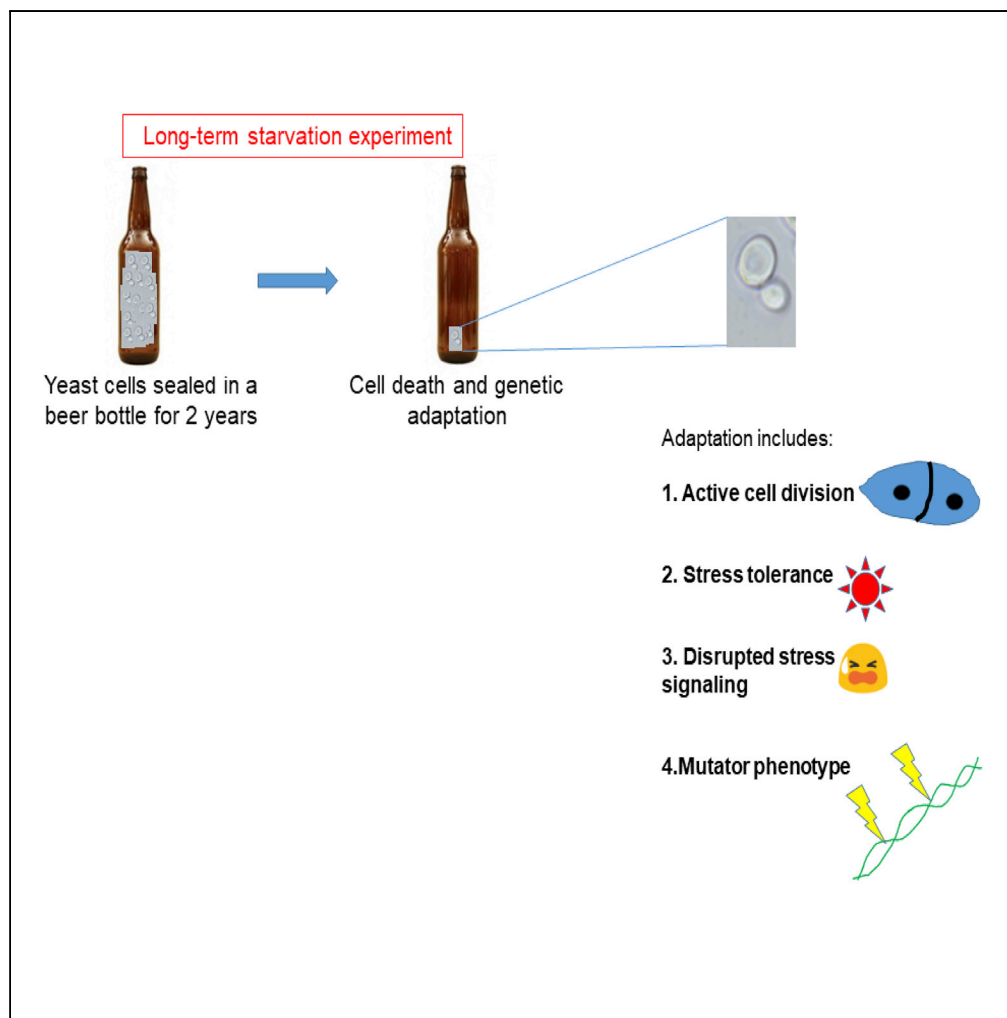**Article**

Eukaryotic Adaptation to Years-Long Starvation Resembles that of Bacteria

Tzemach Aouizerat, Daniel Gelman, Amir Szitenberg, ..., Amijai Saragovi, Ronen Hazan, Michael Klutstein

ronenh@ekmd.huji.ac.il (R.H.)
michaelk@ekmd.huji.ac.il (M.K.)

HIGHLIGHTS

Yeast genetically adapts to long-term starvation in a similar way to bacteria

Adaptation to long-term starvation in yeast includes multi-stress tolerance

Adaptation to long-term starvation in yeast includes mutations in TORC

Adaptation to long-term starvation in yeast includes a mutator phenotype

iScience

Article

Eukaryotic Adaptation to Years-Long Starvation Resembles that of Bacteria

Tzemach Aouizerat,[1,4] Daniel Gelman,[1,4] Amir Szitenberg,[2] Itay Gutman,[1] Shunit Glazer,[1] Eli Reich,[1] Miriam Schoemann,[1] Rachel Kaplan,[1] Amijai Saragovi,[3] Ronen Hazan,[1,5,*] and Michael Klutstein[1,5,6,*]

SUMMARY

The **G**rowth **A**dvantage in **S**tationary **P**hase (GASP) phenomenon, described in bacteria, reflects the genetic adaptation of bacteria to stress, including starvation, for a long time. Unlike in stationary phase where no cell division occurs, GASP harbors active cell division, concurrent with genetic adaptation.
Here we show that GASP occurs also in eukaryotes.
Two strains of *Saccharomyces cerevisiae (Sc404 and Sc424)* have been isolated from 2-year-old sealed bottles of beer. These strains presented advantage in survival and growth over the parent during stress. The differences between the strains are irreversible and therefore genetic in origin rather than epigenetic. Direct competition assays show that *Sc404* and *Sc424* outcompete the parent in direct competition. DNA sequencing shows changes of the genome: the TOR complexes are mutated, and DNA repair gene mutations confer a mutator phenotype. The differences between the strains are reflected in a difference in taste between beers brewed from them.

INTRODUCTION

Cells respond to their environment with surprising efficiency by using various mechanisms. The richness of cellular response to environmental cues was first described in bacteria and studied in detail with regard to how bacteria develop resistance and tolerance to antibiotics (Brauner et al., 2016) and other stress conditions (Orruno et al., 2017) (Harms et al., 2016). Among the strategies that exist to create resistance or tolerance, some particularly efficient ones involve growth arrest of bacterial cells. Clearly, starvation, stress, and growth arrest are the conditions that the vast majority of living cells experience in any environment most of the time as reflected from the fact that addition of nutrients to sea water or soil particles would increase the concentration of cells by several logs. Therefore, the understanding of how cells use long-term stationary phase (LTSP) strategies to overcome challenges in their environment for long times is important and has many practical uses. LTSP cultures of bacteria with the added challenge of antibiotics or abiotic stresses have highlighted phenomena exhibited by bacteria facing these conditions. GASP (growth advantage in stationary phase) cells arise in LTSP cultures as resistant mutants that overtake the entire culture in waves (Farrell and Finkel, 2003) (Finkel and Kolter, 1999). It is vital to clarify at this point that there is a difference between regulated stationary phase and GASP. During stationary phase, cell division is inhibited by tight regulation (Gray et al., 2004). In GASP, however, the situation is quite different. During continuous growth of a culture, after nutrients have been depleted from the culture, and the culture has been maintained in stationary phase for several days, without further addition of medium, death phase occurs. At this stage, more than 90% of the cell population dies. In the few percent of living cells, cycles of cell death and cell division occur. Net cell number does not change dramatically for a long time, but cell division does occur (see later discussion). It is at this stage that cells start to genetically adapt (through mutation-selection cycles) to the harsh culture conditions. Researchers have found (Zambrano et al., 1993) (Zambrano and Kolter, 1996), (Brauner et al., 2016) that, when GASP cells are faced with a chemical or physical challenge, resistant mutants start to emerge. Since GASP division rate is quite low (see later discussion), much time is needed for the resistant mutants to take over the entire culture. During that time, secondary mutants arise, with better resistance against the challenge than the first resistant mutants. Those also start the slow process of taking over the culture. Cleverly designed experiments have been able to demonstrate the existence of these mutations—selection waves in planktonic cultures (Zambrano et al., 1993) (Zambrano and Kolter, 1996) (Farrell and Finkel, 2003) (Finkel and Kolter, 1999) and inside agar (Baym et al., 2016). The hallmark of these mutants is the ability of the end point isolated strain in this experiment to successfully compete with and outgrow the original

[1]Institute of Dental Sciences, Faculty of Dental Medicine, The Hebrew University of Jerusalem, P.O.B. 12272, Ein Kerem, Jerusalem 9112001, Israel

[2]Microbial and Metagenomics Division, Dead Sea and Arava Science Center, Masada 8698000, Israel

[3]The Lautenberg Center for Immunology and Cancer Research, The Institute for Medical Research Israel-Canada, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem 9112001, Israel

[4]These authors contributed equally

[5]These authors contributed equally

[6]Lead Contact

*Correspondence:
ronenh@ekmd.huji.ac.il
(R.H.),
michaelk@ekmd.huji.ac.il
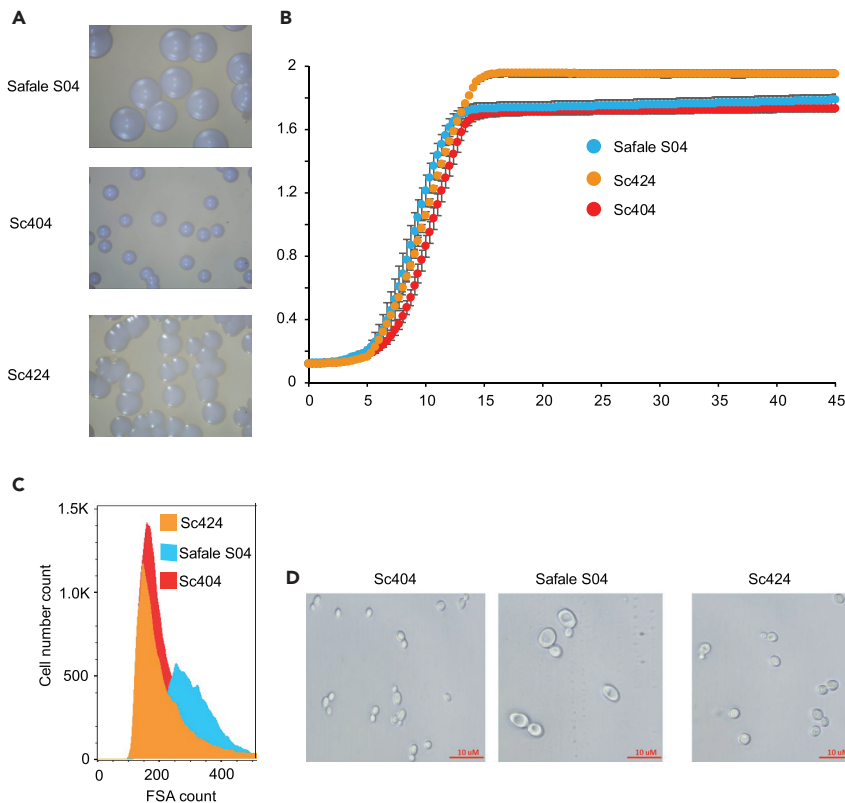(M.K.)
https://doi.org/10.1016/j.isci.2019.08.002

Figure 1. Sc404 and Sc424 Are Yeast Strains Isolated from Long-Term Stationary Phase in Stressful Conditions, Presenting Different Characteristics Than the Parental Strain Safale S-04

(A) Sc404 and Sc424, isolated from 2-year-old sealed bottles of beer, present significantly smaller colonies than Safale S-04, as shown in cell cultures grown for 72 h on solid YPD plates in 30°C.

(B) Growth kinetics of all three strains were tested for 72 h by measuring the turbidity of the cultures in a 96-well plate reader every 20 min. Note that the three strains show similar growth rates in rich YPD medium at 30°C.

Data are represented as mean ± SEM. (C) Flow cytometry for cell size: cell size quantification was preformed using flow cytometry, based on forward scatter and side scatter measurements. (D) microscope images of cells from the different strains. Scale bar is 10 μM.

parental strain in a direct competition experiment. This hallmark gave the phenomenon its name: GASP (growth advantage in stationary phase) (Finkel, 2006).

Bacterial strains exhibiting GASP have been sequenced, and mutations repeatedly found in GASP have been isolated (Zambrano et al., 1993) (Zinser and Kolter, 2004) (Zinser and Kolter, 1999) (Zinser and Kolter, 2000) (Zinser et al., 2003). Surprisingly, many of these genes were found to be related to generalized stress responses such as the RNA polymerase subunit Sigma S (RpoS, stationary phase Sigma factor). Evidently, strains harboring these mutations alone are able to successfully compete with their naive parental strains (Zambrano et al., 1993). It is important to note that, classically, GASP mutants are also resistant to different stresses in addition to being more fit for competition.

Indeed, several mechanisms of drug and stress resistance and some tolerance mechanisms have been demonstrated and documented in eukaryotes (Casalinuovo et al., 2004) (Bojsen et al., 2017) (Huang et al., 2001) (Huang and Houghton, 2001). However, to the best of our knowledge, the GASP phenotype was not described in eukaryotes.

Here we studied the dynamics of *Saccharomyces cerevisiae* yeast cells in long-term stress conditions, as a model for how eukaryotic cells develop stress resistance and tolerance in LTSP cultures. To this end, we looked for isolated environments where yeast cells remained in starvation conditions and where natural GASP survivors might have evolved. One such environment was found in "old" unfiltered beer bottles
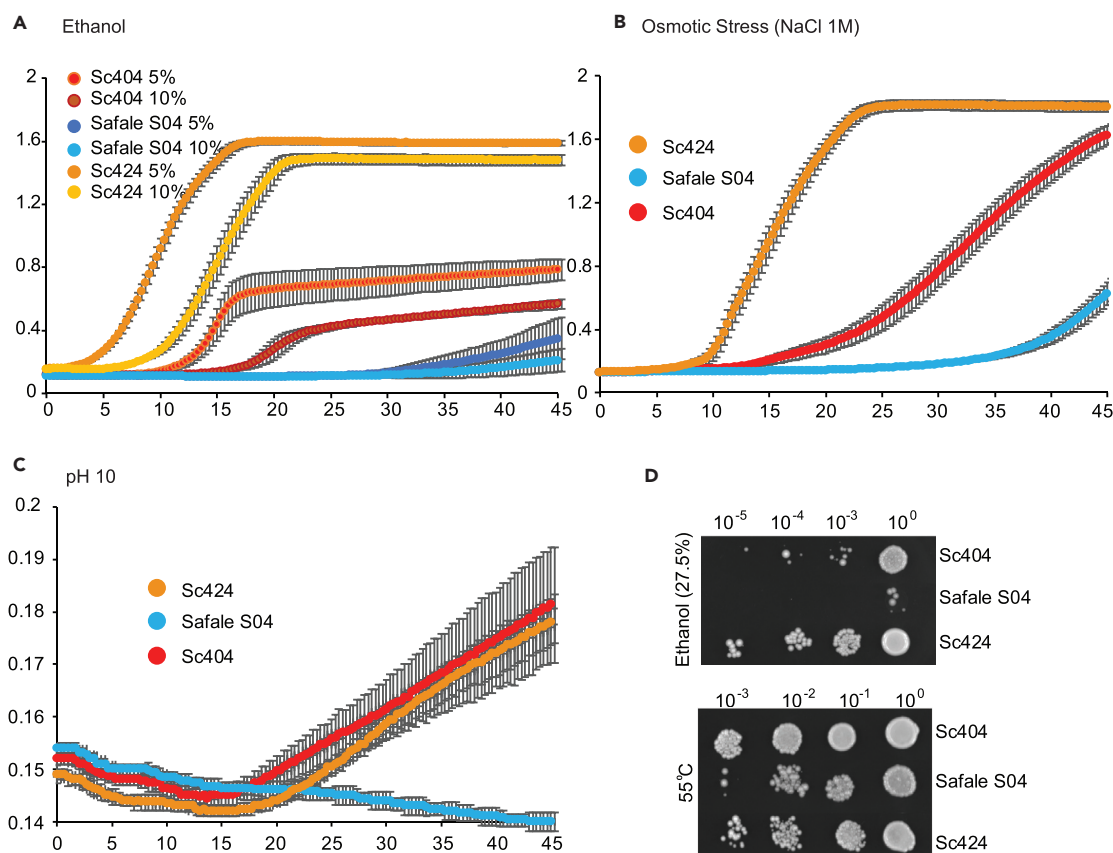
Figure 2. Sc404 and Sc424 Show Increased Ability to Tolerate Various Stressful Environments Compared with Safale S-04

The growth kinetics of Sc404, Sc424, and Safale S-04, represented by measurements of the cultures turbidity in a 96-well plate reader every 20 min for 48 h, were monitored in different stressful conditions.

(A–D) These conditions include (A) ethanol in 5%(v/v) and 10%(v/v), (B) high alkaline environment (pH = 10), and (C) medium with high osmotic pressure (YPD containing 1 or 2 M NaCl). In all tested conditions, the growth of Sc404 and Sc424 was affected significantly less than Safale S-04, which was inhibited almost completely. Data are represented as mean $\pm$ SEM. (D) Sc404, Sc424, and Safale S-04 were exposed to identical sublethal conditions, where Sc404 and Sc424 presented noticeably better tolerance than Safale S-04. The conditions include high temperature (55°C for 10 min) and ethanol (27.5% v/v for 1 h). The cultures had been grown in the same conditions to equal optical density and, after exposure to the stress conditions, spotted on YPD plates.

left for 2 years in a Jerusalem beer brewery, where yeast cells endured harsh conditions, which include low nutrients, high ethanol concentration, and mild temperature (>10°C, <25°C for two years). By comparison of the yeast cells isolated from these bottles with their parental strains, we were able to demonstrate that GASP does indeed appear in eukaryotic LTSP cultures. We have recently shown (Aouizerat et al., 2019) that yeast can survive for thousands of years as micro-colonies in the nanopores of ancient pottery. The GASP mechanism shown here could significantly contribute to such survival.

RESULTS

Isolation of GASP Yeast Strains from Aged Beer Bottles

To determine whether the GASP phenomenon occurs in eukaryotes, we have isolated two strains of *Saccharomyces cerevisiae* that sustained a long period of stressful environment, from two different 2-year-old sealed bottles of beer. The isolated strains were termed *Sc404* and *Sc424*, and their phenotypes in various conditions were characterized and compared with the parental commercial beer strain, *Saccharomyces cerevisiae Safale S-04* (Fermentis Division of S.I.Lesaffre, France). *Sc404* and *Sc424* showed significantly smaller colonies when growing on rich YPD plates than the parental strain, and smaller cells both in micrographs and by fluorescence-activated cell sorting (FACS) analysis (Figures 1A, 1C, and 1D). In addition, we have found that, although *Sc424* and *Sc404* presented similar growth rates in optimal conditions of rich media and optimal temperature, they showed different growth parameters in comparison with the commercial strain in a nutrient-depleted filtrate
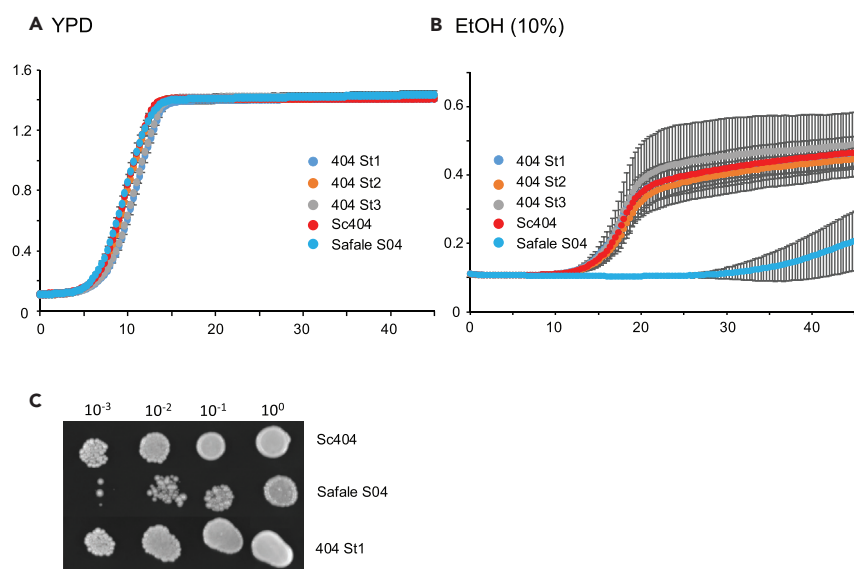
Figure 3. Sc404 Cells Maintain Their Stress-Driven Characters after Serial Dilutions

(A–C) 404St strains were evolved by diluting overnight cultures of Sc404 through a period of 6 days (see Methods). The 404St strains present similar qualities to those of Sc404 such as in (A) growth in YPD and (B) in the presence of ethanol (10% v/v), represented by measurements of the culture turbidity in a 96-well plate reader every 20 min for 48 h. Data are represented as mean ± SEM. (C) 404St strains together with Sc404 and Safale S-04 were grown to the same optical density and exposed to high temperature (55°C for 10 min) and then spotted on YPD plates. In these conditions, 404St strains maintained their advantage in stress conditions over the parental strain Safale S-04.

of the beer from which it was isolated (Figures 1B and S1). These differences have prompted us to further examine the growth of both starvation-adapted stains in various conditions, to understand better the changes that the strains have gone through during adaptation, and check whether this adaptation is similar to GASP.

Resistance and Growth in Stressful Environments

To investigate stress adaptation, we compared the growth of Sc404 and Sc424 and the parental strain in various stress conditions as one of the hallmarks of GASP in bacteria is their ability to tolerate and survive better such conditions. In all prolonged stress conditions tested: 5% or 10% of ethanol, high osmolality levels (1 M NaCl), and alkaline conditions (pH 10), Sc404 and Sc424 presented significantly better growth over Safale S-04 (Figures 2A–2C). Moreover, Sc404 and Sc424 were also able to tolerate better an acute stress for a short time. This included high ethanol concentration (27.5% EtOH) and high temperature (55°C) (Figure 2D). Importantly, the tested stress conditions extend beyond the environment of the beer bottle from which Sc404 and Sc424 were isolated.

GASP Predominantly Consists of Genetic Changes Rather Than Epigenetic Changes

The increased ability of LTSP isolates to withstand stress could originate from permanent genetic changes such as mutations, deletions, and loss or gain of heterozygosity, as previously described in bacteria, or from changes that are epigenetic and reversible in nature such as the changes leading to persisters formations (Fisher et al., 2017).

Researchers have demonstrated the predominant genetic changes in bacterial GASP strains, by showing that the modified strains have maintained their phenotype even after repeated rounds of serial passage through log-phase growth. The Sc404 and Sc424 strains have been passed through a similar process of serial passages for six consecutive days on rich medium (YPD), to select against epigenetic modifications. The yeast strains created in this process were termed 404St1, 404St2, and 404St3; and 424St1, 424St2, 424St3; and 424St4, and their phenotypes were tested and compared with Sc404. Even after serial passages on rich medium, the 404St and 424St strains still showed a significant advantage over the original Safale S-04 strain both in growth kinetics and survival in stressful environments (Figures 3 and S2). It is interesting to note that despite all 404St and 424St showing a significant advantage over the original Safale S-04 strain,
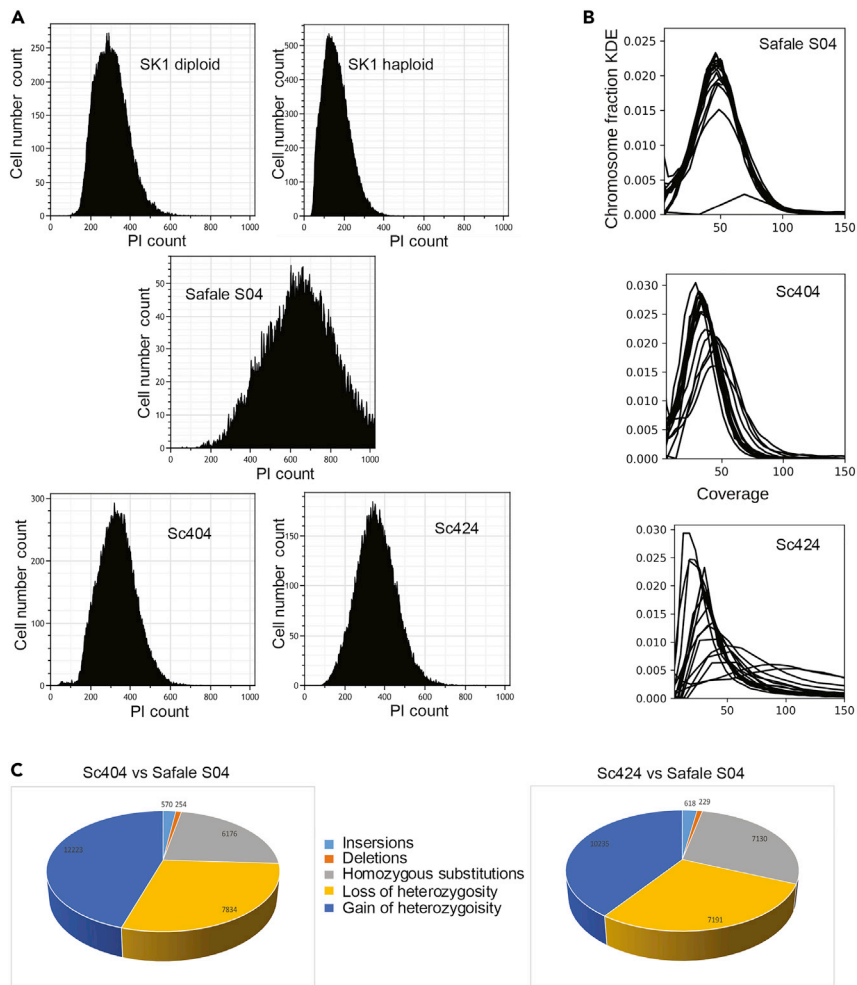
Figure 4. Genomic Analysis of *Safale S-04*, *Sc404*, and *Sc424*

(A) Flow cytometry for DNA content: DNA content was evaluated by flow cytometry following permeabilization and fixation using methanol and treatment with propidium iodide before measurements.

(B) Average sequencing coverage per chromosome in the three sequenced strains shows uniform coverage in all chromosomes in strains *Safale S-04 and Sc404*. *Sc424* shows an over-representation of chromosomes I, III, and VI.

(C) All genomic differences between *Safale S-04*, *Sc404*, and *Sc424* were divided to appropriate categories according to the change caused by the mutation. The relative abundance of these categories is presented in the pie chart. The list of genomic changes can be found on Tables S1, S2, and S3.

there is some phenotypic variability among the tested strains. It is not clear if this variability originates from experimental conditions or if it expresses epigenetic variation among the progeny of *Sc404* and *Sc424*. We conclude that genetic changes, which accumulated during a long starvation period, are the predominant factor in the ability of *Sc404* and *Sc424* to tolerate stress, rather than epigenetic changes.

Genomic Sequence Analysis and Validations

To examine the changes the survivor strains underwent during stress adaptation, we first investigated the genome content of the parental and survivor strains by FACS (Figure 4A). According to our analysis, the parental strain is tetraploid and the survivor is diploid (*Sc404*), or close to diploid (*Sc424*, a little higher than diploid). To test whether chromosomes are present in homolog pairs in the survivors, we performed a sporulation test. Sc404 and Sc424 sporulated efficiently, as did Safale S-04 (Figure S3). It is possible that at least one round of meiosis occurred during adaptation, although stress-related non-meiotic ploidy reduction has also been observed in many fungi (Gerstein et al., 2017) (Hickman et al., 2015). It is important to note that this reduction in ploidy does not necessarily lead to homozigotization of all mutations. Rather,

Functional Category	# Genes in List	# Genes in Category	p Value	Fold Enrichment
DNA repair	38	191	6.4×10^{-5}	2
Protein localization to pre-autophagosomal structure	7	9	6.7×10^{-5}	7.7
RNA splicing	22	111	3.3×10^{-3}	2
Phosphorelay signal transduction system	4	5	8.8×10^{-3}	7.9
Budding cell apical bud growth	7	20	1.2×10^{-2}	3.5
Chromatin remodeling	13	59	1.3×10^{-2}	2.2
Replicative cell aging	10	39	1.4×10^{-2}	2.5
Late nucleophagy	6	17	2.3×10^{-2}	3.5
Positive regulation of actin cytoskeleton reorganization	4	7	2.6×10^{-2}	5.6
Steroid biosynthetic process	7	25	3.5×10^{-2}	2.8

Table 1. Selected Functional Enrichment Categories of Genomic Changes in Sc404 vs. Safeale S-04
Genes where genomic changes occur (see Tables S1 and S2) were subjected to functional enrichment analysis using the DAVID tool (https://david.ncifcrf.gov/summary.jsp). Names of selected categories with their enrichment statistics are listed.

we find many heterozygous mutations in the genome of Sc404 and Sc424 (see later discussion). Furthermore, we sequenced the genomes of *Safale S-04 Sc404* and *Sc424* (Accessions GeneBank:SRR6706477, GeneBank:SRR6706475, and GeneBank:SRR6706476 correspondingly, and Tables S1 and S2 showing genomic changes and Table S3 showing heterozygosity) and determined the differences between these strains. An analysis of chromosome copy numbers showed that in *Safale S-04* and *Sc404* there was no over-representation of any chromosome in the genome (Figure 4B and Table S4). In contrast, the genome of *Sc424* shows an over-representation of chromosomes I, III, and VI (Figure 4B and Table S4), a result that is consistent with previous reports of a possible adaptive duplication of chromosomes (Gerstein et al., 2017) (Hickman et al., 2015). The summary of genomic changes is presented in Figure 4C. The main mechanism (more than 65% of genomic changes in both strains) of change in the genomes of both *Sc404* and *Sc424* are changes in heterozygosity (loss of heterozygosity and gain of heterozygosity, LOH + GOH). As shown in Figure 4C the genomes of *Sc404* and *Sc424* also contain deletions and insertions compared with *Safale S-04*. The sequencing results were also validated by Sanger sequencing in several representative loci, with complete identity to the predicted sequence by next generation sequencing (data not shown).

A functional annotation (https://david.ncifcrf.gov/) performed on all changed genes in *Sc404* and *Sc424* shows an enrichment in DNA repair, cell cycle, metabolism, autophagy, aging, and meiosis genes (Tables 1, 2, and S5). Many genes with mutations are mutated in both *Sc404* and *Sc424* (see Figure 5A). (Genes included are ones with a SNP mutation in the "type" column and "false" in the "heterozygote" column in Tables S1 and S2. For a detailed list of genes included in this analysis, see tab "genes included in 5A" in Tables S1 and S2, and the functional enrichment of these common genes) Tables 3 and S5 show enrichment for the same functional categories as earlier. These results suggest that convergent evolution is at play in this genetic adaptation process.

Stress response can be heavily influenced by the ability of cells to activate cellular signaling. One of the major complexes contributing to this signaling is the TOR complex (Lushchak et al., 2017) (Gonzalez and Hall, 2017). In our genomic analysis we noticed that mutations occurred in the TORC1 and TORC2 complexes (see Tables S1 and S2). Specifically, the TOR1 protein has 20 SNPs between *Sc404* and *SafAle S-04*, the LST8 gene has 5 SNPs, and the SLM1 has 1 SNP. TOR1 also has 20 SNPs between *Sc424* and *SafAle S-04* (the second tab in Tables S1 and S2 now enables the search of more genes with SNPs in both strains). In accordance with the mutations in TORC1 and TORC2, *Sc404* was more sensitive to Rapamycin than *Safale S-04*. Incubation of the *Sc404* and *Sc424* yeast strains in growth medium containing 2.2 μM Rapamycin

Functional Category	# Genes in List	# Genes in Category	p Value	Fold Enrichment
Positive regulation of transcription, DNA-templated	8	17	1.2×10^{-3}	4.4
Double-strand break repair	12	37	1.2×10^{-3}	3
DNA repair	35	191	1.8×10^{-3}	1.7
Budding cell apical bud growth	8	20	3.5×10^{-3}	3.7
Peptidyl-serine phosphorylation	10	32	5.1×10^{-3}	2.9
Replicative cell aging	11	39	6.6×10^{-3}	2.6
Chromatin remodeling	14	59	8.3×10^{-3}	2.2
Methylation	18	87	9.7×10^{-3}	1.9
Autophagy	15	67	1.0×10^{-2}	2.1
Mannose metabolic process	4	5	1.0×10^{-2}	7.5

Table 2. Selected Functional Enrichment Categories of Genomic Changes in Sc424 vs. Safeale S-04

Genes where genomic changes occur (see Tables S1 and S2) were subjected to functional enrichment analysis using the DAVID tool (https://david.ncifcrf.gov/summary.jsp). Names of selected categories with their enrichment statistics are listed.

resulted in marked growth inhibition of *Sc404* (note that *Sc404* plateaus at a lower OD than *Safale S-04*). Interestingly, *Sc424* shows no such sensitivity to Rapamycin (Figure 5B). To investigate whether these and additional mutations affected transcription of TOR subunits, we performed qRT-PCR analysis on all TOR components mRNA in rich medium and medium containing ethanol. The results (Figure S4) show no significant differences between the expression levels of TOR components in *Sc404* and *Safale S-04* except for the gene SLM1. It is unlikely that a modest elevation in SLM1 induction alone will lead to a global change in transcriptional control of TOR complexes.

The occurrence of many mutations in the yeast genome may be explained by a phenotype with a higher mutation rate, which was also previously described as occurring during the domestication process of beer yeast (Gallone et al., 2016) and GASP in bacteria (Avrani et al., 2017). We have thus looked at the sequence divergence of our survivor in DNA repair genes. We found (Table 4) that many DNA repair genes, in most DNA repair pathways, are changed. Notable are mutations in genes that were shown to contribute to phenotypes with a higher mutation rate before such as the gene EXO1 (Tran et al., 1999). Many genes involved in homologous recombination (HR) were also mutated such as RAD50, MRE11, RAD51, and RAD54 (Lisby and Rothstein, 2015). Other genes that are components of DNA damage checkpoints such as CHK1 and MEC1 were changed during starvation (Sanchez et al., 1999) (Sanchez et al., 1996). In support of these results, we found that the survivor strains are slightly more sensitive when exposed to ethyl methanesulfonate (EMS), relative to the parental strain (Figure 5C) as expected from a phenotype with a higher mutation rate.

To measure more accurately the mutation rate in the adapted strains, we performed a fluctuation test for the ability of the yeast to grow in a medium containing 5-Fluoroorotic acid (5-FOA) (Green et al., 1976) (Lang, 2018), (Lang and Murray, 2008) (see Methods). With this assay we can estimate the relative abundance of mutations in the strain of origin and its two adapted decadents. Assuming that the rate of mutations in wild-type yeast strains for growth in 5-FOA is on average 5.5×10^{-8} mutations per genome per generation (Lang and Murray, 2008), the results (Figure 5D) point to an elevation of about 3-fold in *Sc424* and about 6-fold in *Sc404*. These results confirm that the basic mutation level in the adapted strains is higher than in the original strain.

Direct Competition Assays

To determine if *Sc404* and *Sc424* are able to outcompete *Safale S-04*, which is a major hallmark of GASP in bacteria, specific primers were designed to allow the identification and quantification of *Safale S-04*
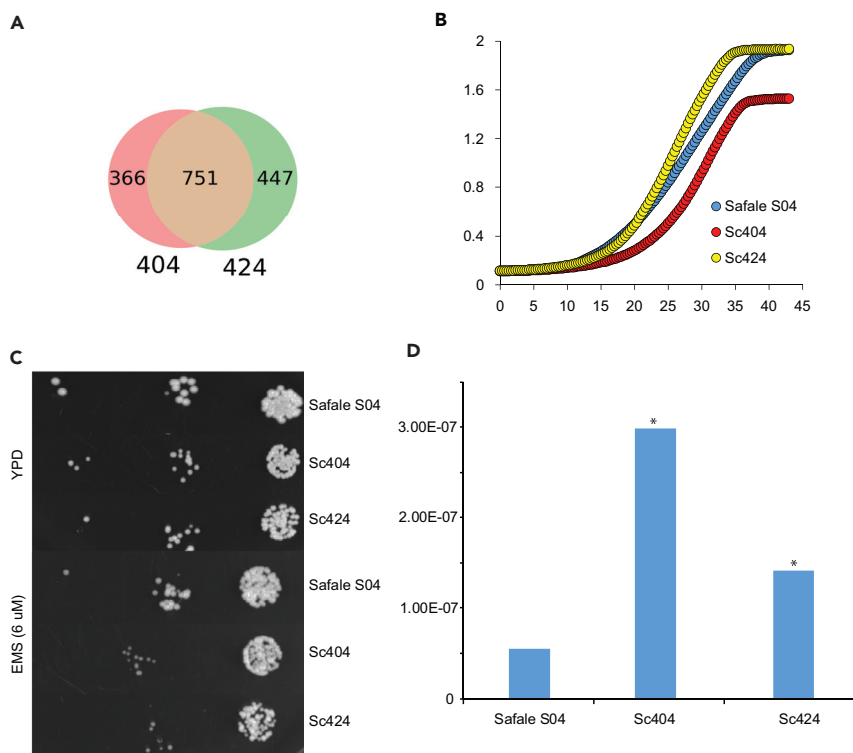
Figure 5. Genomic Analysis of *Safale S-04*, *Sc404*, and *Sc424*

(A–C) (A) The number of genes with genomic changes in *Sc404* and *Sc424* compared with *Safale S-04*—note that 751 genes are changed in both strains. (B) The growth kinetics of *Sc404*, *Sc424*, and *Safale S-04*, represented by measurements of the cultures turbidity in a 96-well plate reader every 20 min for 48 h, were monitored in YPD with 2.2 μM Rapamycin. Data are represented as mean ± SEM. (C) EMS resistance: Overnight yeast cultures were diluted to various concentrations and then spotted on agar plates containing 6 μM of EMS in drops containing 3 mL each. Note that *Sc404* is more sensitive to the drug than *Safale S-04*.

(D) Fluctuation test: a fluctuation test for the ability of the three strains to grow on 5-FOA-containing medium (see Methods). The calculated level of mutations per strain is shown. Note that the level of mutations in *Sc404* and *Sc424* is more than in *Safale S-04*.

differentially from the survivor strains in each competition experiment. Equal numbers of cells had been mixed together in both rich medium and stressful conditions (5% EtOH), and the subpopulations were then monitored for several passages. In each passage the population was diluted 400-fold and re-grown. In each passage samples were taken for DNA extraction and qPCR. Results are presented in Figure 6. These results show that, in both rich medium and in 5% EtOH, *Sc404* and *Sc424* took over the culture after five to six passages. The enhanced capacity of the survivor strains in direct competition in YPD and high ethanol conditions shows that they manifest real GASP capabilities.

Yeast GASP and Cell Divisions

In the previously described bacterial GASP cultures, the growth of new mutants in the culture occurred in waves, each mutant presumably arising from the previous culture and almost entirely taking over the culture owing to growth advantage. This mode of adaptation thus requires active cell division. To test whether active cell division indeed occurs during prolonged starvation in yeast, we subjected the starved cultures (after 2 years of starvation in the same medium as in the starved culture) to live cell imaging. In all fields filmed (see Videos S1 and S2), active cell division was observed (see Figure 7). We also quantified the number of dividing cells in the population, and the average length of the cell cycle during starvation conditions (Figure 7). These measures were used in our estimation of the number of mutations per dividing cell, the number of available mutations in the culture per generation, and the number of total mutations that accumulated in the culture during these 2 years of starvation (see later discussion and Figure 7). To test whether mutations that arise during the course of adaptation indeed increase their

Functional Category	# Genes in List	# Genes in Category	p Value	Fold Enrichment
Double-strand break repair	10	37	5.2×10^{-4}	4.1
DNA repair	26	191	7.2×10^{-4}	2.1
Phosphorylation	26	212	3.2×10^{-3}	1.8
Replicative cell aging	9	39	3.4×10^{-3}	3.5
Intracellular signal transduction	10	48	3.7×10^{-3}	3.1
Chromatin remodeling	11	59	4.8×10^{-3}	2.8
Budding cell apical bud growth	6	20	8.4×10^{-3}	4.5
Protein localization to pre-autophagosomal structure	4	9	1.8×10^{-2}	6.7
Regulation of transcription from RNA polymerase II promoter	15	115	1.9×10^{-2}	2
Late nucleophagy	5	17	2.3×10^{-2}	4.4

Table 3. Selected Functional Enrichment Categories of Shared Genomic Changes in Both Sc404 and Sc424 vs. Safeale S-04

Genes where genomic changes occur (see Tables S1 and S2) were subjected to functional enrichment analysis using the DAVID tool (https://david.ncifcrf.gov/summary.jsp). Names of selected categories with their enrichment statistics are listed.

representation in the culture (eventually representing the vast majority of the culture) owing to selective power, we sequenced by Sanger sequencing two representative loci (in the coding sequence of ALK1 and in the coding sequence of ERV46). These loci were selected on the basis of having conserved primers that can be used for amplifying all strains: Sc404 Sc424 and SafAle S-04, while having multiple SNPs between the strains in the amplified regions themselves (For the primer sequences, see Methods section, primer design paragraph). 30 independent clones from the original starved cultures were sequenced. The results show no SNPs in both regions in all 30 clones of *Sc404* (700- and 1,000-bp-length regions). In the colonies of *Sc424* we found 10 SNPs in 30 colonies in each of the 700- and 1,000-bp regions we sequenced. The SNPs were found each in 1 of the 30 colonies (unrelated SNPs). This number of SNPs amounts to a ratio of ~4 × 10^{-4} of SNPs diverting from the dominant sequence in the culture. These results show that the mutations that are specific to each strain and arose in one clone during the course of the genetic adaptation are now present in almost all the independent clones that we checked. This means these mutations were selected for in the culture, and it supports the appearance and selection of secondary mutations in waves in the culture. It thus seems that GASP in yeast follows the same basic principles and dynamics as the parallel process in bacteria.

Beer Production from GASP Yeast Strains

Understanding the mechanism of yeast stress tolerance may contribute to more efficient ethanol fermentation and beer production. *Sc404* and *Sc424* have acquired modifications that improve their ability to tolerate these exact stresses, compared with the well-known commercial *Safale S-04*. This fact has made *Sc404* and *Sc424* perfect candidates for the production of beer. *Sc404*, *Sc424*, and *Safale S-04* have been used simultaneously for producing beers in the same conditions. The beers that were brewed together under the same conditions were tasted by professional beer tasters (of the Israeli beer association). The three beers differ considerably in their taste characteristics, as shown in the scoring results (Figure S5). Of note is that fact that the tastes and aromas of Sc404 and Sc404 are closer to each other in comparison with those of beer brewed from *Safale S-04.*

DISCUSSION

In the course of their lifespan, cells may find themselves in a variety of different environments and have therefore evolved specific mechanisms to deal with specific acute stress conditions. These mechanisms may become a burden when the cell is exposed to a chronic form of stress. Following this line of thought, an interesting adaptation mechanism to chronic starvation has been described in bacteria. This mechanism was termed GASP (Growth Advantage in Stationary Phase), as the resulting survivors outgrow the parental

TFB4	MCM3	CHK1	SIN3
LIF1	MSH6	DPB3	TFB5
REV1	MCM2	MLH1	RAD2
ACT1	TUP1	RPT6	SMC5
DNA2	MCM7	POL2	RFC4
MSH3	TEL1	LIG4	NUP84
NDK1	RPB1	OGG1	MCM6
MLH3	UNG1	DPB2	SMC6
PAN2	NUP133	GRR1	KU70
HTA1	MCM5	TOR1	PHO85
RAD59	RFA1	SMC3	REV3
SCC2	RAD51	POL1	MSH2
TFB1	SMC1	DOT1	HTB1
RFA2	APN2	EXO1	CKA1
CUL3	SIR2	THO2	RAD17
MEC1	POL3	HAT1	DPB4
SLX4	RVB1	DMC1	SGS1
NTG2	CKB2	RPT4	SSN6
CDC28	MRE11	SUB2	PAN3
RAD52	RDH54	ESA1	RAD54
RAD50			

Table 4. Changed DNA Repair Genes
A list of genes related to DNA repair that are changed in Sc404 and Sc424 vs. Safale S-04.

strain in stressful conditions. Here we describe that a similar adaptation mechanism exists in eukaryotes. As eukaryotic cells are often exposed to the same kinds of stress as bacteria, this may come as no surprise, although the mechanism itself may differ somewhat.

In the same manner as in bacteria, GASP yeast survivors outgrow their parent strain in the stressful conditions it adapted to (see Figure 6). Interestingly, GASP yeast also outgrow the original strain in YPD medium (rich medium), suggesting that YPD poses an adaptive challenge to yeast (perhaps due to fast nutrient exhaustion). The adaptation is composed of a predominantly genetic component rather than an epigenetic one (Figure 3). This point is reflected by the extent of genetic differences between the two strains (Figures 4 and 5). Since multiple ways for stress adaptation exist, including persistence, which contains a major epigenetic component (Sun et al., 2016) (Bojsen et al., 2016) (Knoechel et al., 2014) (Guler et al., 2017) (Rosenberg et al., 2018), this result constitutes a novel and interesting angle to adaptation under stress. This observation could suggest that a prolonged exposure to stress causes a shift toward a genetic adaptation and that in the first stages of adaptation epigenetic forms of adaptation would be prevalent. This hypothesis has been suggested with experimental evidence in bacteria (Levin-Reisman et al., 2017) and merits further experimental exploration in yeast and other eukaryotes in future work. Previous work has addressed the core gene mutations needed for adaptation to environmental stress, and a set of 16 regulators has been identified (Chasman et al., 2014). Interestingly, of the three main genes in this core, HOG1 and PDE2 are mutated in both Sc404 and Sc424 and MCK1 is mutated only in Sc424. Of the remaining 13 genes, 4 (RIM101, GBP2, RIM15, and SWC5) are mutated in both Sc404 and Sc424 and 1 gene (NPR2) is mutated only in Sc424. This shows a high degree of conservation with the critical core of genes needed for genetic adaptation to stress. It should be noted that the fact of using a different experimental approach than ours
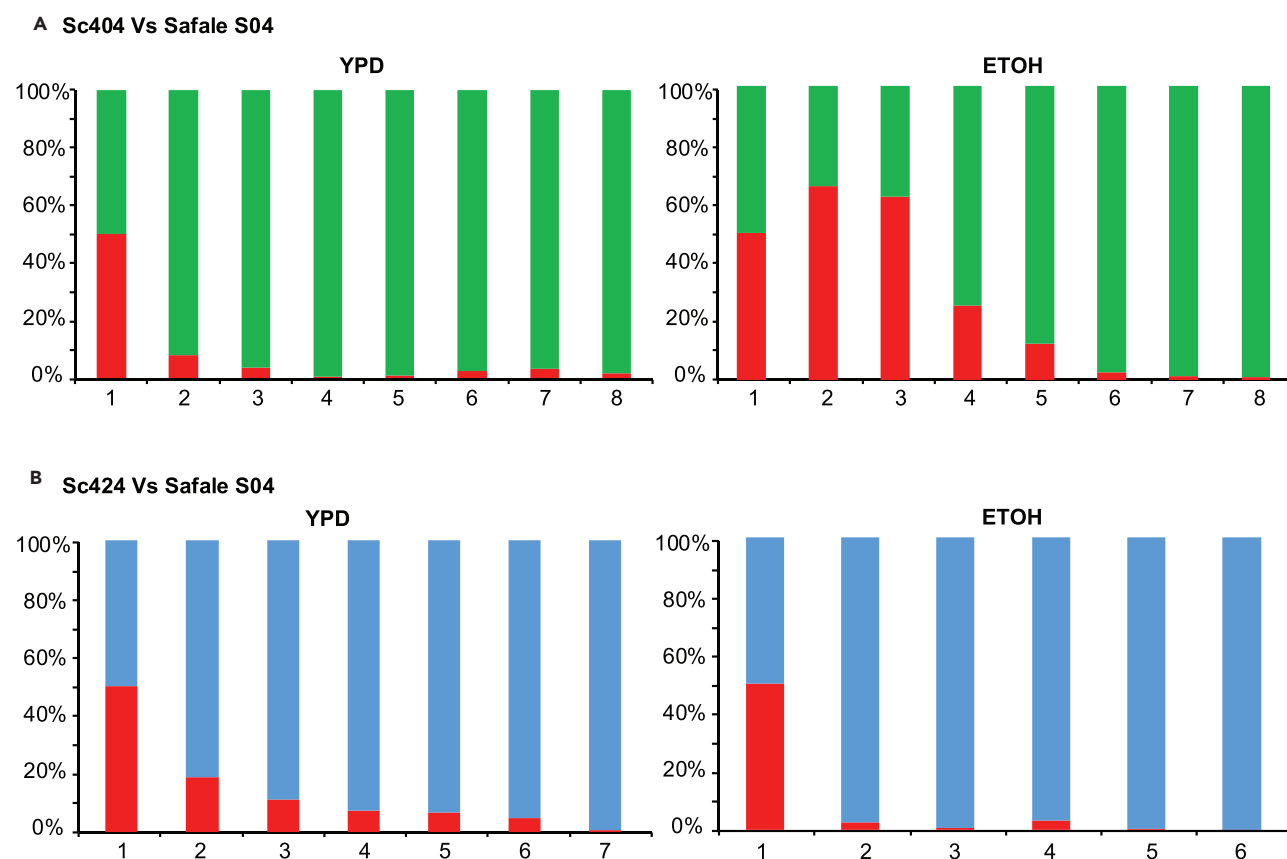
A **Sc404 Vs Safale S04**

B **Sc424 Vs Safale S04**

Figure 6. Direct Competition Assay

(A and B) The relative fractions of *Sc404* (green) (A), *Sc424* (blue) (B), and *Safale S-04* (red) in the culture (after starting from 50-50) was measured at different conditions by qPCR after each dilution (see Methods). Note that in both YPD and Ethanol conditions, *Sc404 and Sc424* take over the culture. Data presented in each experiment are the average of three biological replicates.

(mainly screening of single gene deletion libraries), could underestimate the overlap between the conditions tested by the authors and our starvation conditions.

Our genomic analysis revealed transformative changes in *Sc404* and *Sc424* compared with *Safale S-04*. These changes follow previously described adaptations in eukaryotes, with similarities to GASP in bacteria. Loss and gain of heterozygosity play important roles during eukaryote and yeast evolution (Hope et al., 2017), (Polyak et al., 2009), and the GASP adaptation process is no different. This loss and gain of heterozygosity could in principle, at least in part, originate by cycles of meiosis performed by the yeast cells, which may lead to the reduction in ploidy observed in the survivor strains (Figure 4), although mitotic mechanisms for LOH seem more likely than meiotic mechanisms (Hope et al., 2017), (Polyak et al., 2009). Finally, we also see the adaptation process operating to mutate specific processes that have important downstream influences on life in stressful conditions, such as DNA repair pathways. Mutations in DNA repair genes may manifest in a phenotype with a higher mutation rate, in a similar manner to hyper-mutability described in bacteria under starvation conditions (Avrani et al., 2017). One particularly interesting system that is under the influence of the adaptation process is the TOR complexes (Lushchak et al., 2017) (Gonzalez and Hall, 2017). It seems that TOR1 has a large number of SNPs in both adaptive strains. Additional mutations and changes in the regulation of transcription of the complexes subunits exist (LST8 and LSM1, see earlier text), which make it hard to predict the status of the entire complex and merit additional research. However, this situation bears a remarkable similarity to the mutagenesis of RpoS found in bacteria under GASP conditions (Zambrano et al., 1993) and shows that changes in stress signaling may be required for GASP adaptation.

The dynamics of the population are also similar to bacterial GASP. We show (Figure 7) that cells divide under starvation conditions, presumably feeding off the remains of their deceased brethren. We also show
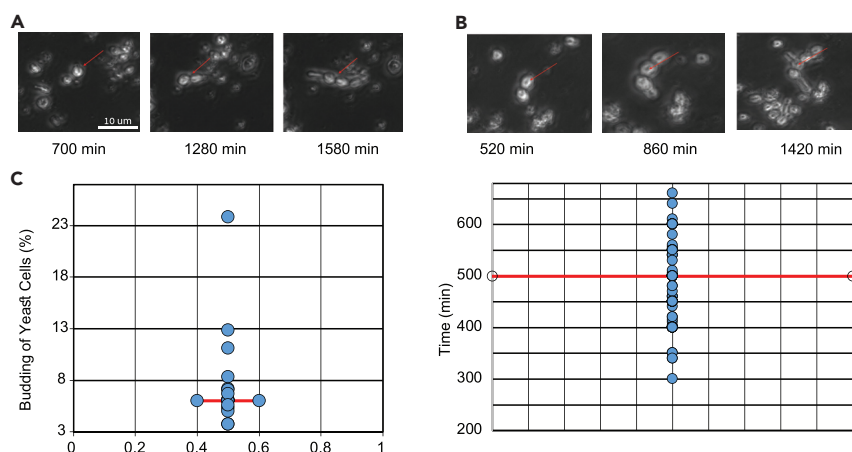
Figure 7. Cell Divisions

(A and B) Examples of still images from live cell imaging experiments of starving cells. Note that cells are dividing (arrows point to the mother cells). See Videos S1 and S2. Scale bar is 10 µM. (C) Percentage of cells that divide in each of the fields imaged by live cell imaging. The red line marks the median level.

(D) Time of division of each dividing cell from bud emergence to the daughter bud emergence. The red line marks the median level.

that these divisions together with strong selective pressure give rise to the high prevalence of specific mutations in the starving population. This leads to the conclusion, as is the situation in bacterial GASP (Avrani et al., 2017), that a mutant outgrows the others in the culture and takes over the vast majority of the population, only to wait for the presentation of a secondary mutant and its selection. From our movies, we devised a mathematical model (see Methods) for the calculation of the number of mutations in the population during the starvation period. Our estimation amounts to the accumulation of the 170,262 mutations for *Sc404* and 79,456 mutations for *Sc424* in 2 years of starvation. This amount of mutations is largely sufficient for the accumulation of approximately 30,000 changed nucleotides in each of the survivors' genomes (after selection). These results also show that dynamic processes and the accumulation of many mutations govern the behavior of cells during starvation, even after years without nutrients. This is indeed the situation in most ecological environments on earth, where microorganisms live with a poor supply of nutrients, competing for available resources and adapting to the situation in a dynamic manner.

We have shown here that GASP can occur in unicellular eukaryotic organisms, but in principle, this adaptation could also operate in multicellular organisms. One intriguing example occurs in tumors. The interior of tumors is characterized by being an extremely stressful environment (Cairns and Mak, 2017). Hypoxia, low pH, and nutrient unavailability all restrict the ability of cells to proliferate. In the same way that yeast cells adapt to a stressful environment in a beer bottle, cancer cells may adapt to harsh conditions in the interior of a tumor. This adaptation may explain the more aggressive phenotype of tumors when they re-grow after surgical excision (Goldfarb and Ben-Eliyahu, 2006). The principle of adaptation through GASP may therefore have broad implications, and the principles shown here in yeast may be conserved in other species and systems.

Limitations of the Study

Our study is limited to the start and end time points of the adaptation process. In the future, a careful time course for the progress of the adaptation needs to be performed. Another limitation comes from the fact that we characterized (albeit to a great degree of detail) only two clones from the adaptation process. In the future, more clones should be analyzed to assess the reproducibility of the course of genetic adaptation.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

iScience

CellPress

SUPPLEMENTAL INFORMATION

ACKNOWLEDGMENTS

AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS

The authors declare they have no conflict of interest.

REFERENCES

Aouizerat, T., Gutman, I., Paz, Y., Maeir, A.M., Gadot, Y., Gelman, D., Szitenberg, A., Drori, E., Pinkus, A., Schoemann, M., et al. (2019). Isolation and characterization of live yeast cells from ancient vessels as a tool in bio-archaeology. MBio 10.

Avrani, S., Bolotin, E., Katz, S., and Hershberg, R. (2017). Rapid genetic adaptation during the first four months of survival under resource exhaustion. Mol. Biol. Evol. 34, 1758–1769.

Baym, M., Lieberman, T.D., Kelsic, E.D., Chait, R., Gross, R., Yelin, I., and Kishony, R. (2016). Spatiotemporal microbial evolution on antibiotic landscapes. Science 353, 1147–1151.

Bojsen, R., Regenberg, B., and Folkesson, A. (2017). Persistence and drug tolerance in pathogenic yeast. Curr. Genet. 63, 19–22.

Bojsen, R., Regenberg, B., Gresham, D., and Folkesson, A. (2016). A common mechanism involving the TORC1 pathway can lead to amphotericin B-persistence in biofilm and planktonic Saccharomyces cerevisiae populations. Sci. Rep. 6, 21874.

Brauner, A., Fridman, O., Gefen, O., and Balaban, N.Q. (2016). Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat. Rev. Microbiol. 14, 320–330.

Cairns, R.A., and Mak, T.W. (2017). Fire and water: tumor cell adaptation to metabolic conditions. Exp. Cell Res. 356, 204–208.

Casalinuovo, I.A., Di Francesco, P., and Garaci, E. (2004). Fluconazole resistance in Candida albicans: a review of mechanisms. Eur. Rev. Med. Pharmacol. Sci. 8, 69–77.

Chasman, D., Ho, Y.H., Berry, D.B., Nemec, C.M., MacGilvray, M.E., Hose, J., Merrill, A.E., Lee, M.V., Will, J.L., Coon, J.J., et al. (2014). Pathway connectivity and signaling coordination in the yeast stress-activated signaling network. Mol. Syst. Biol. 10, 759.

Farrell, M.J., and Finkel, S.E. (2003). The growth advantage in stationary-phase phenotype conferred by rpoS mutations is dependent on the pH and nutrient environment. J. Bacteriol. 185, 7044–7052.

Finkel, S.E. (2006). Long-term survival during stationary phase: evolution and the GASP phenotype. Nat. Rev. Microbiol. 4, 113–120.

Finkel, S.E., and Kolter, R. (1999). Evolution of microbial diversity during prolonged starvation. Proc. Natl. Acad. Sci. U S A 96, 4023–4027.

Fisher, R.A., Gollan, B., and Helaine, S. (2017). Persistent bacterial infections and persister cells. Nat. Rev. Microbiol. 15, 453–464.

Gallone, B., Steensels, J., Prahl, T., Soriaga, L., Saels, V., Herrera-Malaver, B., Merlevede, A., Roncoroni, M., Voordeckers, K., Miraglia, L., et al. (2016). Domestication and divergence of Saccharomyces cerevisiae beer yeasts. Cell 166, 1397–1410.e16.

Gerstein, A.C., Lim, H., Berman, J., and Hickman, M.A. (2017). Ploidy tug-of-war: evolutionary and genetic environments influence the rate of ploidy drive in a human fungal pathogen. Evolution 71, 1025–1038.

Goldfarb, Y., and Ben-Eliyahu, S. (2006). Surgery as a risk factor for breast cancer recurrence and metastasis: mediating mechanisms and clinical prophylactic approaches. Breast Dis. 26, 99–114.

Gonzalez, A., and Hall, M.N. (2017). Nutrient sensing and TOR signaling in yeast and mammals. EMBO J. 36, 397–408.

Gray, J.V., Petsko, G.A., Johnston, G.C., Ringe, D., Singer, R.A., and Werner-Washburne, M. (2004). "Sleeping beauty": quiescence in Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev. 68, 187–206.

Green, M.H., Muriel, W.J., and Bridges, B.A. (1976). Use of a simplified fluctuation test to detect low levels of mutagens. Mutat. Res. 38, 33–42.

Guler, G.D., Tindell, C.A., Pitti, R., Wilson, C., Nichols, K., KaiWai Cheung, T., Kim, H.J., Wongchenko, M., Yan, Y., Haley, B., et al. (2017). Repression of stress-induced LINE-1 expression protects cancer cell subpopulations from lethal drug exposure. Cancer Cell 32, 221–237.e13.

Harms, A., Maisonneuve, E., and Gerdes, K. (2016). Mechanisms of bacterial persistence during stress and antibiotic exposure. Science 354, aaf4268-1–aaf4268-9.

Hickman, M.A., Paulson, C., Dudley, A., and Berman, J. (2015). Parasexual ploidy reduction drives population heterogeneity through random and transient aneuploidy in Candida albicans. Genetics 200, 781–794.

Hope, E.A., Amorosi, C.J., Miller, A.W., Dang, K., Heil, C.S., and Dunham, M.J. (2017). Experimental evolution reveals favored adaptive routes to cell aggregation in yeast. Genetics 206, 1153–1167.

Huang, S., and Houghton, P.J. (2001). Mechanisms of resistance to rapamycins. Drug Resist. Updat. *4*, 378–391.

Huang, S., Liu, L.N., Hosoi, H., Dilling, M.B., Shikata, T., and Houghton, P.J. (2001). p53/ p21(CIP1) cooperate in enforcing rapamycin-induced G(1) arrest and determine the cellular response to rapamycin. Cancer Res. *61*, 3373–3381.

Knoechel, B., Roderick, J.E., Williamson, K.E., Zhu, J., Lohr, J.G., Cotton, M.J., Gillespie, S.M., Fernandez, D., Ku, M., Wang, H., et al. (2014). An epigenetic mechanism of resistance to targeted therapy in T cell acute lymphoblastic leukemia. Nat. Genet. *46*, 364–370.

Lang, G.I. (2018). Measuring mutation rates using the Luria-Delbruck Fluctuation assay. Methods Mol. Biol. *1672*, 21–31.

Lang, G.I., and Murray, A.W. (2008). Estimating the per-base-pair mutation rate in the yeast *Saccharomyces cerevisiae*. Genetics *178*, 67–82.

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., and Balaban, N.Q. (2017). Antibiotic tolerance facilitates the evolution of resistance. Science *355*, 826–830.

Lisby, M., and Rothstein, R. (2015). Cell biology of mitotic recombination. Cold Spring Harb. Perspect. Biol. *7*, a016535.

Lushchak, O., Strilbytska, O., Piskovatska, V., Storey, K.B., Koliada, A., and Vaiserman, A. (2017). The role of the TOR pathway in mediating the link between nutrition and longevity. Mech. Ageing Dev. *164*, 127–138.

Orruno, M., Kaberdin, V.R., and Arana, I. (2017). Survival strategies of *Escherichia coli* and Vibrio spp.: contribution of the viable but nonculturable phenotype to their stress-resistance and persistence in adverse environments. World J. Microbiol. Biotechnol. *33*, 45.

Polyak, K., Haviv, I., and Campbell, I.G. (2009). Co-evolution of tumor cells and their microenvironment. Trends Genet. *25*, 30–38.

Rosenberg, A., Ene, I.V., Bibi, M., Zakin, S., Segal, E.S., Ziv, N., Dahan, A.M., Colombo, A.L., Bennett, R.J., and Berman, J. (2018). Antifungal tolerance is a subpopulation effect distinct from resistance and is associated with persistent candidemia. Nat. Commun. *9*, 2470.

Sanchez, Y., Bachant, J., Wang, H., Hu, F., Liu, D., Tetzlaff, M., and Elledge, S.J. (1999). Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. Science *286*, 1166–1171.

Sanchez, Y., Desany, B.A., Jones, W.J., Liu, Q., Wang, B., and Elledge, S.J. (1996). Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. Science *271*, 357–360.

Sun, J., Li, Z., Chu, H., Guo, J., Jiang, G., and Qi, Q. (2016). *Candida albicans* amphotericin B-tolerant persister formation is closely related to surface adhesion. Mycopathologia *181*, 41–49.

Tran, H.T., Gordenin, D.A., and Resnick, M.A. (1999). The 3′–>5′ exonucleases of DNA polymerases delta and epsilon and the 5′–>3′ exonuclease Exo1 have major roles in postreplication mutation avoidance in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *19*, 2000–2007.

Zambrano, M.M., and Kolter, R. (1996). GASPing for life in stationary phase. Cell *86*, 181–184.

Zambrano, M.M., Siegele, D.A., Almiron, M., Tormo, A., and Kolter, R. (1993). Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. Science *259*, 1757–1760.

Zinser, E.R., and Kolter, R. (1999). Mutations enhancing amino acid catabolism confer a growth advantage in stationary phase. J. Bacteriol. *181*, 5800–5807.

Zinser, E.R., and Kolter, R. (2000). Prolonged stationary-phase incubation selects for lrp mutations in *Escherichia coli* K-12. J. Bacteriol. *182*, 4361–4365.

Zinser, E.R., and Kolter, R. (2004). *Escherichia coli* evolution during stationary phase. Res. Microbiol. *155*, 328–336.

Zinser, E.R., Schneider, D., Blot, M., and Kolter, R. (2003). Bacterial evolution through the selective loss of beneficial Genes. Trade-offs in expression involving two loci. Genetics *164*, 1271–1277.

Supplemental Information

Eukaryotic Adaptation to Years-Long

Starvation Resembles that of Bacteria

Tzemach Aouizerat, Daniel Gelman, Amir Szitenberg, Itay Gutman, Shunit Glazer, Eli Reich, Miriam Schoemann, Rachel Kaplan, Amijai Saragovi, Ronen Hazan, and Michael Klutstein
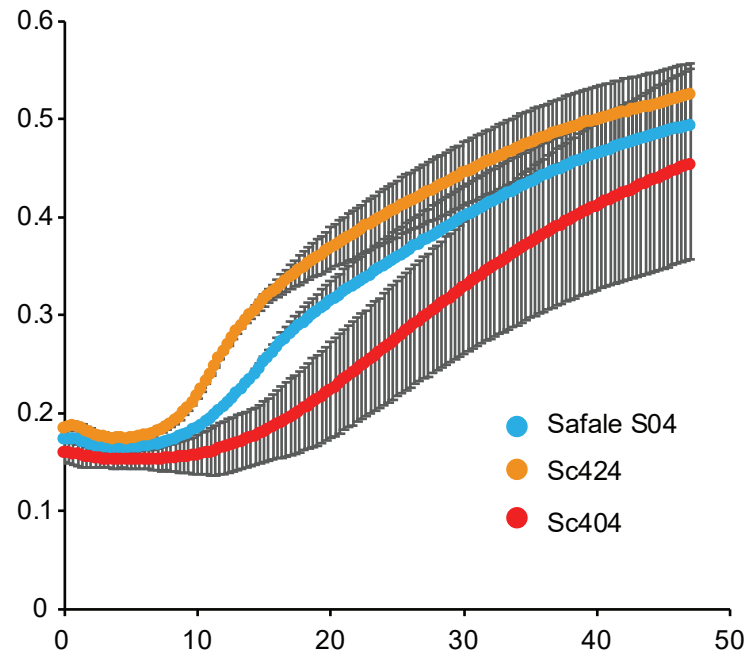
Depleted Medium

Figure S1 related to Figure 1: growth kinetics in nutrient depleted medium: the parental strain and two adapted strains were grown in a nutrient depleted medium (see methods). Note that *Sc424* has an advantage over the parental strain in growth abilities in this nutrient poor environment at 26°C. Data are represented as mean ± SEM
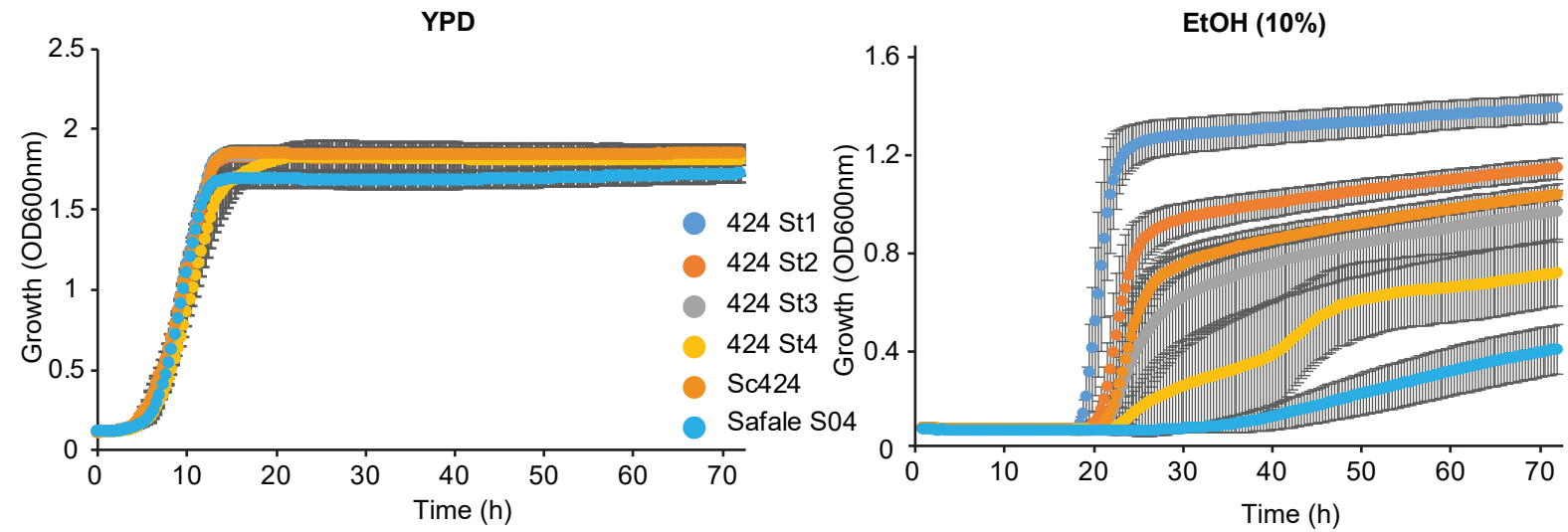
Figure S2 related to Figure 3: *Sc424* **cells maintain their stress-driven characters after serial dilutions:** *424St* strains were evolved by diluting overnight cultures of *Sc424* through a period of six days. The *424St* strains present similar qualities to those of *Sc424* such as in (A) growth in YPD and (B) in the presence of ethanol (10% v/v) , represented by measurements of the cultures turbidity in a 96 well plate reader every 20 minutes for 48 hours. Data are represented as mean ± SEM

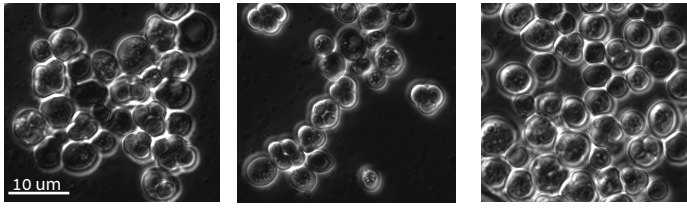Safale S04   Sc404   Sc424

10 um

Figure S3 related to Figure 4: Sporulation: Cells from SPO plates are shown. Note that asci and spores appear in all three strains. Scale bar is 10 µM
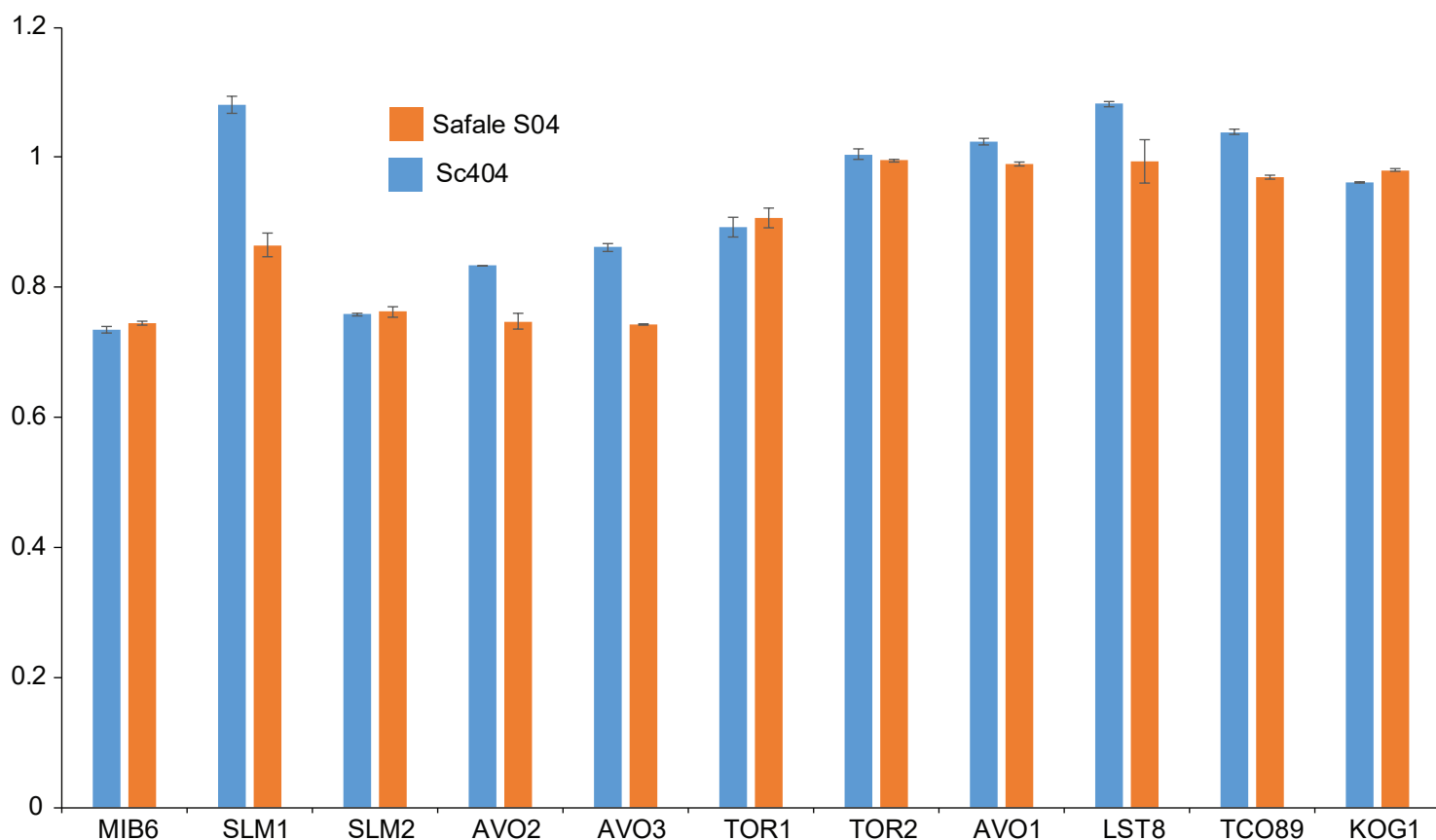
Figure S4 related to Figure 5: expression of TORC mRNAs: RNA was isolated from *Sc404* and *Safale S04* grown in YPD medium and YPD medium containing 5%(v/v) Ethanol. qRT-PCR was performed for TORC genes and the ratio between the levels of the mRNA in YPD and Ethanol were compared and normalized to the level of the actin gene. Results are presented as fold change between Ethanol and YPD conditions. Note that except for SLM1, none of the other genes significantly changed its expression pattern between *Sc404* and *Safale S04*. Data are represented as mean ± SEM
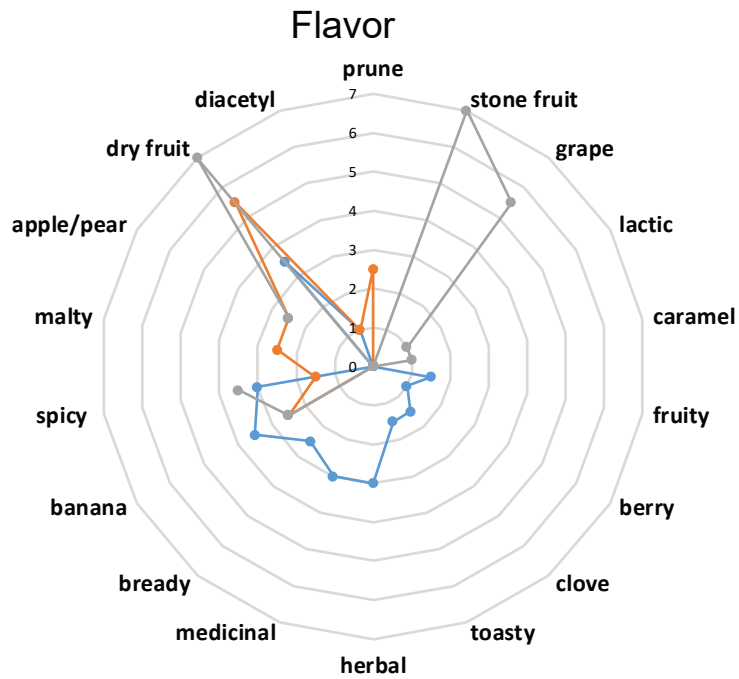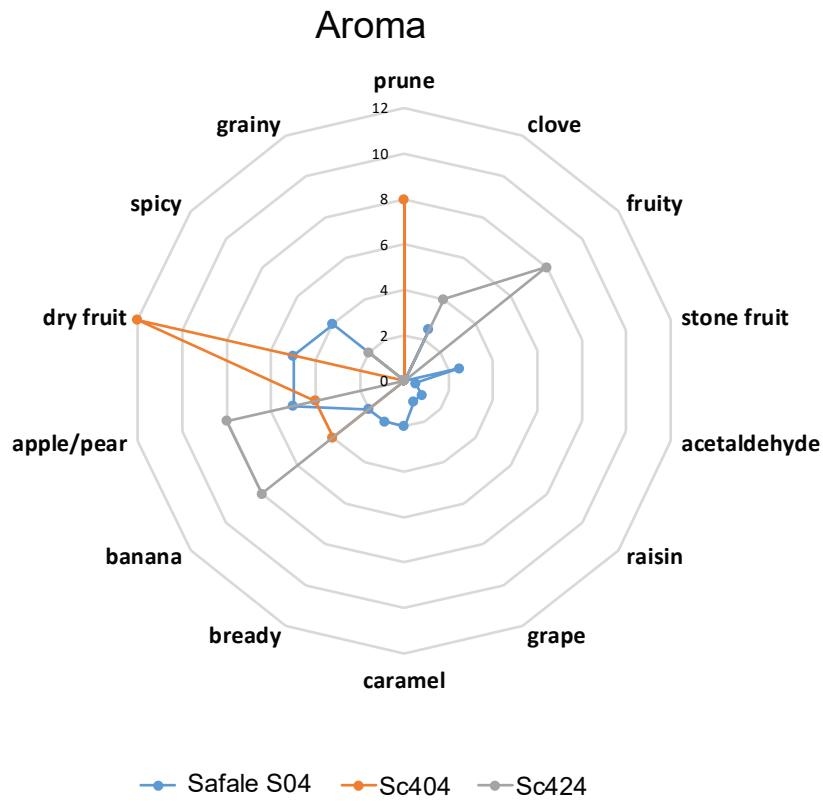
Aroma

Flavor

Figure S5 related to Figure 1: Beer Preparation: *Sc404* and *Safale S04* were used to brew two different beers under the same conditions, and their aroma and flavor profiles were determined, according to the Jerusalem Beer Tasters panel- a group of professional beer tasters.

Transparent Methods:

Strains:

The commercial brewer's *Saccharomyces cerevisiae* strain used in this work, termed *Safale S-04* was purchased from Fermentis (Division of S.I.Lesaffre, France). *Sc404* and *Sc424* derive from this strain, isolated from 2-year-old bottled beers brewed from *Safale S-04*. After two years in starvation conditions, we streaked the culture on YPD plates, and picked a single colony which was used as the basis for further studies. In order to validate the representative nature of the single colony, we have isolated 30 colonies, and checked their growth rate in various conditions. The rate of growth was nearly identical (data not shown). As mentioned above, we have also sequenced two regions in 30 colonies. These results show that the mutations which are specific to each strain are present in almost all the independent clones that we checked, and that the single colonies we picked are representative (see above results section).

S288C, the commonly lab strain was used as a reference strain.

Yeast Growth Conditions:

Unless mentioned otherwise, the yeast strains used in this work were routinely grown from a single colony either in liquid YPD medium (Difco) at 30°C, in aerobic conditions with agitation (250–300 rpm) or on solid YPD medium containing 2% w/v Bacto-agar (Difco) incubated at 30°C.

Nutrient depleted filtrate medium - Nutrient depleted filtrate growth tests were performed in growth media taken from an old beer bottle (2-years-old) at a temperature of 26 degrees. The fluid was filtered through a 0.22 uM filter**.**

Transfers for Testing Epigenetic Effect

Sc404 strain was grown as described in 30ml of liquid YPD, starting from the original sequenced colonies and then separated into three separate sub-cultures. During a period of six days, the culture was diluted by factor 30,000 in intervals of 24 hours, into fresh media with the same volume. The dilution rate of 30,000 was determined experimentally, in order to bring the saturated cultures after back to the starting concentration. The concentration of the culture after 18 hours is in the order of $\sim 10^9$ cells per ml, and after 30,000-fold dilution the concentration will be $\sim 3 \times 10^4$ cells per ml, high enough to prevent a bottleneck effect. The sub-cultures were then frozen and termed *404St1*, *404St2* and etc.

Growth Rate:

Overnight cultures were diluted by factor of 1000, and their growth was monitored turbidimetrically in a 96-well plate reader (Synergy; BioTek, Winooski, VT) at 600 nm with 5 seconds shaking every 20 minutes, during a period of 48 hours. Each result represents an average value of three independent cultures.

Survival Tests:

The yeast strains were grown as described above for 24 hours prior to performing the experiment. The following conditions were tested:

Ethanol stress. Samples of 100 µL of each strain were washed and re-suspended in 100 µL solution containing 27.5% (v/v) ethanol for 1 hour. The cells were then washed, and plated on YPD agar plates.

Heat Shock. Samples of 100 µL of each strain were exposed to 55°c for 10 minutes, and then plated on YPD agar plates.

Stressful Growth Conditions:

Ethanol stress. Ethanol growth tests were performed in solutions containing 80% (v/v) YPD and 20% (v/v) consisting of ethanol and DDW, according to the noted percentage of ethanol in each test.

pH stress. pH growth tests were performed in sterile YPD tittered to different acidity levels as noted, using NaOH and HCl.

Osmotic stress. Osmotic pressure tests were performed in solutions of YPD containing different concentrations of NaCl (0.5M, 1M, 1.5M, 2M).

DNA Extraction

Yeast DNA isolation was performed as previously described (Mann and Jeffery, 1989). Briefly, 10 ml of overnight cultures were centrifuged at 3000 rpm for 5 minutes, and washed in sterile water. The cells were treated with 200 µL of phenol chloroform, 0.3 g of acid washed glass beads and 200 µL of Smash and Grab solution (Mann and Jeffery, 1989) , and broken using vortex for 3 minutes, after which TE buffer was added. The cells were centrifuged, and the aqueous layer containing the DNA was transferred to 1 ml ethanol, washed and suspended in TE buffer. 1 µL of RNase (10 mg/ml DNase- and Protease-free RNase, Thermo Fisher Scientific) was added, and the solution was incubated at 37°C for 5 minutes. 10 µL of ammonium acetate (4M) and 1 ml of ethanol were then added, and the solution was washed, and suspended in 100 µL of TE buffer. The extracted DNA was stored at -20°C.

DNA Quantification

DNA quantification was carried out on a Synergy H1 microplate

reader (BioTek Instruments, Inc., Vermont, USA), using a Take3 micro-volume plate.

Real Time PCR Reactions

The Real Time PCR was performed using the CFX96 real-time PCR detection system
(Bio-Rad, Hercules, CA, USA) in 96 well micro-plates. Amplifications were initiated
by 5 sec at 95°C, followed by 40 cycles consisting of 95°C for 5 sec and 60°C for
30sec. The temperature was then raised by 0.5°C every 5sec from 65°C to 95°C to
obtain the melting temperature for each specific reaction.

Samples of 20µL were prepared from 10 µL iTaq[TM] Universal SYBR Green
Supermix (Bio-Rad, Hercules, CA, USA), 10pmol (1µL of 10µM) of each primer and
1µL DNA template. Ultra-pure water was added to obtain a total volume of 20µL.

Direct competition assay:

Equal numbers of cells from both *Sc404* and *Safale So4* strains were mixed together
in optimal and stressful conditions. 10^3 cells/ml of each culture were mixed and grown
in rich YPD medium, and 10^4 cells/ml of each culture were mixed and grown in YPD
containing 1M of NaCl or 5% (v/v) ethanol. Every three to five days, overnight
cultures of the mixtures were diluted to their initial concentration, and samples were
taken for DNA extraction. The ratio between the different yeast strains subpopulations
was monitored using Real Time PCR Reaction. DNA extracted from samples taken
from the mixture was diluted to a final concentration of 50 ng/µl for the Real Time
PCR reaction.

Primer Design

We quantified the culture by two sets of strain non-specific primers, compared with
Safale S-04 specific primers. For the *Safale S-04* specific primer, the sequences of the
two yeast strains were aligned to identified regions that are present in *Safale S-04* but
not in *Sc404* or *Sc424*. Primers for RT-PCR were then designed from these regions,
using Primer 3 (http://primer3.ut.ee) (Untergasser et al., 2012) (Koressaar and Remm,
2007). The specificity of each set of primers was tested by a Real Time PCR reaction

for each set of primers with both *Sc404* and *Safale S-04* individually and in combination, in different ranges of known DNA concentrations.

Primers:

SafaleOnly – F –GCAATAGGCGCAATCACACT, R- GGGAACAAATAAGCAAGCCG

Both1 - F –AACGAGGGCACCTTCATCTG, R – GGCCACTGTTGAAGATGGGA

Both 2 – F –TGCTTCGACTGGAGCTAACC, R – CGCAAGAGAGATTTTCGCCG

For validation of genomic sequences and their consistency between different clones, we planned two sets of primers: in the coding sequence of ALK1 and in the coding sequence of ERV46. These loci were selected on the basis of having conserved primers that can be used for amplifying all strains: Sc404 Sc424 and SafAle S04, while having multiple SNPs between the strains in the amplified regions themselves. Planned primer sequences:

ALK1 F- ATTTTAAACCCCGCAAAAGG R-TTGATACTGGTATTCGTTTA (700 bp)

ERV46 F-TGATACCTTTATCGTGCATC R-ACATTTCGAAAAAGACGAAC (1000 bp)

RNA extraction and cDNA Synthesis

Yeast RNA isolation was performed using a YeaStar RNA Kit (Zymo Research), according to the manufacturer instructions. The isolated RNA was then purified using RNA Clean&Concentrator^{TM} (Zymo Research). The extracted RNA was stored at -80°C.

cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer instructions. cDNA was stored at -20c.

Quantification of Gene Expression Using cDNA Real Time PCR Reaction

Primer sets for the TOR complex were designed using Primer 3 (http://primer3.ut.ee) (Untergasser et al., 2012) (Koressaar and Remm, 2007). The primers were then used in Real Time PCR with the cDNA synthetized from each yeast strain RNA. For reactions containing cDNA from yeast grown in YPD the concentration of cDNA used was 500 ng/µl. For reactions containing cDNA from yeast grown in YPD

containing 5% of ethanol, the concentration of cDNA used was 1500 ng/µl. The ratio between the gene expression level between optimal and sub-optimal conditions were quantified for each strain, and then compared between the strains.

Primers:

KOG1 - F – CGGTTGAAGTGGACATTCCT, R – GCTCTCCAGCGACGTATCAT

TOR 1 - F – TGGAATTGAAGGCAGTTTCC R – ATCAAATCCCCAATGGATCA

TOR 2 - F – CCTTGAAGCTTTTGCTTTCG R– TCGTTTTCCACCCTTTGAAC

LST8 - F -GCGGCAGCAAATACTAAAGG R – ACACACGAGCAGTGTGATCC

TCO89 - F – TGCAAGTGCAAAGATTCAGG R – CGTGCATTCTGTGAAGCAGT

AVO1 - F – ATCCTCTTTGCATGGGACAC R – TGCTGCCTTCTCCATACCTT

AVO3 - F – CCTCAAGCAACAGTTCGACA R - GGTCGCCCAATTCTTCACTA

AVO2 - F – GGCACGACAAGCATGAACTA R – ATATGGATGGGGGCTCTACC

BIT61 -F– AGCCGGCAAACTCTTGTAGA R – GCATTGTCATTGCTCCTCAA

SLM1 - F – TCAGCACCTGCATAACTTGC R– GCTGCTGCTGTTGTTGTTGT

SLM2 - F– GCCTATCAACAGCAGCAACA R –TTTTGCCGATCGTGATCATA

ACT1- F- CTGCCGGTATTGACCAAACT R- CGGTGATTTCCTTTTGCATT

EMS sensitivity

Ethyl methanesulfonate (Sigma) in a concentration of 0.0097M was added to 25 ml of YPD agar plates before pouring, in various volumes (between 5 to 25 µl). Overnight yeast cultures were then diluted to various concentrations and then spotted on the agar plates in drops containing 3 µl each.

Rapamycin sensitivity

Rapamycin (Glentham Life Sciences) resuspended in DMSO to a concentration of 0.27M was diluted in 100% ethanol to a final concentration of 0.0027M. For controls, an equal volume of DMSO was diluted in ethanol in the same conditions. The overnight yeast cultures were then diluted to a concentration of 10^5 CFU/ml in YPD, and mixed to solutions containing $2.7*10^{-5}$M of rapamycin (resuspended in ethanol as described). The growth was monitored turbidimetrically in a 96-well plate reader (Synergy; BioTek, Winooski, VT) at 600nm with 5 seconds shaking every 20

minutes, during a period of 48 hours. Each result represents an average value of three independent cultures.

Beer Production

4.5 L of autoclaved water were frozen in -20°C a night before the beginning of the brewing.

5 L of water were heated to 70°C on fire. Malt extract was then added to the pre-heated water to obtain a final concentration of 100 gr/L, while thoroughly stirring, and cooked together for 30 min between 63-67°C . The solution was then heated to reach 100°C , and once boiling has occurred 1 gr/L of hops were added. The mixture remained boiling for 45 more minutes, following addition of another 1 gr/L of hops. The mixture was then heated for an additional minute.

The previously prepared ice cold water was then added to the mixture, and the prepared wort was passed into a sanitized fermenter to reach a final volume of 10 L at room temperature.

The wort was left at room temperature for 30 min, and then overnight cultures of yeast were added. Fermentation typically began within 12-48h, and the mixture was left untouched for a week.

Flow cytometry

To visualize cell size, yeast cultures were sampled and analyzed based on their forward scatter and side scatter readings. For DNA quantification, yeast cells were first permeabilized and fixed using ice cold methanol and then labelled with propidium iodide 100ul/ml (Synonym: 3,8-Diamino-5-(3-diethylaminopropyl)-6-phenylphenanthridinium iodide methiodide, 3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium, diiodide) (P4864 SIGMA-ALDRICH) at room temperature prior to measurements. Stained cells were analyzed by Gallios flow cytometer with KALUZA software (Beckman Coulter, Brea, CA) and analyzed using Flowjo analysis software (FLOWJO, LCC).

Fluctuation assays:

Fluctuation assays were performed to determine the rate at which cells mutated to become resistant to 5-FOA. To begin each fluctuation assay, a single colony of each yeast stain was grown overnight to saturation in SC−Ura. The cultures were then diluted 1:10,000 into SC media, and grown overnight. On the following day, each

culture was diluted 1:16 into 5-FOA medium, and dispensed into 96-well plates in a volume of 100uL, to a total amount of 128 wells for each of the yeast strains. The cultures were grown for 2 days at 30° without shaking. Positive turbid wells, suggesting of mutation occurrence during the growth, were determined if the turbidity of the well was greater than 1.15 multiplicity of median for the turbidity of the specific strain, counted using 96-plate reader. The number of mutations was determined as in (Foster, 2006).

DNA Sequencing

Sequencing was performed in the interdepartmental unit at the Hebrew University, Hadassah Campus. Libraries were prepared by using a Nextera XT DNA kit (Illumina, San Diego, CA), and DNA was amplified by a limited-cycle PCR and purified using AMPure XP beads. The DNA libraries were normalized, pooled, and tagged in a common flow cell at 2×500 base-paired-end reads using the Illumina MiSeq platform. The quality of the reads was determined using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Sequencing data pipeline and analysis:

To further investigate ploidy changes and to study the loss of heterozygosity in *Sc404* and Sc424 compared to the commercial strain Safale S04, we analyzed short read data recovered from the three strains. The analysis was made reproducible by recording the scripts in a Jupyter notebook and publishing it on GitHub, along with associated files. Raw sequence reads were trimmed with Trimmotaic V 0.36 (Bolger et al., 2014) to remove illumina adaptors and quality trim sequence windows with low Phred scores (lower than 30). The trimmed reads were used in all downstream analyses.
We published the details of our pieline in the following address on Github: https://github.com/DSASC/Aouizerat_Gelman_et_al_2019 or in Zenodo DOI: DOI: 10.5281/zenodo.3268716

Ploidy of Safale, Sc404 and Sc424:

To reconstruct the ploidy of the three isolates, we used Jellyfish V2.2 (Marcais and Kingsford, 2011) to compute a kmer distribution. Smudgeplot v0.1.3 (https://github.com/tbenavi1/smudgeplot/tree/v0.1.3) was then used to determine the

number of genome copies by cross referencing the coverage of heterozygote kmers with the frequency of the rare variant in each heterozygote kmer. We further investigated ploidy of each chromosome, by mapping the read data of the three strains to the genome assembly of the wild type strain S288C (Assembly R46, GCA_000146045.2) using BWA v0.7 (Li and Durbin, 2009). We accounted for GC content bias using DeepTools v2 (Ramirez et al., 2016).

Gain and loss of heterozygosity in Sc404 and Sc424:

To identify gain and loss of heterozygosity in Sc404 and Sc424, compared to the Safale S04 isolate, polymorphisms within the BWA alignment of each sample were detected using FreeBayes v1.2 . We made sure to consider only polymorphism in the sequence data of each sample and ignore changes in comparison with the reference genome assembly. Gain of heterozygosity in Sc404 or Sc424 was defined as a polymorphism that did not exist in Safale S04, and loss of heterozygosity was the lack of polymorphism in a Sc404 or Sc424 sequence locus that contained polymorphism in Safale S04 . To identify genes in which polymorphism was gained or lost, we carried out a functional annotation of the reference genome proteins, using eggnog-mapper v1 (Huerta-Cepas et al., 2017).

Shared and unique polymorphism between Sc404 and Sc424:

To identify shared and unique polymorphism between Sc404 and Sc424, we first produced a de-novo assembly of Safale S04 to serve as a reference. We selected the celera assembler v-wgs-8.3 (Huson et al., 2001), which produced superior assemblies compared to some more modern assemblers. This was confirmed by producing blobplots (Laetsch and Blaxter, 2017) and comparing assembly statistics. An example comparison between assemblies of different programs is available on the GitHub repository. Safale S04 genes were identified using Augustus V3 (Keller et al., 2011) and were functionally annotated with eggnog-mapper v1 (Huerta-Cepas et al., 2017). Sc404 and Sc424 reads were mapped to the SefAle genome, and polymorphisms identified, as described above.

Model for mutation rate:

From the fluctuation test, we could estimate the rate of mutations per genome per

generation in both strains (3×10^{-7} for Sc404, 1.4×10^{-7} for Sc424). This mutation rate was measured in both strains between two time points using a fluctuation assay (see above). Using our data from the movies we filmed (Figure 7), we could estimate the percentage of dividing cells in the culture (6% of cells on average). This measure then directs towards calculating the rate of mutations in dividing cells only (1.8×10^{-8} for Sc404, 8.4×10^{-9} for Sc424). We then counted the amount of cells in the original culture using a hemocytometer (4.5×10^{7} cell per ml). Using this count, and assuming no selection or clonal interference, we could then estimate the total number of mutations in the entire culture (100 ml) per generation (81 mutations for Sc404, 38 mutations for Sc424) which could arise if no selection was applied. Using data from our movies (Figure 7), we estimate that the cell cycle in starvation conditions is 500 minutes long on average. This amounts to 2102 generations in two years of starvation, and to the accumulation of the 170262 mutations for Sc404 and 79456 mutations for Sc424 with no selection. Since in reality around 30000 mutated nucleotides were found in each of the strains, this model shows the strength of selection and clonal interference in these starved and stressed cultures.

Supplemental references:

Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics *30*, 2114-2120.

Foster, P.L. (2006). Methods for determining spontaneous mutation rates. Methods Enzymol *409*, 195-213.

Huerta-Cepas, J., Forslund, K., Coelho, L.P., Szklarczyk, D., Jensen, L.J., von Mering, C., and Bork, P. (2017). Fast Genome-Wide Functional Annotation through Orthology Assignment by eggNOG-Mapper. Mol Biol Evol *34*, 2115-2122.

Huson, D.H., Reinert, K., Kravitz, S.A., Remington, K.A., Delcher, A.L., Dew, I.M., Flanigan, M., Halpern, A.L., Lai, Z., Mobarry, C.M.*, et al.* (2001). Design of a compartmentalized shotgun assembler for the human genome. Bioinformatics *17 Suppl 1*, S132-139.

Keller, O., Kollmar, M., Stanke, M., and Waack, S. (2011). A novel hybrid gene prediction method employing protein multiple sequence alignments. Bioinformatics *27*, 757-763.

Koressaar, T., and Remm, M. (2007). Enhancements and modifications of primer design program Primer3. Bioinformatics *23*, 1289-1291.

Laetsch, D.R., and Blaxter, M.L. (2017). KinFin: Software for Taxon-Aware Analysis of Clustered Protein Sequences. G3 (Bethesda) *7*, 3349-3357.

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics *25*, 1754-1760.

Mann, W., and Jeffery, J. (1989). Isolation of DNA from yeasts. Anal Biochem *178*, 82-87.

Marcais, G., and Kingsford, C. (2011). A fast, lock-free approach for efficient parallel counting of occurrences of k-mers. Bioinformatics *27*, 764-770.

Ramirez, F., Ryan, D.P., Gruning, B., Bhardwaj, V., Kilpert, F., Richter, A.S., Heyne, S., Dundar, F., and Manke, T. (2016). deepTools2: a next generation web server for deep-sequencing data analysis. Nucleic Acids Res *44*, W160-165.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., and Rozen, S.G. (2012). Primer3--new capabilities and interfaces. Nucleic Acids Res *40*, e115.