating the genetic material necessary for the creation of new genes. Paradoxically, analysis of contemporary polyploids suggests that increased ploidy is an inherently unstable state. To shed light on this apparent contradiction and to determine the effects of nascent duplications of the entire genome, we generated isogenic polyploid strains of the budding yeast *Saccharomyces cerevisiae*. Our data show that an increase in ploidy results in a marked decrease in a cell's ability to survive during stationary phase in growth medium. Tetraploid cells die rapidly, whereas isogenic haploids remain viable for weeks. Unlike haploid cells, which arrest growth as unbudded cells, tetraploid cells continue to bud and form mitotic spindles in stationary phase. The stationary-phase death of tetraploids can be prevented by mutations or conditions that result in growth arrest. These data show that whole-genome duplications are accompanied by defects that affect viability and subsequent survival of the new organism.

THE generation of complete genome sequences has revealed that many organisms contain a large number of duplicated genomic regions. This is particularly evident in *Saccharomyces cerevisiae* where duplications compose 50% of the genome. The genes within these paralogous segments are often found to be in the same order and orientation with respect to the centromere. These features led Wolfe and others to propose that the duplications found in *S. cerevisiae* are the remnants of an ancient whole-genome duplication rather than the result of many sporadic partial duplications of smaller genomic regions (Wolfe and Shields 1997; Gibson and Spring 2000). Recent sequencing of the genome of *Kluyveromyces waltii* showed that it is related to the genome of *S. cerevisiae* by a 1:2 mapping in the manner expected for the postulated whole-genome duplication (Kellis *et al.* 2004). The lack of complete correspondence between duplicated regions is a consequence of sequence divergence that has occurred by mutations or deletions over time. The evolution of genomes by polyploidization rather than by small duplications supports Ohno's proposal that whole-genome duplications spawn the evolution of new genes (Ohno *et al.* 1968).

Although whole-genome duplications would seem to be an efficient mechanism for evolving new genes, the sudden doubling of every gene may present physiological difficulties for the nascent polyploid organism. Studies of polyploids made in the laboratory indicate that organisms with whole-genome duplications have problems in genomic stability, gene regulation, and development (Mayer and Aguilera 1990; Guo *et al.* 1996; Matzke *et al.* 1999; Lin *et al.* 2001; Pikaard 2001; Storchova and Pellman 2004). Polyploidization can induce a flurry of genetic and epigenetic events that include DNA sequence elimination and gene silencing (Song *et al.* 1995; Mittelsten Scheid *et al.* 1996; Feldman *et al.* 1997; Ozkan *et al.* 2001; Shaked *et al.* 2001). Genome-wide transcription analysis in yeast demonstrates that there are genes whose expression is repressed by increases in ploidy (Galitski *et al.* 1999).

The budding yeast *S. cerevisiae* provides the opportunity to study the consequences of polyploid formation under laboratory conditions and without the extensive selection that must inevitably take place during evolutionary time. Although *S. cerevisiae* is normally haploid or diploid, its ploidy can be manipulated at will because cells with a ploidy content $>2n$ are viable and can mate with other cells to create organisms of yet higher ploidy. Haploid yeast cells are either *MAT***a** or *MAT*α and mate with cells of the opposite mating type to form diploids that are *MAT***a**/*MAT*α. Although these *MAT***a**/*MAT*α dip-

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TABLE 1

***S. cerevisiae* strains used in this study**

Strain	Relevant genotype
AAy16	*MAT*α[a] *cln3::LEU2*
AAy19	*MAT*α/α/α/α[a] *cln3::LEU2/cln3::LEU2/cln3::LEU2/cln3::LEU2*
AAy111	*MAT*α[b]
AAy112	*MAT*α/α[b]
AAy113	*MAT*α/α/α[b]
AAy114	*MAT*α/α/α/α[b]
AAy115	*MAT***a**/α/α/α[b]
AAy307	*MAT*α/α *leu2::hisG/leu2::hisG his3::hisG/his3::hisG ura3-52/URA3 his1::LEU2/HIS1*
AAy327	*MAT*α/α/α/α *leu2::hisG/leu2::hisG/leu2::hisG/leu2::hisG his3::hisG/his3::hisG/his3::hisG/his3::hisG ura3-52/ura3-52/ura3-52/URA3 his1::LEU2/HIS1/HIS1/HIS1*
AAy414	*MAT*α *URA3 HIS3*
AAy417	*MAT*α/α/α/α *ura-52/ura3-52/ura3-52/URA3 his3::hisG/his3::hisG/his3::hisG/HIS3*

[a] Σ1278b background: *ura3-52 leu2::hisG his3::hisG.*
[b] Σ1278b background: *ura3-52 leu2::hisG::LEU2 his3::hisG.*

loids do not mate, they can easily be converted to mating-type homozygotes (*MAT***a**/*MAT***a** or *MAT*α/*MAT*α) that are fully capable of mating with haploids to produce triploids or with diploids to produce tetraploids. The ease with which cells of higher ploidy can be generated raises the question: "What limits the ploidy of cells?"

In this report, we take advantage of the facility with which one can create isogenic polyploid yeast strains to determine the consequences of nascent whole-genome duplications. Polyploids show chromosome instability during exponential growth but do not manifest any other obvious growth defects. However, in stationary phase, recently constructed tetraploids show a striking loss of viability. Whereas haploids survive for months, newly formed tetraploids die rapidly. This death is not due to the increased cell size of polyploids, but rather to their failure to arrest growth during stationary phase. The inviability of nascent tetraploid cells in stationary phase suggests that whole-genome duplications may require additional genetic and/or epigenetic changes to permit their survival.

MATERIALS AND METHODS

Strains, plasmids, and media: Strains used in this study are listed in Table 1. All strains were derived from *S. cerevisiae* strain Σ1278b (also known as MB1000; Grenson *et al.* 1966; Brandriss and Magasanik 1979). Isogenic ploidy series were constructed as previously described (Galitski *et al.* 1999). Cells were cultured in synthetic complete (SC) media or YPD prepared using conventional methods (Guthrie and Fink 1991). Yeast transformation was accomplished by the lithium acetate method (Gietz *et al.* 1992).

The original strain from which the polyploid series was constructed was a haploid *MAT*α *ura3-52 his3::hisG leu2::hisG* strain (Galitski *et al.* 1999). To make many of our comparisons, we transformed the ploidy series to Leu$^+$. Therefore, strains AAy111–AAy115 are all Leu$^+$ (*e.g.*, AAy114 is *MAT*α/α/α/α *ura3-52/ura3-52/ura3-52/ura3-52 his3::hisG/his3::hisG/his3::hisG/his3::hisG leu2::hisG/leu2::hisG/leu2::hisG/leu2::hisG::LEU2*). The *cln3::LEU2* disruption was generated using plasmid pBSSK-*cln3::LEU2* (kindly provided by A. Amon). This plasmid was digested with *Pvu*II and gel purified prior to transformation. Positive transformants were verified by PCR and by comparison of cell size relative to wild-type cells.

Calcofluor white (Fluorescent Brightener 28), menadione, paraquat (methyl viologen), hydrogen peroxide, sorbitol, NaCl, and LiCl were obtained from Sigma Chemical. All were added at the designated concentration to cooled (<60°), premixed SC agar (2%). Plates were dried at room temperature and used for spot assays within 48 hr.

Viability measurement: Cells were inoculated at low density (optical density at $600_{nm} < 0.05$) into the appropriate media and continuously grown at 30°. At the indicated intervals, 100 μl of culture was sampled and serially diluted. Five microliters of each dilution was spotted sequentially onto YPD plates and grown overnight at 30° prior to visualization. The colony-forming units per milliliter of culture (CFU per milliliter) were determined by counting a sufficient number of colonies (50–200) from two duplicate spots and multiplying the average by the appropriate dilution factor. For each time point, the mean values from at least three experiments are plotted. Error bars in all figures represent standard deviations.

Heat-shock sensitivity: Yeast cultures were grown overnight at 30° in SC. Saturated cultures were then diluted to optical density (OD) $600_{nm} = 0.1$ into SC prewarmed to 30° and grown into log (OD $600_{nm} = 0.8$–1.0) or stationary phase (2.5–4 days of incubation). Samples were collected and serially diluted in water in a thin-walled 96-well PCR plate. Diluted samples were subjected to 50° heat shock in a 96-well PCR block for 2 hr and mixed by pipette in 10-min intervals. Samples were then spotted onto YPD plates as described above to assess survival. The percentage viable is (the number viable after heat shock/number viable before heat shock) × 100.

Zymolase sensitivity: Cultures were grown as in heat-shock assay and samples were collected. A 1-OD 600_{nm} unit of cells was collected. Cells were pelleted, washed in water, and resuspended in 1 ml of 0.15 mg/ml zymolase 100T (Seikagaku) in 10 mm Tris-HCl, pH 7.5, 1 mm dithiothreitol. Samples were agitated on a roller at 30°. After 2 hr, the OD 600_{nm} was measured to assess the amount of cell lysis.

Microarray analysis: For the diauxic-shift time course, cells were grown overnight in SC and diluted the next morning to an optical density of $600_{nm} < 0.05$. Cells were then incubated at 30° and the growth measured by OD 600_{nm}. The glucose content of the media was measured using Diastix reagent strips (Bayer). Cells were collected during mid-logarithmic phase at an optical density ~1.0. This sample was designated as $t = 0$. All other time points reflect the number of hours after glucose depletion. RNA was extracted using hot acidic phenol and mRNA was isolated using the PolyA tract kit (Promega, Madison, WI). A total of 2 μg of mRNA was labeled for each sample as previously described (Wodicka *et al.* 1997), hybridized to the Y36100 oligonucleotide array set (Affymetrix), and data were extracted. Scan-to-scan variations in intensity were corrected by applying a scaling factor to each experiment so that the median value of all experiments was equal. To evaluate the relative change in expression in each strain throughout the time course, a ratio was created by dividing the expression value in all time points by the $t = 0$ value on a gene-by-gene basis. Cluster analysis was performed using Cluster and visualized with TreeView (Eisen *et al.* 1998). Genes were considered differentially regulated if their expression

fluctuated at least twofold during the time course. The statistical significance of the overlap between regulated genes in different experiments was calculated using a hypergeometric distribution. *P*-values were calculated using the online hypergeometric distribution calculator at http://www.alewand.de/stattab/tabdiske.htm. Raw data and supplementary materials are available at http://jura.wi.mit.edu/fink_public/duplication.

DAPI staining: Cultures were grown as in heat shock assay and samples collected at the indicated intervals. Cells were collected by low speed centrifugation, washed in 1× phosphate buffered saline (PBS), and fixed in 80% ethanol for 10 min. Cells were collected again, washed two times in 1× PBS, and resuspended in 1× PBS containing a final concentration of 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma Chemical). Samples were incubated at room temperature in the dark for 30 min prior to visualization.

Chromosome loss: Genotypes of strains used in these experiments are shown in Table 3A. Strains were grown in liquid SC to log phase (12 hr) or stationary phase (40 hr). At each stage, 100 μl of culture was sampled and serially diluted. Ten microliters of each dilution was spotted sequentially onto YPD and SC + 5-fluoroorotic acid (5-FOA) plates. Cells that were able to grow on SC + 5-FOA were patched onto SC −leucine plates. The number of cells that were able to grow on SC + 5-FOA but unable to grow on SC −Leu was divided by the number of CFU/ml formed on YPD. This provided an estimate of the number of cells that had lost a single chromosome V as compared to the total number of viable cells.

Starvation in water: Cultures were grown in SC at 30°. At the indicated intervals, cells were collected from 10 ml of culture by centrifugation. Spent SC was removed into a sterile test tube. Cells were washed with 10 ml of room temperature water and resuspended in their spent SC or in 10 ml of sterile water. All strains were then incubated at 30° and their viability was monitored as described.

Indirect immunofluorescence: All strains were grown in liquid SC media at 30° for 7 days. Cells were fixed with formaldehyde (3.7%) for 90 min. Fixed cells were washed three times with solution B (1.2 M sorbitol, 100 mM KH_2PO_4 × K_2HPO_4 at pH 7.5) and resuspended in 250 μl of solution B with 0.4 mg/ml lyticase, 2 μl/ml β-mercaptoethanol, and 3 μl/ml phenylmethylsulfonyl fluoride (PMSF). Cells were then incubated at 30° and monitored for spheroplast formation under the microscope. Spheroplasts were gently harvested and washed with solution B supplemented with PMSF. Variable dilutions of spheroplasts were placed on slides covered with 2% polyethylenimine and placed in a humid chamber for 20 min. Cells were then washed twice with solution B and fixed with methanol for 5 min. Dried slides were washed twice with solution B and blocked overnight at 4° using 4% nonfat milk in solution B. The primary antibody YOL1/34 (Jackson Laboratories) was resuspended in milk solution at 1:10 and applied to slides. After 2 hr at room temperature in a humid chamber, the unbound antibody was aspirated and slides were washed 10 times with solution B. FITC-conjugated anti-rat secondary antibody (1:50) was then applied to the samples and incubated for 2 hr at room temperature in the dark. Slides were washed 10 times with solution B and incubated with DAPI (0.5 μg/ml) in solution B supplemented with PMSF (3 μl/ml) for 15 min in the dark. Slides were washed once in solution B and overlaid by mounting solution (90% glycerol, 1× PBS, 5 mg/ml phenylenediamine) and covered with coverslips. Cells were viewed using a Nikon ECLIPSE E600 fluorescence microscope (Nikon). Spindle formation was analyzed in cells that stained positively for DAPI to normalize for cell viability. Statistical significance was calculated using a heteroscedastic *t*-test, which is a two-sample Student's *t*-test that compares the means of two ranges of data with unequal variances.

RESULTS

Polyploids die in stationary phase: An isogenic set of strains varying in ploidy from haploid to tetraploid was compared for viability in liquid media by monitoring their ability to form colonies when plated. Cells of higher ploidy are viable during exponential growth, but are unable to survive prolonged incubation after cell multiplication has ceased (as measured by direct cell count, plating, and turbidity). Haploids enter stationary phase and remain viable for weeks, whereas tetraploids begin to lose viability after 4 days and are completely inviable after 10–15 days (Figure 1, A and B). These cells not only fail to form colonies but also fail to mate. The loss of viability associated with polyploidy is not strain or mating type specific, as it is observed in isogenic ploidy series of both mating types (*MAT***a** → *MAT***a**/**a**/**a**/**a** and *MAT*α → *MAT*α/α/α/α) as well as strains derived from both the Σ1278b and S288c backgrounds (not shown). However, *MAT***a**/**a**/α/α tetraploids survive somewhat better than *MAT*α/α/α/α tetraploids (Figure 2A). This increase in viability appears to be a consequence of heterozygosity at the mating-type locus because *MAT*α/α/α/α tetraploids carrying the *MAT***a** locus on a plasmid are more viable than *MAT*α/α/α/α tetraploids carrying the vector alone (Figure 2B). Throughout this study, haploids were compared with tetraploids; however, triploids show defects similar to those observed in tetraploids.

The inability of polyploid cells to survive during stationary phase is not specific to SC. When grown in YPD medium instead of SC, tetraploids still die more rapidly; however, both haploids and tetraploids remain viable for a longer period of time on YPD (Figure 3A). The nutritional requirements that result from the auxotrophic markers used for strain construction (uracil and histidine) are not responsible for the differences in viability between haploids and tetraploids because replacement of the mutant alleles of these markers with functional alleles by integrative transformation does not restore viability to the tetraploids (Figure 3B). Moreover, supplementing stationary-phase cultures with uracil and histidine does not alter the difference in viability between haploids and tetraploids (Figure 3C).

Polyploid cells do not exhibit increased sensitivity to stress: Tetraploids do not manifest any obvious defects in morphology or development. They mate with cells of the opposite mating type and the resulting zygotes undergo meiosis to produce ascospores and viable meiotic products. Moreover, tetraploids are no more sensitive to a variety of environmental stresses than are haploids (Table 2). Tetraploid cells do not exhibit an increased sensitivity to osmotic stress (sorbitol), salt stress (Na^+), toxic ions (Li^+), or calcofluor white, but they are more thermosensitive. Tetraploids are also no more sensitive or resistant to oxidative stress (paraquat, menadione, and H_2O_2) than are isogenic haploids. This finding distin-

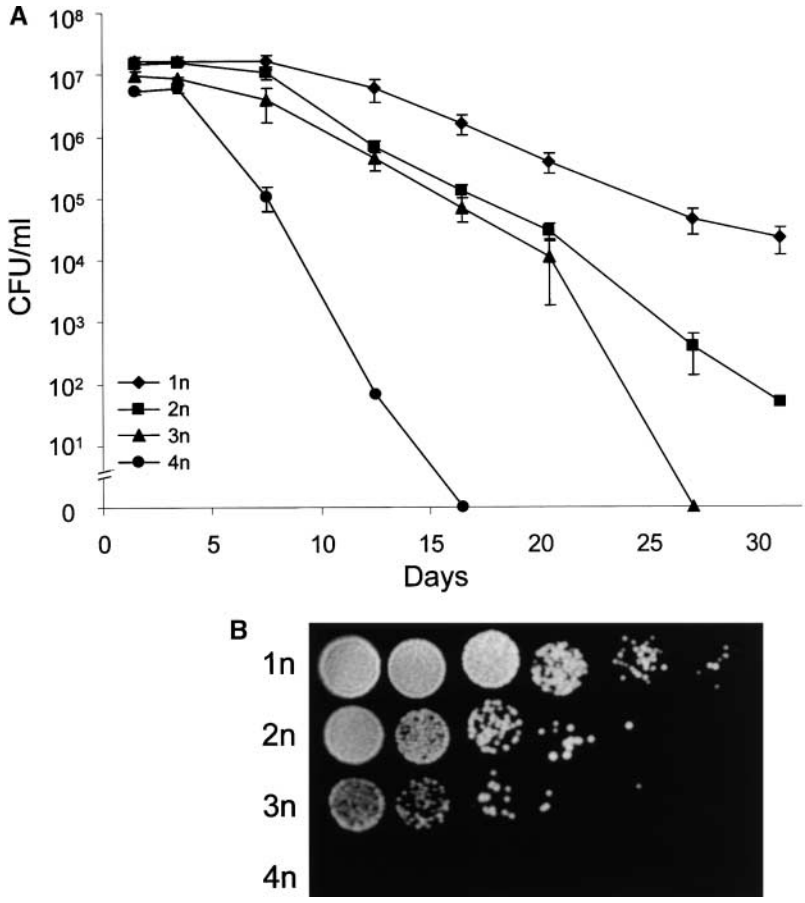

FIGURE 1.—Survival during stationary phase decreases with an increase in ploidy. (A) Strains of increasing ploidy were grown into stationary phase and monitored for viability. CFU/ml was measured for 1*n MAT*α (◆), 2*n MAT*α/α (■), 3*n MAT*α/α/α (▲), and 4*n MAT*α/α/α/α (●). CFU, colony forming units. (B) Strains of increasing ploidy (1*n*–4*n*) were grown for 16.5 days, serially diluted, and spotted onto YPD. The spots from left to right represent fivefold dilutions of each culture.

guishes the stationary-phase defect in polyploids from previous studies that have identified oxidative stress as a component of stationary-phase survival (FABRIZIO *et al.* 2001).

Increased chromosome loss in polyploids: Diploids and tetraploids with a genetically marked chromosome V (Table 3A) were constructed and used to monitor the loss of this chromosome during growth. Increased chromosome instability has been reported in strains of high ploidy (MAYER and AGUILERA 1990). These findings were confirmed in our isogenic strains. Cells in exponential growth display a 357-fold increase in the frequency of chromosome loss as compared to isogenic diploid cells (Table 3B). This high proportion of cells that have lost chromosome V is maintained even after the cells have entered stationary phase. However, tetraploid cells show no increase in the frequency of mitotic recombination ($\sim 10^{-5}$ Ura^- Leu^+ segregants) in either exponential or stationary phase.

Tetraploids acquire transcriptional and physiological signatures of stationary phase: To determine whether tetraploids recognize the environmental conditions that trigger stationary-phase entry, we used whole-genome arrays to analyze the transcription profile of tetraploids during the growth period in which glucose is exhausted. As yeast cells exhaust the glucose in their medium, they undergo the diauxic shift, a switch from fermentation to respiration, and transition to stationary phase. Previous studies have identified a pattern of changes in gene expression that define a transcriptional signature of the diauxic shift (DERISI *et al.* 1997). To analyze our strains, samples were taken for microarray analysis as the glucose concentration decreased. The analysis of the transcription profiles in tetraploid cells demonstrates that they manifest the key features associated with the diauxic shift. Tetraploid cells regulate 51 of 77 genes ($P = 6.6 \times 10^{-25}$) in a manner similar to that described by DeRisi and colleagues in their study of the diauxic shift (Figure 4A; DERISI *et al.* 1997). Furthermore, these genes are those expected to be elevated during the transition to respiration. These similarities are especially noteworthy considering that different media, strains, and microarray platforms were used in the two studies.

Tetraploid cells also acquire the transcriptional and physiological attributes that are signatures of stationary phase. In yeast, *SNZ1* is specifically upregulated during the postdiauxic and stationary phases of growth and can be used as a transcriptional marker for the entry to stationary phase (PADILLA *et al.* 1998). Our microarray analysis demonstrates that haploids and tetraploids both upregulate *SNZ1* >3.5-fold in the period following the diauxic shift (Figure 4B). Stationary-phase cells have also been shown to acquire thermotolerance (WERNER-WASHBURNE *et al.* 1993) and resistance to cell-wall-degrading enzymes such as zymolase (DE NOBEL *et al.* 1990;

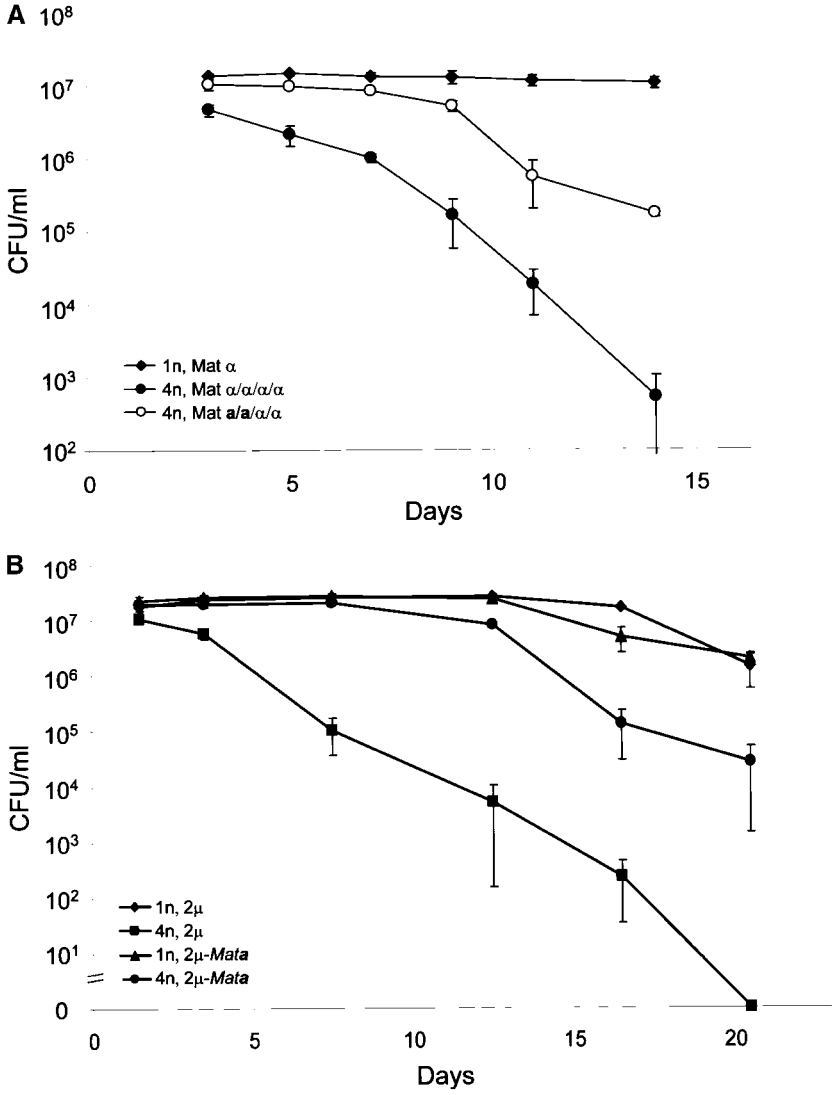

FIGURE 2.—Mating-type heterozygosity in tetraploids suppresses death during stationary phase. (A) Mating-type homozygotes and heterozygotes are compared for their ability to remain viable in stationary phase. Strains are labeled as follows: 1*n MAT*α (◆), 4*n MAT*α/α/α/α (●), and 4*n MAT***a**/**a**/α/α (○). (B) Haploids and tetraploids carrying a 2μ or 2μ-*MAT***a** plasmid were grown into stationary phase and their viability monitored (CFU/ml). Strains are labeled as follows: 1*n MAT*α, 2μ (◆); 4*n MAT*α/α/α/α, 2μ (■); 1*n MAT*α, 2μ-*MAT***a** (▲); and 4*n MAT*α/α/α/α, 2μ-*MAT***a** (●).

SOBERING *et al.* 2002). Tetraploid cells acquire zymolase resistance, but are more thermosensitive than isogenic haploids (Table 2). The failure to acquire thermotolerance is not a manifestation of a broader sensitivity to stress because tetraploids are not more sensitive to osmotic stress, salt stress, or toxic cations.

Tetraploids show no obvious deficit in the transcription of genes known to be required for exponential or stationary-phase growth. For example, the *ubi4*, *rvs161*, *ard1*, and *bck1* mutants are hypersensitive to starvation (WHITEWAY and SZOSTAK 1985; FINLEY *et al.* 1987; CROUZET *et al.* 1991; COSTIGAN and SNYDER 1994), but tetraploid cells are not defective in the expression of these genes (Figure 4B). Moreover, many of these mutations confer additional phenotypes (*e.g.*, *ubi4*) that are not displayed by tetraploid cells.

A comparison of haploids and isogenic tetraploids shows that they have very similar transcription profiles throughout the transition to stationary phase. Of the 224 genes upregulated greater than twofold in haploids, tetraploids similarly upregulate 148 of them ($P = 6.9 \times 10^{-228}$). In addition, of 272 genes downregulated greater than twofold in haploids, tetraploids similarly downregulate 194 of the same genes ($P = 7.6 \times 10^{-251}$; Figure 4C). Despite these similarities, the transcriptional changes in these two strains are not identical. Tetraploids fail to induce 76 genes that are upregulated greater than twofold in haploids and they do not downregulate 78 genes that are repressed greater than twofold in haploids. Thus, we cannot eliminate the possibility that one or more of the genes whose regulation differs in tetraploids is responsible for their failure to survive in stationary phase.

Tetraploids fail to arrest the cell cycle in stationary phase: Previous studies have shown that nutrient deprivation prevents the initiation of a new cell division cycle (PRINGLE and HARTWELL 1981). In an asynchronous culture, cells that have not passed Start arrest in G_1 as unbudded cells, whereas cells that have already committed to a new cell cycle continue through one cell division before arresting in G_1. The resulting stationary-phase culture is composed of a homogeneous population of

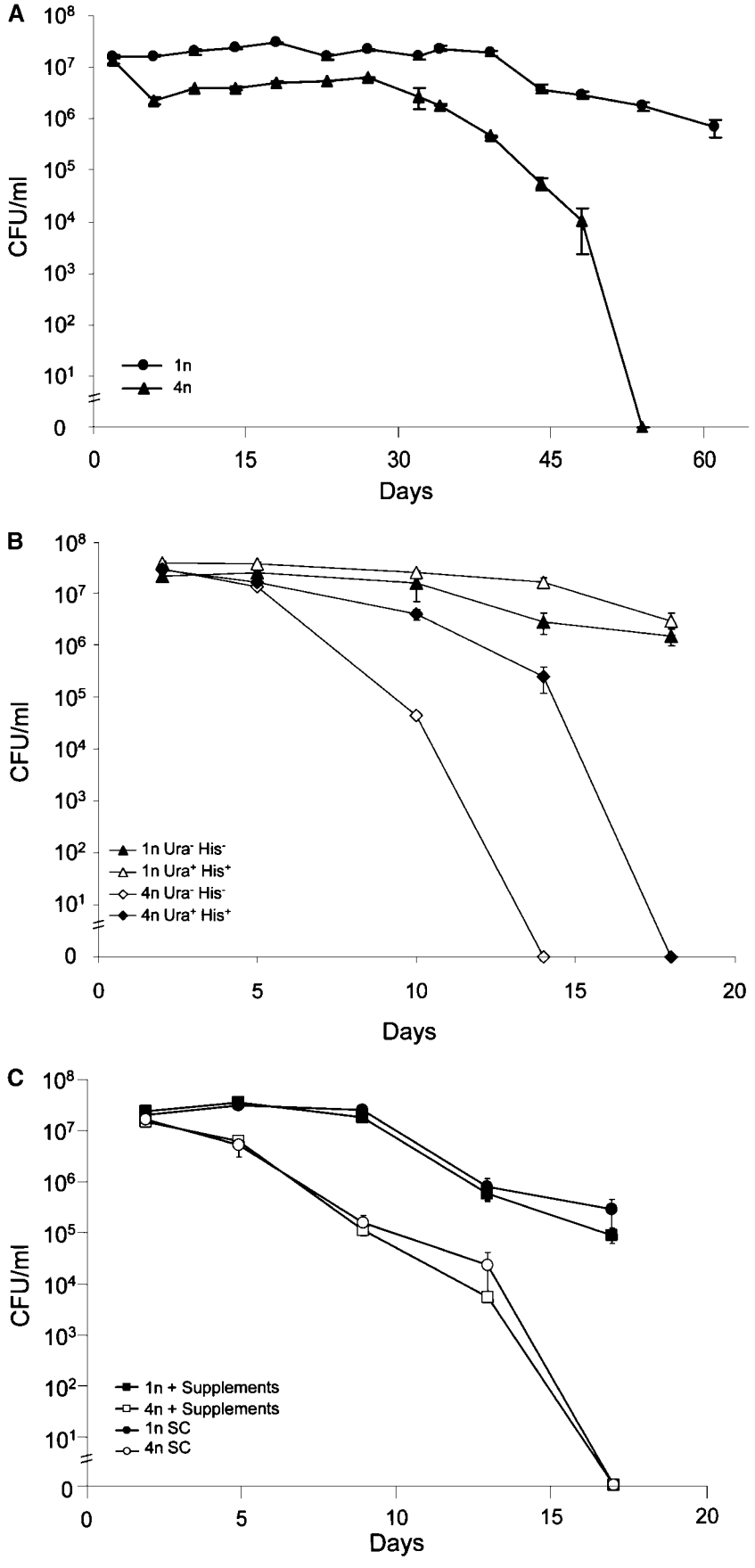

FIGURE 3.—Polyploids die in stationary phase on several different media. (A) Survival in YPD. Haploid and tetraploid strains were grown into stationary phase in YPD and monitored for viability. CFU/ml was measured for 1*n* *MAT*α (●) and 4*n* *MAT*α/α/α/α (▲). (B) Survival in synthetic medium. Prototrophic and auxotrophic strains were compared for their viability in stationary phase. CFU/ml was measured for 1*n* *MAT*α Ura$^-$ His$^-$ (▲), 1*n* *MAT*α Ura$^+$ His$^+$ (△), 4*n* *MAT*α/α/α/α Ura$^-$ His$^-$ (◇), and 4*n* *MAT*α/α/α/α Ura$^+$ His$^+$ (◆). (C) Survival in spent medium supplemented with nutritional requirements. Spent media was supplemented with 0.2 mM uracil and 0.3 mM histidine during stationary phase. CFU/ml was measured for 1*n* *MAT*α + uracil + histidine (■), 4*n* *MAT*α/α/α/α + uracil + histidine (□), 1*n* *MAT*α spent SC (●), and 4*n* *MAT*α/α/α/α spent SC (○).

TABLE 2

Tetraploids are not generally sensitive to stress

Stress	1*n*	4*n*
Paraquat	+++++	+++++
Menadione	+++++	+++++
H_2O_2	++++	++++
Sorbitol	+++++	+++++
NaCl	+++++	+++++
LiCl	+++++	+++++
Calcofluor white	+++++	+++++
Growth at 37°	++	++
Heat shock at 50°	+++++	+
Zymolase resistance	+++++	++++

Haploids and tetraploids were examined for their response to a variety of stresses. Growth was scored in a range (+ to +++++) relative to a control plate (no stress). Cells were exposed to the following: oxidative stress (paraquat 1 μM–1 mM, menadione 10 μM–1 mM, H_2O_2 2 mM–6 mM), high osmolarity (sorbitol 1–2 M), salt stress (NaCl 1–100 mM), toxic ions (LiCl 10 mM), calcofluor white (0.025–0.1 mg/ml), continuous growth at high temperatures (37°), heat-shock resistance in stationary phase (50°), and resistance to 0.15 mg/ml zymolase 100T in stationary phase.

unbudded cells with a single nucleus. To evaluate the composition of haploid and tetraploid cultures during stationary phase, we examined cells for their nuclear staining and budding status.

Haploid cells arrest early in stationary phase as a homogeneous population of unbudded cells with a single nucleus (Figure 5A). Calculation of the budding index in a haploid culture shows that only 0.16% (n = 641) of haploid cells continue to bud after 2.5 days of growth at 30°. After 4 days, all haploids (n = 672) arrest without a bud (Figure 5B). By contrast, a population of stationary-phase tetraploids is heterogeneous, consisting of budded and unbudded cells (Figure 5A); 1.3–5.9% of tetraploid cells are budded during a 5.5-day incubation (n = 653 at 2.5 days, n = 627 at 4 days, and n = 1232 at 5.5 days; Figure 5B). Continued formation of buds in stationary phase is also a property of Σ1278b *MAT***a**/**a**/**a**/**a** tetraploids and of S288c *MAT***a**/**a**/**a**/**a** tetraploids. Interestingly, the DAPI staining in tetraploid nuclei becomes diffuse as appreciable numbers of cells begin to die. Ultimately, tetraploid cells completely fail to stain with DAPI (Figure 5A, days 7 and 9).

The frequency of budded tetraploid cells underestimates the number of cells that have initiated the cell cycle. Cells at very early stages of the cell cycle may have no observable bud. Moreover, in flocculent strains such as Σ1278b it is difficult to distinguish between two adherent cells and a cell with a large bud. For this reason, we counted only those cells with a small bud. This procedure ignored cells later in cell division, which is potentially a large component of the population. A second measure of cell cycle progression in tetraploids was obtained by visualizing the mitotic spindles in stationary-phase haploid and tetraploid cells. The fraction of cells that fail to arrest in G_1 can be estimated by measuring the number of cells with duplicated spindle pole bodies, since spindle pole body duplication is required during mitotic growth (Byers and Goetsch 1975; Adams and Kilmartin 2000). In haploids, a small percentage of cells (1.8 ± 1.2) possess mitotic spindles after 7 days of growth. Strikingly, a much larger percentage of isogenic tetraploids (18.5 ± 3.7; P = 0.01) possess mitotic spin-

TABLE 3

Frequency of chromosome V loss in diploid and tetraploid strains

A. Strain	Chromosome V
*MAT*α/α	*URA3* ● *his1::LEU2* *ura3-52* ● *HIS1* (+/−)
*MAT*α/α/α/α	*URA3* ● *his1::LEU2* *ura3-52* ● *HIS1* *ura3-52* ● *HIS1* *ura3-52* ● *HIS1* (+/−/−/−)

B. Time	*MAT*α/α	*MAT*α/α/α/α	Fold increase
0	$<10^{-6}$	$<10^{-6}$	—
Log	1.91×10^{-5}	6.82×10^{-3}	357×
Stationary	1.14×10^{-5}	1.45×10^{-3}	127×

Chromosome loss rates represent the frequency of Leu$^-$ 5-FOA^R segregants in a population (see materials and methods) of logarithmically growing cells (Log) and stationary cells (Stationary). The averages of two independent experiments are shown.

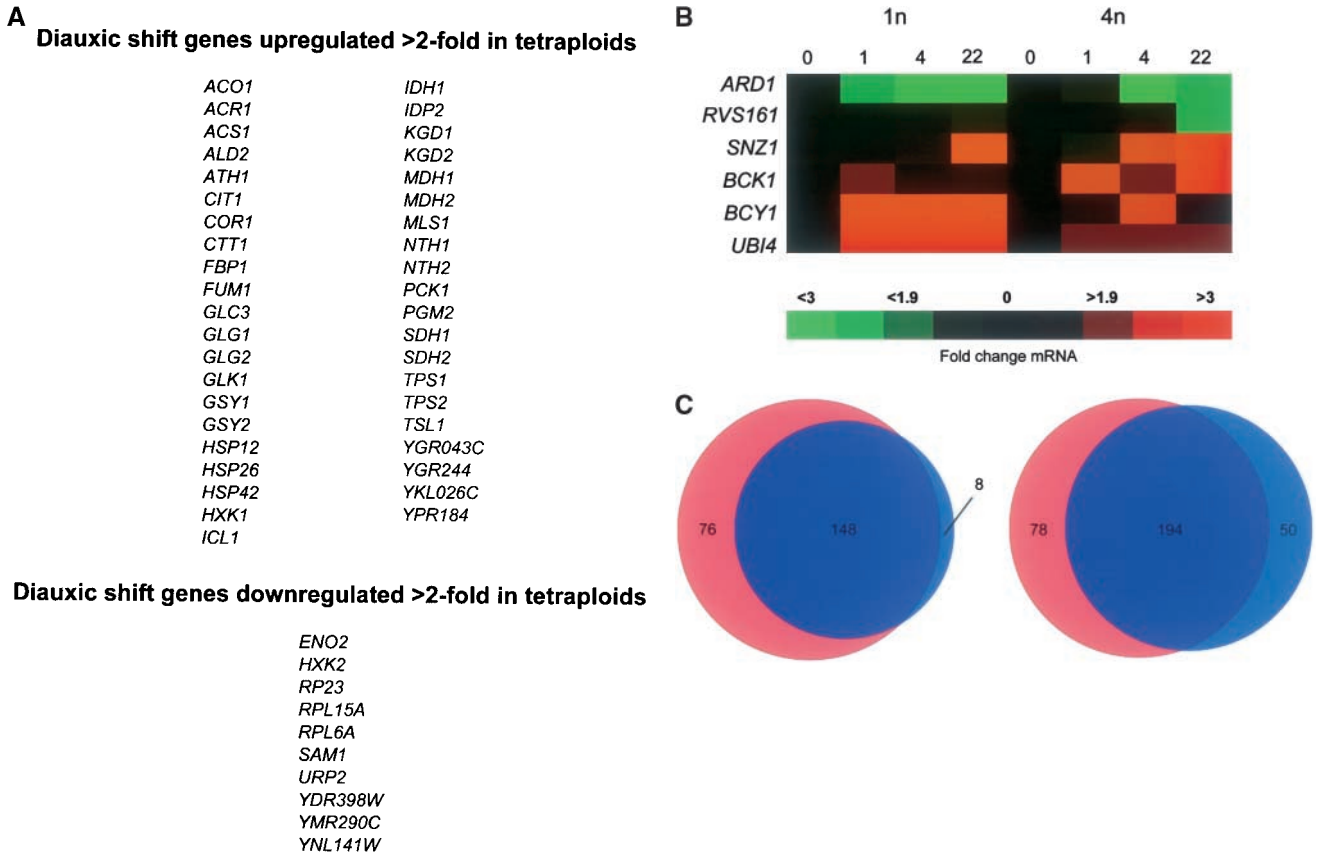

FIGURE 4.—Microarray analysis reveals that tetraploid cells have the transcriptional signatures of the diauxic shift and stationary phase. (A) Comparison of genes induced and repressed in tetraploids during the diauxic shift to those identified in a previous study of the diauxic shift (DERISI *et al.* 1997). (B) Gene expression changes of genes important for stationary-phase viability (*ARD1*, *RVS161*, *BCK1*, *BCY1*, *UBI4*) and a transcriptional marker of stationary phase, *SNZ1*, after 1, 4, and 22 hr in culture. Increased expression relative to log phase is shown in red and decreased expression is shown in green. (C) Overlap of haploid and tetraploid genes that are upregulated (left) and downregulated (right) more than twofold during the postdiauxic phase of growth. Haploid genes are shown in red and tetraploid genes are shown in blue. Similarly regulated genes are shown in purple.

dles at the same stage, suggesting that tetraploid cells fail to arrest in G_1 (Figure 5C and Table 4). Interestingly, *MAT*α/α/α/α tetraploids that overexpress *MAT***a** appear to achieve cell cycle arrest. In stationary phase fewer mitotic spindles are observed in *MAT*α/α/α/α, 2μ-*MAT***a** tetraploids (7.2 ± 1.9%) than in *MAT*α/α/α/α tetraploids (18.5 ± 3.7%; $P = 0.018$), suggesting that decreased cell cycle entry is responsible for the increased viability of *MAT*α/α/α/α, 2μ-*MAT***a** tetraploids (Table 4).

Starvation in water suppresses polyploid death: If stationary-phase death in tetraploids is a consequence of their failure to arrest growth in response to nutrient limitation, then a treatment that leads to growth arrest should rescue the cells. Incubation in water rather than medium completely deprives yeast cells of nutrients and subjects them to extreme starvation. To test the effects of extreme starvation on tetraploid viability, cells were grown into stationary phase, washed, and then transferred to water or returned to the conditioned medium in which they had been growing.

Starvation in water completely suppresses the loss of viability of stationary-phase tetraploids. Tetraploid cells that were transferred to water after 2–5 days of growth in SC remain viable, even after 21 days of incubation in water (Figure 6A). By contrast, tetraploids that are returned to conditioned medium die after 12–14 days. Haploids remain viable in both water and conditioned medium, although incubation in conditioned medium results in a slight decrease in viability. In addition, haploids remain viable when transferred to the conditioned medium collected from tetraploids, demonstrating that the inviability observed in stationary-phase tetraploids is not due to the accumulation of a toxic compound produced by tetraploid cells (not shown).

The analysis of the spindles and microtubules in tetraploids incubated in water demonstrates that these cells achieve cell cycle arrest. In contrast to the large percentage of mitotic spindles observed in tetraploids that remain in synthetic media (18.5 ± 3.7), mitotic spindles are observed in far fewer tetraploid cells that were incubated in water (2.3 ± 1.9%; $P < 0.01$; Figure 6B and Table 4).

Tetraploid cells remain metabolically active in stationary phase: The metabolic activity of stationary-phase cells as assayed by whole-genome arrays demonstrates

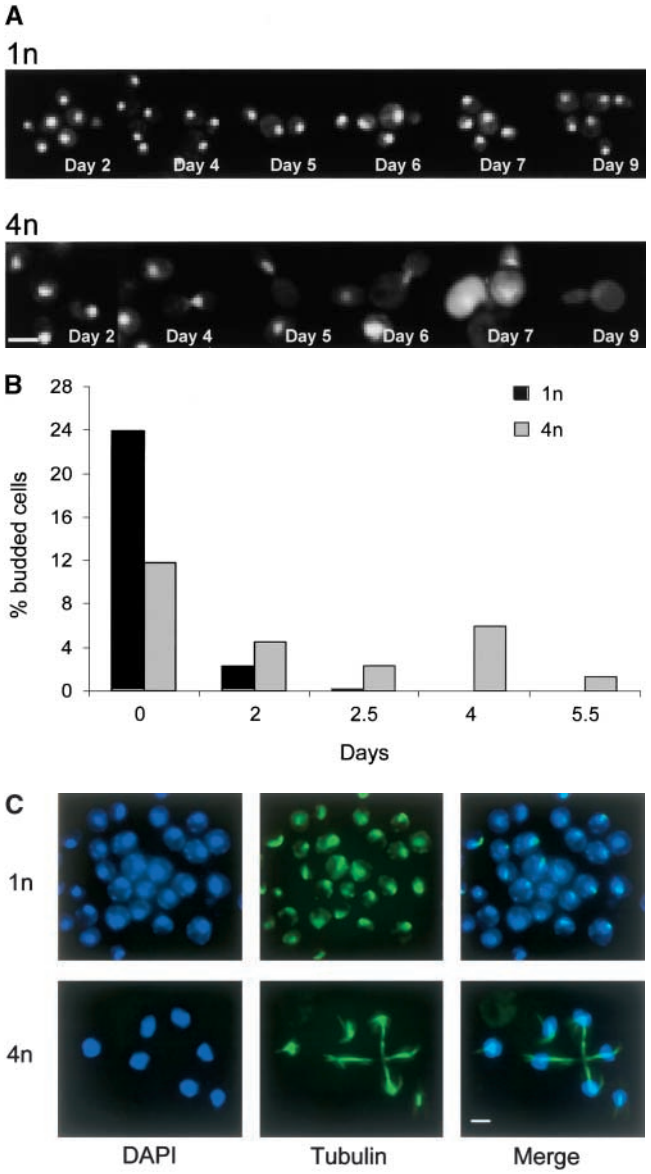

FIGURE 5.—Tetraploids fail to arrest in G_0 during stationary phase. (A) Cells were treated with DAPI to stain their nuclei and visualized under the microscope (×600). Haploids arrest as unbudded cells with a single nucleus, indicating G_0 arrest. Tetraploids fail to enter G_0 and form buds with nuclei at the junction between mother and daughter cells. Bar, 5 μm. (B) Budding index was calculated using DAPI-stained cells. Mother-daughter pairs that contained a single or "bow-tie" shaped nucleus were used to distinguish dividing cells. (C) Staining of microtubules in stationary-phase cultures by indirect immunofluorescence. Mitotic spindles are present in tetraploids, indicating that tetraploids fail to arrest in G_0. Cells were grown for 7 days. Bar, 4 μm.

that global transcription is markedly higher in stationary-phase tetraploids as compared to haploids at an equivalent stage of growth. After 3–5 days in SC, the stationary-phase-associated expression of *SNZ1* significantly increases in both haploid and tetraploid cells. At this point, 4952 genes were expressed greater than twofold more in tetraploids than in haploids in two/two independent experiments, whereas only 9 genes were expressed greater than twofold more in haploids than in tetraploids. Interestingly, a comparison of the global transcription in tetraploids starved in water to those that are grown in SC reveals that 2597 genes are expressed at least twofold lower in the tetraploids grown in water relative to those grown in SC. The high level of transcriptional activity observed in tetraploids grown in SC suggests that these cells remain active throughout stationary phase. Furthermore, the decrease in transcriptional activity observed in tetraploids starved in water may explain why these cells do not enter mitosis and are able to remain viable in stationary phase.

TABLE 4

Microtubule staining reveals mitotic spindles in wild-type tetraploids, but not in haploids, *cln3* tetraploids, or tetraploids incubated in water

Strain	% cells with mitotic spindles/total cells
*MAT*α WT	1.8 ± 1.2
*MAT*α/α/α/α WT	18.5 ± 3.7
*MAT*α/α/α/α, 2μ-*MAT***a**	7.2 ± 1.9
*MAT*α, H_2O	1.6 ± 0.5
*MAT*α/α/α/α, H_2O	2.3 ± 1.9
*MAT*α, *cln3*	1.9 ± 0.3
*MAT*α/α/α/α, *cln3*/*cln3*/*cln3*/*cln3*	5.4 ± 2.3

Comparison of mitotic spindle formation in haploids and tetraploids. Wild-type tetraploids show a significantly large percentage of mitotic spindles relative to wild-type haploids, *cln3* tetraploids, and tetraploids incubated in water. All strains were grown for 7 days in stationary phase. WT, wild type.

Deletion of the G_1 cyclin *CLN3* rescues polyploid viability in stationary phase: The increased mitotic arrest that is observed under conditions that rescue tetraploid viability suggests that inappropriate cell cycle progression is responsible for the loss of viability in stationary-phase tetraploids. If so, then mutations like *cln3* that retard entry into the cell cycle should also rescue tetraploid viability during stationary phase. *CLN3* regulates the transcription of *CLN1* and *CLN2*, which together control passage from the G_1 to the S phase of the cell cycle (TYERS *et al.* 1993; STUART and WITTENBERG 1995). Strains deleted for *CLN3* are viable but delayed for cell cycle entry, as more time is required to accumulate the levels of the other G_1 cyclins, *CLN1* and *CLN2*, which are necessary to trigger the passage through Start (RICHARDSON *et al.* 1989). To determine the effect of impeded cell cycle entry on stationary-phase survival, isogenic *cln3* haploids and *cln3*/*cln3*/*cln3*/*cln3* tetraploids were constructed.

Deletion of *CLN3* suppresses the stationary-phase death observed in tetraploid cells. *CLN3*/*CLN3*/*CLN3*/*CLN3* tetraploids die after 16–18 days in culture, whereas *cln3*/*cln3*/*cln3*/*cln3* tetraploids remain completely viable after >21 days in culture (Figure 7, A and B). Moreover, staining of the spindles and microtubules in *cln3* strains by indirect immunofluorescence reveals that

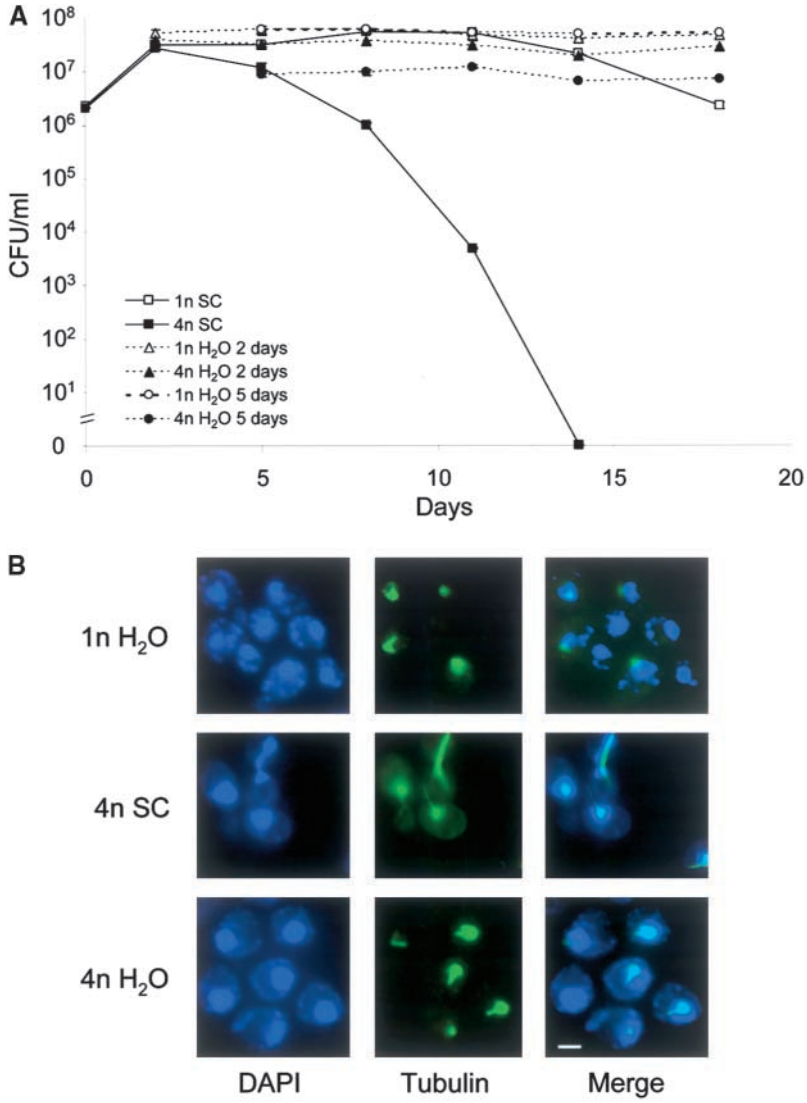

FIGURE 6.—The stationary-phase death in tetraploids is suppressed by incubation of postdiauxic cultures in water. (A) Strains were grown in SC for 2 or 5 days and transferred to water or reintroduced to spent SC media. Viability was monitored after strains were transferred from spent SC to water or from spent SC to spent SC. CFU/ml were measured for haploids reintroduced to spent SC (*MAT*α SC, □), tetraploids reintroduced to spent SC (*MAT*α/α/α/α, ■), haploids transferred to water after 2 days of growth (*MAT*α, △), tetraploids transferred to water after 2 days of growth (*MAT*α/α/α/α, ▲), haploids transferred to water after 5 days of growth (*MAT*α, ○), and tetraploids transferred to water after 5 days of growth (*MAT*α/α/α/α, ●). (B) Staining of microtubules by indirect immunofluorescence in strains incubated in SC and water. Mitotic spindles are present in tetraploids, whereas haploids remain arrested after 7 days in culture. Bar, 5 μm.

cln3/cln3/cln3/cln3 tetraploids possess far fewer mitotic spindles (5.4 ± 2.3%) than wild-type tetraploids do (18.5 ± 3.7%; $P = 0.01$; Figure 7C and Table 4), suggesting that rescue by the deletion of *CLN3* is due to impeded cell cycle entry in tetraploids. The suppression of stationary-phase death in *cln3/cln3/cln3/cln3* strains demonstrates that the loss of viability in wild-type tetraploids is not a consequence of the increased cell size associated with the increase in DNA content. Wild-type haploids measure 4.16 ± 0.61 μm × 3.53 ± 0.47 μm, whereas isogenic tetraploids measure 7.24 ± 0.69 μm × 5.95 ± 0.79 μm. However, *cln3/cln3/cln3/cln3* tetraploid cells are even larger (10.18 ± 0.87 μm × 7.48 ± 0.63 μm) than *CLN3/CLN3/CLN3/CLN3* tetraploids (Figure 7D and Table 5).

DISCUSSION

The comparison of newly formed polyploids with their haploid progenitors has revealed that nascent polyploids have a defect in stationary-phase viability. One possible explanation for the inviability of stationary-phase tetraploids is that these cells accumulate abnormalities during log phase that make them inviable in stationary phase. Our studies on isogenic diploid and tetraploid cells show that tetraploids have increased chromosome instability during exponential growth. These data agree with a previous study indicating that tetraploids experience a 1000-fold increase in chromosome loss rates relative to diploids (MAYER and AGUILERA 1990). However, this increase in chromosome instability *per se* cannot account for their inviability in stationary phase. First, of the population of tetraploid cells that enter stationary phase, both euploid and aneuploid members can be rescued by starvation in water. Second, tetraploids lacking *CLN3* function survive stationary phase as well as their haploid counterparts. Third, mating-type heterozygosity increases tetraploid viability in stationary phase. Under all these conditions, the strains had the same prehistory and, therefore, the same genetic constitution. If chromosome

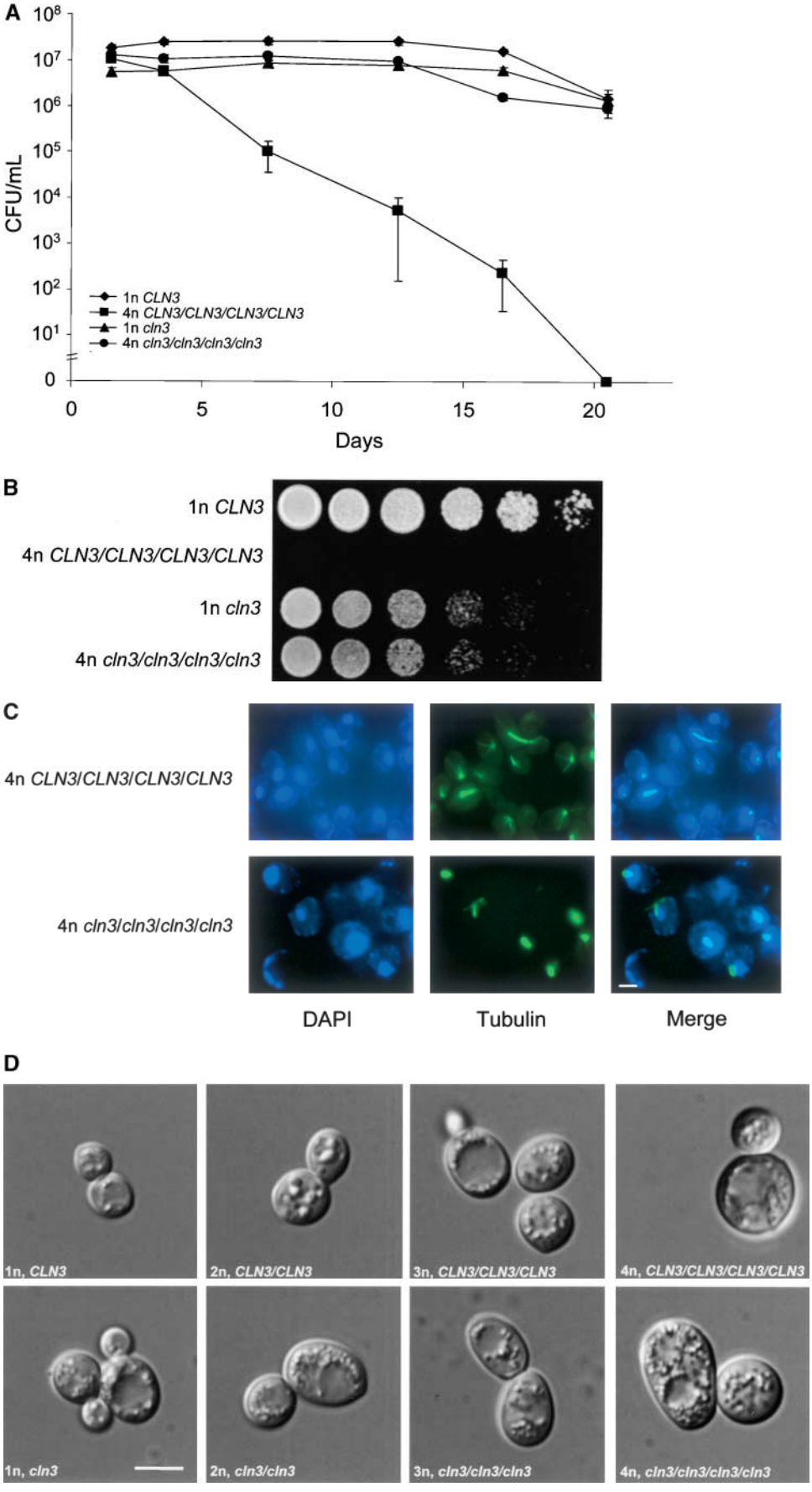

FIGURE 7.—Deletion of *CLN3* in tetraploids suppresses stationary-phase death and decreases entry into mitosis. (A) *cln3* haploids and tetraploids were grown into stationary phase and measured for their viability (CFU per milliliter). Viability was measured for: 1*n MAT*α wild-type (◆), 4*n MAT*α/α/α/α wild-type (■), 1*n MAT*α *cln3* (▲), and 4*n MAT*α/α/α/α *cln3*/*cln3*/*cln3*/*cln3* (●). (B) Wild-type and *cln3* haploids and tetraploids were grown for 12.5 days in stationary phase and serially diluted onto YPD. (C) Staining of microtubules by indirect immunofluorescence in wild-type and *cln3*/*cln3*/*cln3*/*cln3* tetraploids. Cultures were grown for 7 days. Bar, 5 μm. (D) Cell size comparison of wild-type and *cln3*/*cln3*/*cln3*/*cln3* cells. Bar, 5 μm.

TABLE 5

Increased cell size in *cln3* strains

Strain	Size (μm; length × width)
1*n CLN3*	4.16 ± 0.61 × 3.53 ± 0.47
2*n CLN3/CLN3*	5.25 ± 0.67 × 4.16 ± 0.52
3*n CLN3/CLN3/CLN3*	6.24 ± 0.66 × 4.89 ± 0.61
4*n CLN3/CLN3/CLN3/CLN3*	7.24 ± 0.69 × 5.95 ± 0.79
1*n cln3*	5.95 ± 0.65 × 4.55 ± 0.64
2*n cln3/cln3*	8.05 ± 0.78 × 6.08 ± 0.66
3*n cln3/cln3/cln3*	8.99 ± 0.82 × 6.53 ± 0.67
4*n cln3/cln3/cln3/cln3*	10.18 ± 0.87 × 7.48 ± 0.63

imbalances were responsible for the striking difference in viability, then none of these conditions would have rectified the problem.

Our experiments suggest that tetraploid cells die because they fail to arrest mitotic growth in stationary phase. First, tetraploids continue to bud in stationary phase, whereas haploids arrest as unbudded cells. Under these conditions the number of tetraploid cells does not increase, so the initiation of buds is not the prelude to a normal cell division. Second, a large number of tetraploid cells display mitotic spindles in stationary phase, whereas virtually all isogenic haploids arrest with no discernible mitotic spindles. Third, all conditions that increase tetraploid viability in stationary phase, such as mating-type heterozygosity and starvation in water, also increase mitotic arrest in stationary-phase tetraploids. Finally, deletion of *CLN3*, a G_1 cyclin critical for cell cycle entry, dramatically increases the viability of tetraploids during stationary phase. Importantly, a much lower percentage of *cln3/cln3/cln3/cln3* tetraploids display mitotic spindles during stationary phase, suggesting that impeded entry into an abnormal cell cycle leads to increased survival of stationary-phase tetraploids.

Our data suggest that polyploid cells fail to detect the nutrient conditions that normally signal entry into stationary phase in spent medium. Indeed, when tetraploids were incubated in water, the cells remained completely viable. A comparison of the transcription profile of tetraploids grown in spent medium with that of cells starved in water shows that water imposes a much greater reduction in overall transcription as compared with spent medium. Moreover, tetraploid cells do not bud or show abnormal mitotic spindles when placed in water. These data suggest that in spent medium tetraploids continue to enter the cell cycle because they fail to sense the depletion of nutrients.

Even on spent medium tetraploid cells display the transcriptional and physiological signatures that are indicative of transition through the diauxic shift and entry to stationary phase. Despite these indications that tetraploids are capable of sensing nutrient depletion, the transcriptional profiles of haploids and tetraploids are not completely identical; >150 genes are differentially regulated between haploids and tetraploids. It is possible that one or more of the genes that are inappropriately regulated in tetraploids is important for cell cycle arrest or stationary-phase survival.

Whole-genome duplications impose a selective disadvantage upon the newly formed polyploid cells. These cells rapidly lose chromosomes during exponential growth and fail to survive in stationary phase. The current models for the evolution of new genes posit an initial whole-genome duplication followed by mutation of one of the duplicated copies to acquire a new function. For the duplication to survive, the positive selective pressures must be balanced against the negative effects of those duplications. Whole-genome duplications could occur either by endoreduplication or by mating of diploids homozygous at the *MAT* locus. Since tetraploids heterozygous at the *MAT* locus survive stationary phase better than those homozygous at the *MAT* locus, our study would favor the latter mechanism.

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