RESEARCH ARTICLE

Stress Alters Rates and Types of Loss of Heterozygosity in *Candida albicans*

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ABSTRACT **Genetic diversity is often generated during adaptation to stress, and in eukaryotes some of this diversity is thought to arise via recombination and reassortment of alleles during meiosis. *Candida albicans*, the most prevalent pathogen of humans, has no known meiotic cycle, and yet it is a heterozygous diploid that undergoes mitotic recombination during somatic growth. It has been shown that clinical isolates as well as strains passaged once through a mammalian host undergo increased levels of recombination. Here, we tested the hypothesis that stress conditions increase rates of mitotic recombination in *C. albicans*, which is measured as loss of heterozygosity (LOH) at specific loci. We show that LOH rates are elevated during *in vitro* exposure to oxidative stress, heat stress, and antifungal drugs. In addition, an increase in stress severity correlated well with increased LOH rates. LOH events can arise through local recombination, through homozygosis of longer tracts of chromosome arms, or by whole-chromosome homozygosis. Chromosome arm homozygosis was most prevalent in cultures grown under conventional lab conditions. Importantly, exposure to different stress conditions affected the levels of different types of LOH events, with oxidative stress causing increased recombination, while fluconazole and high temperature caused increases in events involving whole chromosomes. Thus, *C. albicans* generates increased amounts and different types of genetic diversity in response to a range of stress conditions, a process that we term "stress-induced LOH" that arises either by elevating rates of recombination and/or by increasing rates of chromosome missegregation.**

IMPORTANCE **Stress-induced mutagenesis fuels the evolution of bacterial pathogens and is mainly driven by genetic changes via mitotic recombination. Little is known about this process in other organisms. *Candida albicans*, an opportunistic fungal pathogen, causes infections that require adaptation to different host environmental niches. We measured the rates of LOH and the types of LOH events that appeared in the absence and in the presence of physiologically relevant stresses and found that stress causes a significant increase in the rates of LOH and that this increase is proportional to the degree of stress. Furthermore, the types of LOH events that arose differed in a stress-dependent manner, indicating that eukaryotic cells generate increased genetic diversity in response to a range of stress conditions. We propose that this "stress-induced LOH" facilitates the rapid adaptation of *C. albicans*, which does not undergo meiosis, to changing environments within the host.**

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Stress-induced mutagenesis fuels the evolution of bacterial pathogens, resistance to antibiotics, tumor progression, and resistance to chemotherapy, all of which occur under stress and are driven by genetic changes (reviewed in references 1 and 2). Although initially controversial because of a lack of distinction between the stress conditions and the conditions used to select for the mutations, the idea that stress-induced mutagenesis induces a hypermutagenic state in subpopulations of bacterial cells is now widely accepted (2). Several studies in *Escherichia coli* and *Saccharomyces cerevisiae* focused on genome alterations leading to adaptation in starving or aging cells (3). Importantly, in subsequent starvation episodes, organisms carrying stress-induced genomic rearrangements exhibited a fitness advantage relative to the parental strain (4).

Loss of heterozygosity (LOH) reveals genetic variability in diploid organisms by exposing the phenotypes associated with recessive alleles and often has detrimental outcomes for an organism. For example, LOH is a prerequisite for the initiation and the development of cancer (5) via inactivation of tumor suppressor genes (two-hit hypothesis) (6); indeed, LOH has been associated with breast (7), skin (8), and colorectal (9) cancer. In addition, allele-specific gene expression or allelic imbalance plays a critical biological role in human variability (10). Finally, increased recombination in response to stress (fitness-associated recombination [FAR] [11, 12]) is thought to promote the evolution of complex traits by accelerating the rate of adaptation (13).

LOH events may reveal genetic diversity that affects fitness under stress conditions and/or unstressed conditions. The extent of LOH can range from short LOH tracts that form via gene conversion or double crossovers to long LOH tracts which arise via a single crossover event or by nonreciprocal events such as break-induced replication (BIR), and generate a region of homozygosity extending from the site of recombination to the telomere.

Whole-chromosome (whole-Chr) LOH, a product of chromosome nondisjunction that most frequently occurs because of defects in centromere/kinetochore/mitotic spindle function, usually results in aneuploidy, an imbalance in the number of chromosomes. Most aneuploid strains grow less well than their euploid parental strain. However, under specific stress conditions such as extreme temperature, nutrient shortage, and exposure to chemotherapeutic (14) or antifungal (15–17) drugs, some aneuploidies provide a strong fitness advantage. Strains monosomic for a specific chromosome often undergo reduplication of the remaining homolog, yielding a disomic, homozygous chromosome (18). If the two alleles in the heterozygous parental strain provide a differential benefit under a given stress, cells that retain the more beneficial allele after LOH may exhibit a growth advantage over cells that do not undergo LOH.

Candida albicans is a commensal fungus that resides in the human oral cavity, the gastrointestinal tract, and the genitourinary tract. Within the host, it competes with other microbes for nutrition in different body niches and adapts to different temperatures, different pH ranges, and different levels of oxidative stress, for example, when it encounters immune cells such as phagocytes (for a review, see reference 19). Under conditions of weakened immunity or imbalance in the commensal flora, *C. albicans* becomes an opportunistic pathogen with a disease spectrum ranging from mild superficial infections, such as oral thrush and vaginitis, to severe, life-threatening bloodstream infections such as disseminated candidiasis. These different candidal infections involve colonization of, and thus adaptation to, different host environmental niches and growth conditions, including elevated temperatures in the febrile host. Treatment with antifungal drugs is sometimes accompanied by the rapid acquisition of drug resistance, which can arise by chromosome rearrangements and/or chromosome missegregation (15, 16).

How *C. albicans* adapts to abrupt changes in environmental conditions is not well understood. *C. albicans* is a highly heterozygous diploid that reproduces primarily via mitotic division (20, 21). It possesses a mating locus (*MAT*) (22) and undergoes a parasexual cycle, in which diploids mate (23, 24) to form tetraploids that subsequently undergo "concerted chromosome loss," producing near-diploid progeny that are often trisomic and/or homozygous for one or more whole chromosomes. Multiple short-range recombination events occur within a subset of parasexual progeny, yielding recombinant chromosomes (25, 26). Homothallic mating further enriches the repertoire of routes that *C. albicans* can use to generate the wide range of genetic diversity observed in clinical isolates (27–29). Importantly, since no meiosis has been detected in *C. albicans*, genetic diversity is assumed to arise only via mitotic events.

In *C. albicans*, LOH events, as well as whole-Chr and segmental aneuploidies, have been observed in strains growing under selection for antifungal drug resistance (15–17). For example, homozygosis of hyperactive alleles of positive regulators of drug efflux pumps (15, 30) or of genes encoding drug targets, such as *ERG11* (31), or increased copy numbers of these genes provide a selective advantage in the presence of these drugs (32). In contrast, growth on poor carbon or nitrogen sources sometimes selects for LOH due to loss of whole chromosomes or chromosome segments (18, 33).

In *C. albicans*, little is known about the frequency of genetic changes that arise spontaneously and whether the rate of appearance of those changes is affected by exposure to stress. Furthermore, rates of spontaneous LOH have been measured only at one genomic locus (*GAL1*) (34, 35). Since the types of events that arise at different loci are often influenced by local features of the DNA (e.g., proximity to direct or inverted repeats or transposons can lead to gross chromosomal rearrangements, chromosome aberrations, and overall genome instability in *S. cerevisiae* [36–38]), it is important to determine both the rates and the types of LOH events that occur most frequently and to ask if there are chromosome regions with especially high or low rates of LOH in general. Furthermore, since candidal infections are treated with antifungal drugs and antifungal drug resistance can cause significant clinical complications, it is important to understand how *C. albicans* cells respond to physiologically relevant stresses, including antifungal drug stress.

Here, we measured rates of LOH and the distribution of types of LOH events that appeared in the absence and in the presence of physiologically relevant stresses. Importantly, we found that stress affected the rates and types of LOH events in a manner that was proportional to the degree of stress. Thus, it appears that *C. albicans* generates increased genetic diversity in response to a range of stress conditions but that it employs more than one mechanism to do so.

RESULTS

Establishment of a system to measure LOH rates and types in *Candida albicans*. We first set out to measure LOH rates at a number of different loci. A critical issue in the measurement of LOH rates is that selection pressure for the marker used should not influence the interpretation of results. We used two counterselectable markers, which greatly facilitate the detection of rare events (39): *GAL1* (34, 40), which we were able to use only at its native locus on Chr1, and the *URA3* marker, which could be inserted at different loci across the genome. We asked if marker selection affected rates using strain YJB9318, in which one copy of *GAL1* was replaced with the *URA3* gene. LOH rates calculated for each marker were very similar (see Fig. S1A in the supplemental material), indicating that the two different selection conditions could be used interchangeably and that they did not exert any obvious bias on the LOH rate.

In *S. cerevisiae* and in limited analyses in *C. albicans*, LOH is influenced by chromosomal position, including proximity to centromeres (39, 41). We used 18 different strains, each one heterozygous for *URA3* at a different locus, with each chromosome arm represented at least once (Fig. 1C; also see the supplemental material). We then compared LOH rates across the genome and as a function of distance from the centromere and other genome features. LOH rates were similar between independent isolates carrying the same marked locus but were different between different marked loci, with LOH rates ranging from ~10^{-6} to ~10^{-7}/cell division (Fig. 1D; also see Table S1 in the supplemental material). LOH rates at different loci generally correlated with increased distance from the centromere ($R^2 = 0.3$, Fig. S2A) and did not correlate with the distance of the loci from the nearest major repeat sequence (Fig. S2B). The most obvious outlier was the *ERG13* locus, which is on the right arm of ChrR adjacent to the rDNA repeats (Fig. 1C; Table S1). This is consistent with the results of Andersen et al. (39), who found nonstereotypical LOH rates for loci near the rDNA repeats in *S. cerevisiae*. Importantly, the rate of reversion of a point mutation in *C. albicans* (Fig. 1B; also see the

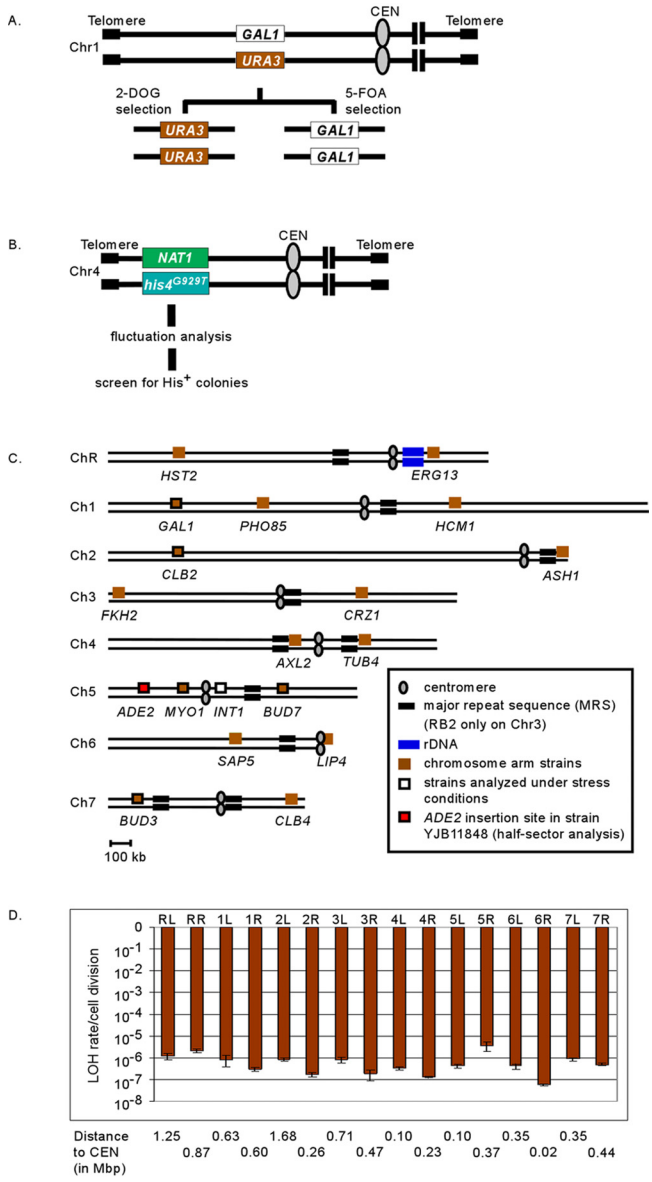

FIG 1 Determination of LOH and point mutation rates. (A) Configuration of Chr1 for comparing LOH rates using selection for loss of *GAL1* or *URA3*. One copy of *GAL1* was replaced with *URA3*. Fluctuation analysis was performed using selection on either 2-deoxygalactose (2-DOG) or 5-fluoroorotic acid (5-FOA). (B) Configuration of Chr4 for measuring the rate of point mutations that revert the *his4 G929T* point mutation at the native *HIS4* locus (18). Fluctuation analysis selected for the appearance of rare His^+ colonies. (C) Positions of *URA3* insertions (one per strain) used to measure LOH rates. In addition, the position of *ADE2*, used for half-sector analysis (strain YJB11848, which is *ade2*Δ/Δ at the native *ADE2* locus), is shown. (D) LOH rates for markers on all 16 chromosome arms. The distance between the *URA3* marker used and the centromere (CEN) is indicated below.

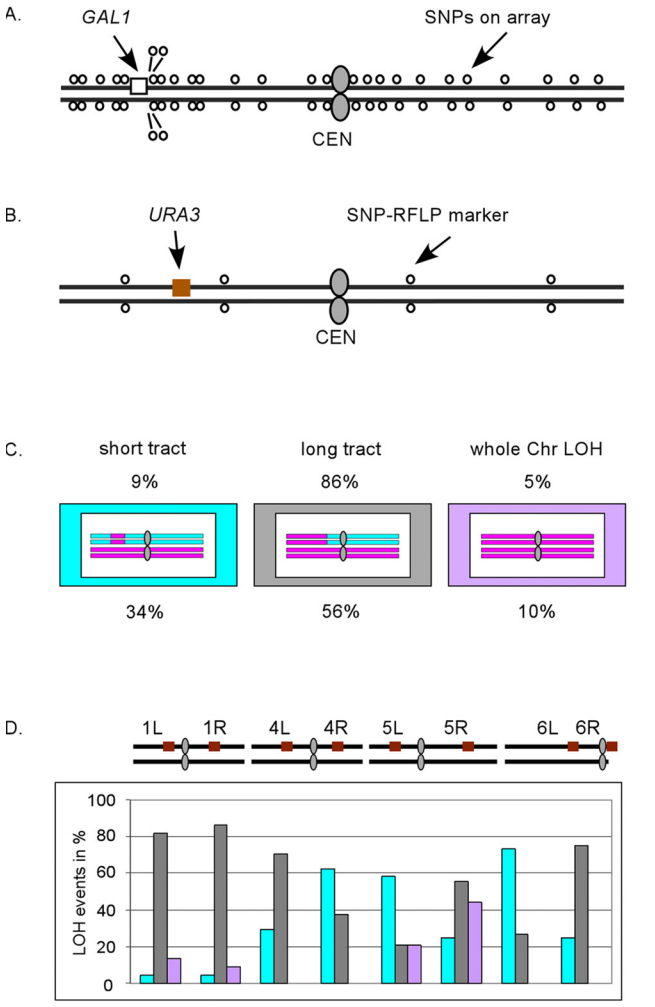

FIG 2 SNP analysis of LOH types. (A and B) Detection of LOH events using an SNP microarray (A) and using SNP-RFLP analysis (B). (C) Total proportion of each type of LOH event (n = 21 post-fluctuation strains for array analysis [above] and 15 to 24 isolates per marker for 8 different markers yielding n = 173 post-fluctuation analysis strains for SNP-RFLP analysis of the *URA3*-marked chromosome [below]). (D) Percentages of LOH types from SNP-RFLP analysis at individual Chr arms for the 8 different marked strains tested (n = 15 to 24 for each strain).

supplemental material) was 5.9×10^{-10} ($\pm$ 2.1×10^{-10}) per generation, which is much lower than rates of LOH measured in this study (see Discussion).

We next determined the types of LOH events using whole-genome single nucleotide polymorphism (SNP) microarray analysis for a set of 21 isolates derived from YJB9318 (*GAL1/gal1*::*URA3*) (34) (Fig. 2A). All 21 Gal^- isolates exhibited alterations on Chr1. Importantly, no SNP changes were detected on other Chrs. Long LOH tracts involving homozygosis of Chr1L, including SNP loci starting between *GAL1* and *CEN1* and extending to the left telomere (Fig. 2C), were most common. Short LOH tracts surrounding and including the *GAL1* locus were less frequent, and only one example of whole-Chr LOH was detected (Fig. 2C).

Since the SNP microarray analysis described above detected only changes on Chr1 (the marked chromosome), we next analyzed types of LOH events that arose at 8 different loci using 4 SNP-RFLP (restriction fragment length polymorphism) markers located on the same Chr as the *URA3* marker that had been lost (42) (Fig. 2B). For each locus studied, we collected 24 5-fluoroorotic acid-resistant (5-FOA^r) isolates taken from the 20 independent cultures used to determine LOH rates. When SNP-RFLP markers were distal to the marker that underwent LOH (as they were for *URA3* inserts on Chr1, -4, -5, and -6), the analysis

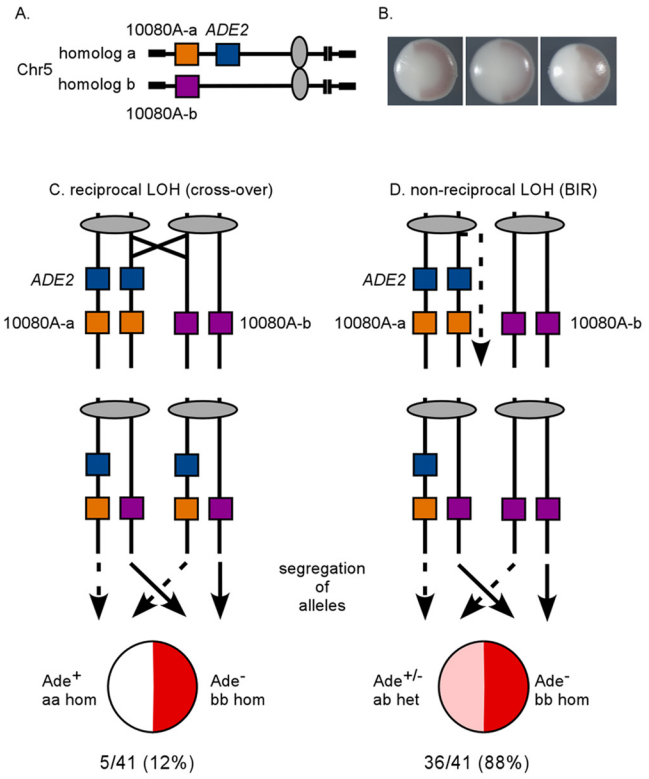

FIG 3 Half-sector analysis to distinguish reciprocal versus nonreciprocal LOH events. (A) Configuration of Chr5 in strain YJB11848, which has one copy of *ADE2* inserted on the left arm of Chr5 and distal SNP marker 10080A. (B) Examples of half-sectored colonies detected on MIN medium supplemented with uridine, histidine, and adenine. (C and D) Reciprocal (C) and nonreciprocal (D) LOH yield half-sectored colonies in which the phenotype of the white sector is homozygous or heterozygous, respectively. Adapted from the work of Andersen et al. (39).

distinguished between short LOH tracts (LOH of only *URA3* and/or *URA3* and the marker closest to it), long LOH tracts (loss of both markers on the same chromosome arm as *URA3*), and whole-Chr events (LOH of all SNP markers on the chromosome). The SNP-RFLP analysis, like the SNP microarray study, found that long LOH tracts were the most frequent type of LOH event (Fig. 2C; see also Table S2 in the supplemental material).

Long LOH tracts can arise by reciprocal (single crossovers [XO]) or nonreciprocal (BIR) recombination. We used half-sector analysis (see the supplemental material) with a strain carrying a heterozygous *ADE2* marker (YJB11848) to distinguish between these two mechanisms (Fig. 3C and D), Interestingly, 88% of the long-tract LOH events arose by a nonreciprocal mechanism such as BIR (see Table S3 in the supplemental material). The remaining 12% arose by a reciprocal recombination event, most likely a single crossover (Fig. 3C).

Stress conditions cause elevated rates of LOH and aneuploidy. LOH and aneuploidy are found frequently in strains resistant to azole antifungal drugs (17, 43) and can arise multiple times in an individual patient (32). Furthermore, at least some aneuploidies clearly confer a fitness advantage over the parental strain in the presence of azoles and, perhaps surprisingly, exert little, if any, fitness cost in the absence of the drugs (16). In addition, in *S. cerevisiae*, while point mutations can increase the fitness of aneuploid strains (44), different combinations of aneuploid chromosomes also can confer a fitness advantage under different types of stress conditions (14).

We asked if physiologically relevant stress conditions that *C. albicans* regularly encounters in the host (increased temperature [39°C, mimicking moderate fever in the host], oxidative stress [hydrogen peroxide, mimicking the production of reactive oxygen species by the host's immune cells], or antifungal stress [fluconazole, the most widely and commonly used antifungal drug, at subinhibitory concentration]) affected the rate at which LOH and/or aneuploidy arises. Importantly, because stress conditions can be selective for specific aneuploidies or LOH events, these analyses were performed using six different parental strains, three strains with *URA3* inserted at different positions on Chr5 and three strains with *URA3* inserted on Chr1, -2, or -7 (Fig. 1C; location of *URA3* is indicated by brown boxes with black frame; see also Table S6 in the supplemental material). Chr 5 has been the focus of several studies on genome instability (15, 17, 45), and Chr1 (~3.2 Mbp), Chr2 (~2.2 Mbp), and Chr7 (~0.95 Mbp) were chosen to account for differences in Chr size that potentially could influence LOH rates and events. Furthermore, by measuring LOH rates using several different strains with markers on different chromosomes, there is less likelihood that selection pressure on a given gene or chromosome region will affect the general conclusions.

A critical point is that the *URA3* marker does not have a known role in the *in vitro* survival of *C. albicans* cells under heat, oxidative, or antifungal stress. In addition, we performed the stress experiments with strain YJB9318, which has *URA3* inserted at the *GAL1* locus, and strains were analyzed as described above but with selection for *GAL1* loss (on 2-deoxygalactose [2-DOG]) or *URA3* loss (on 5-FOA) after exposure to the stress condition. Importantly, similar to the results under nonstress conditions, LOH rates of the two markers under stress conditions were not different (see Fig. S1A and Table S4 in the supplemental material), and thus any change in LOH rate is independent of the counterselectable marker used and can be attributed to the particular stress condition.

Strikingly, LOH rates were higher under stress conditions than under unstressed conditions. Fold changes for LOH rates were variable and yet lower on average for temperature stress (ranging from a 1- to a 40-fold increase) and intermediate for oxidative stress (from a 3- to a 72-fold increase). Fluconazole exposure resulted in the most dramatic increases in LOH rate, with up to a 285-fold increase (Fig. 4; see also Table S5 in the supplemental material). While strain YJB9834 appeared to be an outlier with respect to LOH rates in all three stresses (Table S5 and Fig. 4), these differences were not statistically significant (Kruskal-Wallis; $P = 0.45$ for each stress). Additional statistical analyses were performed for each stress to determine whether LOH rates are significantly different between control and stress conditions. While there was no statistically significant difference observed for temperature stress ($U = 54$; $P = 0.125$), for both H_2O_2 and fluconazole stress control LOH rates were statistically different from the stress LOH rates ($U = 0$; $P < 0.001$).

Growth under stress conditions alters the proportion of different LOH events that are recovered. We next analyzed the types of LOH events that accompanied the loss of *URA3* during exposure to stress. For each of the three stress conditions, we analyzed 24 5-FOA^r isolates derived from each of the strains carrying a

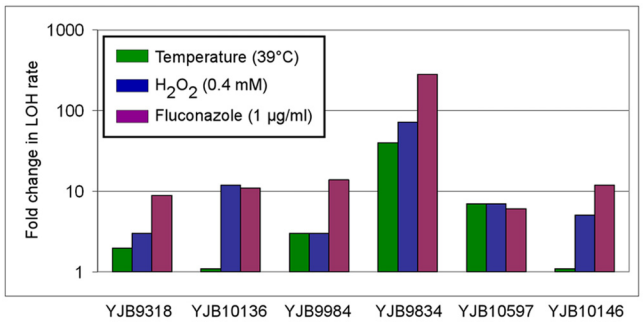

FIG 4 LOH rate fold changes in stressed cells. The graph shows fold increases in LOH rates in stressed cells for 6 different strains each with *URA3* inserted at a different locus (Fig. 1C; see also Table S6 in the supplemental material). The *y* axis shows fold change in LOH rate (note that the *y* axis is on a logarithmic scale).

marked locus, using SNP-RFLP analysis. Interestingly, the proportions of short LOH tracts, long LOH tracts, and whole-Chr LOH differed depending upon the stress condition. In strains exposed to 39°C, a stress that did not cause a statistically significant increase in LOH rate (see above), the proportion of whole-Chr LOH increased ~6-fold (Fig. 5A; see also Table S2 in the supplemental material). In contrast, in cells exposed to H_2O_2, the proportion of short LOH tracts increased almost 2-fold (Fig. 5A; Table S2). Finally, exposure to fluconazole, which caused the most dramatic increase in LOH rate, resulted in a ~5-fold increase in the proportion of whole-Chr LOH (Fig. 5A). The rate of LOH did not correlate with the type of LOH events seen: both febrile temperature (no increase in LOH rate) and an azole antifungal (285-fold increase in LOH rate) resulted in an increased proportion of whole-Chr LOH. Furthermore, exposure to different stresses triggered different mechanisms of genome alteration: oxidative stress caused an increased proportion of short LOH tracts, while the other two stresses caused an increased proportion of whole-Chr LOH, presumably due to elevated chromosome nondisjunction.

Increased H_2O_2 stress causes increased LOH rates and is accompanied by a shift in LOH types. The effects of stress on LOH rates in the experiments above appeared to be proportional to the degree to which cells were stressed (with H_2O_2 < temperature < fluconazole, measured as reduction in growth rates and levels of viability [see Fig. S1B and S2C to S2F in the supplemental material]), with the three types of stresses having very different physiological consequences. Thus, we next asked if LOH rates were directly related to the degree of stress using a single type of stress. To determine the degree to which H_2O_2 stress affected cell growth, we first performed growth curves and fluctuation analysis using a range of H_2O_2 concentrations. YJB9318 grew as well as did the no-stress control at up to 1 mM H_2O_2 (Fig. S1B), which suggests that the 0.4 mM stress condition used above (and in several other *C. albicans* studies [46, 47]) should be considered a mild stress condition. Growth decreased at 3 mM H_2O_2 and stopped completely at 10 mM H_2O_2. Fluctuation analysis was performed using the same H_2O_2 concentrations as those for the growth curves, and FUN-1 staining was performed to correct for nonviable cells. Percent dead cells ranged from 0.6% in the no-stress controls to 7% in 10 mM H_2O_2 (data not shown).

Importantly, the rate of LOH increased linearly with the increased level of H_2O_2 used ($R^2 = 0.9$) (Fig. 5B). This linear relationship was also observed for different temperatures and a range of fluconazole concentrations (see Fig. S2C to S2F in the supplemental material). In addition, SNP-RFLP analysis was performed for 24 5-FOA^r isolates after fluctuation analysis for 1 mM, 5 mM, and 10 mM H_2O_2 stress to determine the LOH events accompanying *URA3* loss. Strikingly, SNP-RFLP analysis revealed a shift in LOH types from 100% short LOH tracts for no stress and 0.4 mM H_2O_2 stress to mostly long LOH tracts for 1 mM, 5 mM, and 10 mM H_2O_2 stress (Fig. 5C). Since the SNP-RFLP analysis fol-

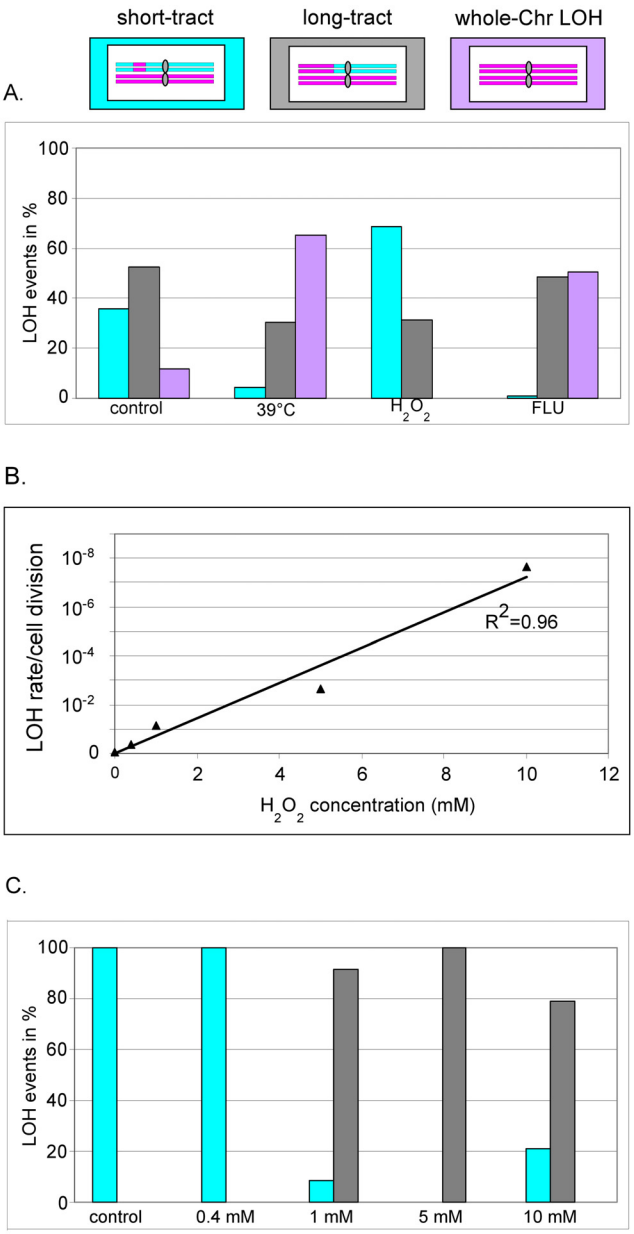

FIG 5 Effect of stress on types and rates of LOH events. (A) Short-tract, long-tract, and whole-Chr events are diagrammed above and framed with a color corresponding to the type: cyan for short-tract, light gray for long-tract, and lavender for whole-Chr events. (B) LOH rates in cells exposed to different concentrations of H_2O_2 ($R^2 = 0.96$). (C) Types of LOH events in cells exposed to different H_2O_2 concentrations shift from primarily short-tract events to primarily long-tract events. Color scheme is as in panel A.

lowed only two markers per Chr arm, we cannot rule out the possibility that long LOH tracts were due to two separate short LOH events. The most parsimonious explanation is that the large increase in the proportion of these events is due to increases in single crossovers and/or BIR events. Perhaps, the proportion of double-strand breaks (DSBs) relative to single-strand nicks increases with increasing H_2O_2 concentration. Thus, for a single type of stress, it is clear that increasing levels of stress result in increased LOH rates and a shift in the types of events that occur.

DISCUSSION

In asexual organisms, mitotic recombination is a major mechanism of genome evolution (48, 49). Here, we exploited the naturally high levels of heterozygosity in *C. albicans* to measure LOH events that reveal genetic diversity at the population level. Importantly, we found that LOH rates increased with increased levels of stress and that different stress conditions caused increases in different types of LOH events, many of which have been seen in clinical isolates (reviewed in reference 50) as well as lab isolates that were passaged through a mammalian host (34). Increasing rates of LOH in the presence of stress may facilitate the rapid adaptation of *C. albicans* populations to changing environments within the host, and the types of LOH events that arise likely reflect the stress conditions to which the population was exposed.

Rates and types of LOH in *C. albicans*. LOH rates measured here for *C. albicans* are similar to those reported previously for *S. cerevisiae* (39, 51). Similarly, the rate of reversion of a point mutation in *C. albicans* was comparable to rates of point mutations in *S. cerevisiae*, *Neurospora crassa*, and *E. coli* (51, 52). Importantly, *C. albicans* LOH rates are several orders of magnitude more frequent than point mutation rates. While we do not have strains that allow us to directly compare point mutation and LOH rates at the same locus, the data are consistent with the idea that LOH events are much more frequent than point mutations.

The types of LOH events that arise in *C. albicans* are independent of the marker employed or the method used to identify LOH events (Fig. 2A to 2C) and yet are locus dependent both in the absence and in the presence of stress (Fig. 2D). Similarly, in *S. cerevisiae*, locus-specific effects on the types of LOH have been observed (39). Thus, despite the lack of meiosis, *C. albicans* undergoes a range of recombination events that resemble those seen in an organism with a conventional sexual cycle.

It is not clear if whole-Chr homozygosis or long LOH tracts occur more efficiently or whether they provide more benefit (or less fitness cost) during growth under stress conditions. For example, chromosome nondisjunction, which causes whole-Chr LOH, may be less costly to the cell than activation of the recombination machinery, which is involved in generating short and long LOH tracts. In addition, whole-chromosome events may provide a larger opportunity to provide a selective advantage and yet can also incur a larger fitness cost (14, 44, 53, 54).

The influence of stress on rates and types of LOH events. Different stress conditions clearly increase the levels of different types of LOH. We propose that this is directly related to the type of DNA damage inflicted by the stress condition. Temperature stress causes chromosome loss (55), as well as aneuploidy (56), and we speculate that this occurs through a limitation in heat shock protein chaperones and cochaperones Sgt1, Sti1, and Cdc37, which have roles in the assembly of functional kinetochores (reviewed in reference 57) and spindle pole bodies/centrosomes (58) and the function of mitotic checkpoints (59).

The mechanism by which fluconazole treatment causes whole-Chr LOH is less obvious. However, fluconazole-resistant (Flu^r) strains are often aneuploid (16, 17), consistent with the idea that fluconazole affects chromosome segregation, either directly or indirectly. For example, we imagine that alterations in ergosterol biosynthesis may affect nuclear membrane fluidity, which would then impact the function of the spindle pole bodies embedded in the nuclear membrane (reviewed in reference 60) and/or the ability of cytoplasmic dynein to properly associate with the cortex and execute proper nuclear separation (61). Such defects would indirectly affect chromosome segregation mechanisms, resulting in polyploidy and/or aneuploidy.

All organisms are constantly exposed to oxidative stress as a normal by-product of respiratory metabolism (62, 63). In *S. cerevisiae*, oxidative stress caused an increase in double-strand breaks (DSBs) (64, 65) and a ~10-fold increase in mitotic recombination rates (66). Similarly, H_2O_2 caused a dose-dependent increase in mutation frequency at the human leukocyte antigen class A locus in human T lymphocytes (65). We propose that H_2O_2 in *C. albicans* causes increased LOH rates by generating chromosome breaks that are repaired by recombination, resulting in the linear relationship between LOH rate and H_2O_2 concentration.

Benefits and costs of LOH. The *C. albicans* genome and population structure suggests that the organism is generally clonal (20, 21) and, as we show here, gives rise to long LOH tracts during mitotic growth (Fig. 2D and 5A and C; see also Table S2 in the supplemental material). Interestingly, a recent study revealed that the distribution of genetic variation in clinical isolates of *S. cerevisiae* differs from that of environmental isolates (67). Like *C. albicans*, clinical *S. cerevisiae* isolates exhibited high levels of heterozygosity, reduced sporulation efficiency, and more efficient pseudohyphal formation compared to environmental isolates (67). Strikingly, heterozygous clinical *S. cerevisiae* isolates had long LOH tracts, consistent with long periods of clonality predicted by the facultative asexuality hypothesis (68), where diploid asexual organisms persist by means of mitotic recombination. Evidence for long-tract LOH events in the evolutionary past also can be found in *C. albicans* strain SC5314 (69), as well as in strain WO-1 (69). Thus, it appears that, as in *S. cerevisiae*, the stress of growth within host niches has had an important influence on the genome structure of *C. albicans*.

Relevant to this work, *C. albicans* and other pathogenic *Candida* species can adapt to very high levels of oxidative stress (70, 71) while *S. cerevisiae* laboratory strains cannot. It would be interesting to determine if clinical *S. cerevisiae* isolates, which have LOH patterns more like those seen in *C. albicans*, also adapt to oxidative stress more readily, since adaptation to oxidative stress is thought to be important for survival of an initial oxidative attack, as well as for the subsequent establishment of an infection.

While homozygosis of some alleles may provide a fitness advantage under stress and may not have a major fitness cost as measured under laboratory conditions (16, 72, 73), the fact that clinical *C. albicans* strains carry homozygous regions, and yet heterozygosity remains prevalent at most loci, implies that there is an advantage to the maintenance of heterozygosity during growth in the human host. Whether cells that undergo LOH are eventually lost from the population or whether they regain heterozygosity through rare mating events (74) remains to be determined.

MATERIALS AND METHODS

Determination of LOH rates by fluctuation analysis. To measure LOH rates, fluctuation analysis was essentially carried out as described previously (56). Briefly, strains were streaked for single colonies on either MIN (0.67% yeast nitrogen base without amino acids, 2% dextrose, 1.5% agar) (5-FOA selection) or MIN-Gal (2-DOG selection) with appropriate amino acids and grown for 2 days at 30°C. For nonstress conditions, 20 independent single colonies each were inoculated into 5-ml liquid yeast extract-peptone-dextrose (YPD; 1% yeast extract, 1% Bacto peptone, 2% dextrose, 1.5% agar) cultures and grown for 16 h at 30°C. After 16 h of growth, cells were pelleted, washed once with distilled water, and resuspended in 1 ml distilled water. Appropriate dilutions of each culture were spotted onto YPD for total cell count and onto 5-FOA to determine the proportion of cells that lost the *URA3* marker (5-FOA^r) or onto 2-DOG to determine the proportion of Gal$^-$ cells (2-DOG^r). YPD CFU were counted on day 2, and 5-FOA^r colonies were counted on day 3, which is prior to the time that colonies would have formed "adaptive" mutations on the 5-FOA medium (75). *URA3* and *GAL1* loss rates were determined using the method of the median by Lea and Coulson (76).

Standard deviations were calculated in Excel. A Kruskal-Wallis test was performed to test if LOH rates at the different genomic loci were statistically different from each other. For each Chr arm locus, two independent transformants were analyzed (see Table S6 in the supplemental material) and at least two independent fluctuation analyses were performed for each transformant.

Determination of LOH rates under stress. To determine whether stress alters LOH rates, strains were grown using three different stress conditions that mimic different host environments: increased temperature mimicking a mild fever, oxidative stress (hydrogen peroxide [H_2O_2]) mimicking *C. albicans* interaction with host immune cells, and antifungal drug (fluconazole) stress. Twenty cultures, each inoculated with a single colony, were grown at 30°C in YPD containing 0.4 mM H_2O_2 or 1 μg/ml fluconazole for 16 h. For temperature stress, the cultures were grown in YPD at 39°C for 16 h. In parallel, for each stress experiment, 20 cultures were grown at 30°C in YPD and served as no-stress controls. Fluctuation analysis was carried out at least twice for each strain under each condition. LOH rates were determined as described above. To determine if an increase in severity of a particular stress resulted in increased LOH rates, fluctuation analysis was performed using a range of H_2O_2 concentrations (0.4 mM, 0.6 mM, 0.8 mM, 1 mM, 3 mM, 5 mM, 7 mM, and 10 mM in strain YJB9318); different temperatures for strains YJB9318, YJB9984, and YJB9834; and a range of fluconazole concentrations (0 μg/ml, 0.4 μg/ml, 0.8 μg/ml, 1.0 μg/ml, and 1.4 μg/ml for strain YJB9318). LOH rates were measured as described above, and determination of doubling times, growth curves, and FUN-1 staining are described in the supplemental material. A nonparametric Mann-Whitney *U* test was performed for each stress to test if LOH rates for stressed conditions were statistically different from those for unstressed conditions.

Determination of LOH events. SNP microarray analysis was performed as described elsewhere (26). We previously developed an SNP-RFLP assay for distinguishing short-tract LOH, long-tract LOH, and whole-Chr LOH events in *C. albicans* using a set of 32 SNP-RFLP markers, 4 per Chr and 2 markers per Chr arm (42). To distinguish the LOH events that accompanied the loss of the *URA3* marker, for Chr1, -4, -5, and -6, 24 5-FOA^r isolates obtained after fluctuation analysis were analyzed by SNP-RFLP for the chromosome carrying the *URA3* marker (42). Briefly, PCR was performed in 96-well plate format in a total volume of 25 μl with 5× PCR buffer; 2.5 mM (each) dATP, dCTP, dGTP, and dTTP; 10 μM (each) primer (see Table S7 in the supplemental material); 0.25 μl e2TAK (Takara); and 30 ng of genomic DNA (gDNA) using the following conditions: initial denaturation at 98°C for 3 min; 30 cycles with denaturation at 98°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 1 min; and a final extension time at 72°C for 5 min. For restriction digests, 5 μl of PCR product was digested overnight in a total volume of 15 μl with 1 μl restriction enzyme, 10× restriction buffer, and 10× bovine serum albumin (where indicated) at 37°C or 65°C (for details, see Table S7 in reference 42). Digested PCR products were separated in a 3% agarose gel along with undigested and digested controls, gels were photographed, and restriction patterns were analyzed.

For details of materials and methods not given here, please see Text S1 in the supplemental material.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00129-11/-/DCSupplemental.

Text S1, DOC file, 0.05 MB.
Figure S1, PDF file, 0.127 MB.
Figure S2, PDF file, 0.13 MB.
Table S1, DOC file, 0.076 MB.
Table S2, DOC file, 0.068 MB.
Table S3, DOC file, 0.038 MB.
Table S4, DOC file, 0.032 MB.
Table S5, DOC file, 0.07 MB.
Table S6, DOC file, 0.102 MB.
Table S7, DOC file, 0.139 MB.

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