Candida albicans Genetic Background Influences Mean and Heterogeneity of Drug Responses and Genome Stability during Evolution in Fluconazole

Aleeza C. Gerstein,[a,b]* Judith Berman[a,b]

[a]School of Molecular Cell Biology and Biotechnology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel
[b]Department of Genetics, Cell Biology & Development, College of Biological Sciences, University of Minnesota, St. Paul, Minnesota, USA

ABSTRACT The importance of within-species diversity in determining the evolutionary potential of a population to evolve drug resistance or tolerance is not well understood, including in eukaryotic pathogens. To examine the influence of genetic background, we evolved replicates of 20 different clinical isolates of *Candida albicans*, a human fungal pathogen, in fluconazole, the commonly used antifungal drug. The isolates hailed from the major *C. albicans* clades and had different initial levels of drug resistance and tolerance to the drug. The majority of replicates rapidly increased in fitness in the evolutionary environment, with the degree of improvement inversely correlated with parental strain fitness in the drug. Improvement was largely restricted to up to the evolutionary level of drug: only 4% of the evolved replicates increased resistance (MIC) above the evolutionary level of drug. Prevalent changes were altered levels of drug tolerance (slow growth of a subpopulation of cells at drug concentrations above the MIC) and increased diversity of genome size. The prevalence and predominant direction of these changes differed in a strain-specific manner, but neither correlated directly with parental fitness or improvement in fitness. Rather, low parental strain fitness was correlated with high levels of heterogeneity in fitness, tolerance, and genome size among evolved replicates. Thus, parental strain background is an important determinant in mean improvement to the evolutionary environment as well as the diversity of evolved phenotypes, and the range of possible responses of a pathogen to an antimicrobial drug cannot be captured by in-depth study of a single strain background.

IMPORTANCE Antimicrobial resistance is an evolutionary phenomenon with clinical implications. We tested how replicates from diverse strains of *Candida albicans*, a prevalent human fungal pathogen, evolve in the commonly prescribed antifungal drug fluconazole. Replicates on average increased in fitness in the level of drug they were evolved to, with the least fit parental strains improving the most. Very few replicates increased resistance above the drug level they were evolved in. Notably, many replicates increased in genome size and changed in drug tolerance (a drug response where a subpopulation of cells grow slowly in high levels of drug), and variability among replicates in fitness, tolerance, and genome size was higher in strains that initially were more sensitive to the drug. Genetic background influenced the average degree of adaptation and the evolved variability of many phenotypes, highlighting that different strains from the same species may respond and adapt very differently during adaptation.

KEYWORDS AMR, fitness, ploidy

Address correspondence to Aleeza C. Gerstein, aleeza.gerstein@umanitoba.ca, or Judith Berman, jberman@tauex.tau.ac.il.

* Present address: Aleeza C. Gerstein, Departments of Microbiology and Statistics, The University of Manitoba, Winnipeg, Canada.

In eukaryotic microbes, the responses to severe stresses, including exposure to antimicrobial drugs, can occur through genetic changes that arise within susceptible microbial populations and spread via conventional evolutionary processes or via physiological responses that modulate the ability of cells to survive and grow in the

presence of the stress. Drug resistance, measured as reduced susceptibility, can be assessed as a higher MIC. For fungal pathogens, broth microdilution assays, Etest strips, or disk diffusion assays are assessed after growth in a set range of drug concentrations (1). Antifungal drug tolerance, a property distinct from drug resistance (2, 3), is the ability of some cells in a population to grow slowly in the presence of a drug at concentrations above the MIC. In tolerant strains, the subpopulation of cells that grow (generally from 10 to 90% of cells, depending on the strain) is usually evident when growth is assessed after an extended time frame in the drug (reviewed in reference 4). Hence, tolerant strains have susceptible MIC levels (5, 6) and have often been termed "resistant" in assays in which they are allowed to grow for longer periods of time and/or in spot assays that measure partial growth. Clinically, the failure to clear infections is more likely when the infecting strain is drug resistant. High levels of drug tolerance may influence infection clearance (3, 7), although more studies that use quantitative criteria to definitively measure tolerance levels and distinguish tolerant from resistant isolates are needed (reviewed in reference 4).

Factors that influence the change in antifungal resistance and tolerance levels in pathogenic fungi have not been well elucidated. Unlike prokaryotes, which often acquire new traits horizontally via plasmids, eukaryotic pathogens primarily acquire new traits vertically via *de novo* mutations, chromosome-scale changes in copy number (ploidy) or allele frequency, and recombination events. Changes in either genome-wide ploidy (the number of sets of homologous chromosomes) or aneuploidy (the gain or loss of individual chromosomes) also arise often in laboratory populations subjected to stress (8–10) or passaged through mice (11, 12). Ploidy and aneuploidy variation have also been found in some clinical isolates of *Candida albicans*, *Candida glabrata*, and *Saccharomyces cerevisiae* (13, 14) as well as in environmental isolates of *S. cerevisiae* (15, 16).

Changes in ploidy arise more frequently than point mutations (17). In addition, they are especially prevalent in strains exposed to azole drug stress (18–22). Indeed, exposure to fluconazole promotes karyotypic change by inducing unconventional cell cycle events in a subpopulation of cells (23, 24). Hence, fluconazole can both drive and select for karyotypic variation within and among populations. Furthermore, the frequency of aneuploidy among clinical isolates may be underestimated, as strain isolation methods (i.e., multiple growth cycles in rich medium) may impose a fitness cost that selects against aneuploid isolates. A mechanistic link between drug resistance and specific aneuploidies exists in multiple pathogenic fungal species (e.g., *C. albicans* [22, 25–28] and *Cryptococcus neoformans* [29, 30]; suggested in *Candida auris* [31, 32]). Because fluconazole tolerance and drug resistance are distinct and tolerance is sensitive to inhibitors that do not affect resistance (2, 3), it follows that antifungal tolerance likely evolves via a different subset of genes than resistance, although it is also possible that there is overlap between genes involved in tolerance and resistance.

The importance of genetic background in the link between genotype and phenotype for *de novo*-acquired mutations is becoming appreciated in both laboratory and natural settings (33–35 and references within). For example, the phenotype of deleted or repressed genes can vary significantly in different backgrounds (36–38). Moreover, closely related strains can differ in the classification of genes that are essential for viability under a specific growth condition (39). The same is true of mutations classified as beneficial in one strain background (including different mutations in the same gene [40]), which can be neutral or deleterious in other backgrounds (e.g., references 41 to 44). Different strain backgrounds also may exhibit different mutation rates, thereby affecting the frequency with which genetic variation arises (45, 46). Thus, the genetic background of a population likely influences the mutations available for adaptation and hence the trajectory of evolution. Furthermore, the constraints on variation in the degree of intra- and interpopulation heterogeneity are likely to differ in different genetic backgrounds.

Here, we explored the interplay between genetic background, fitness, drug resistance and drug tolerance, and karyotypic variation by following the evolutionary

TABLE 1 Strains used in this study[h]

Strain no.	Strain name	Clade	*MTL* genotype, patient status (if known), site of isolation, country of origin	Parental fitness		Parental resistance (24 h)	Parental tolerance (72 h)
				24-h OD	72-h OD		
A1	L26[a]	1	**a**/**a**, vaginitis, vagina, USA	1.6	1.8	4	0.26
A2	P87[a]	4	**a**/**a**, HIV, oral, South Africa	1.1	1.3	1	0.10
A3	GC75[a]	4	α/α, healthy, oral, South Africa	0.3	0.7	0.0125	0.27
A4	P78048[a]	1	α/α, bloodstream, Canada	0.5	1.0	0.5	0.22
A5	P57072[a]	2	α/α, bloodstream, USA	1.3	1.7	4	0.91
A6	P34048[a]	3	**a**/α, bloodstream, Turkey	1.1	1.4	1	0.29
A7	P37037[a]	1	**a**/α, healthy, oral, USA	1.0	1.5	1	0.37
A8	P75016[a]	4	**a**/α, bloodstream, Israel	0.7	1.2	0.5	0.32
A9	P75063[a]	4	**a**/α, bloodstream, France	0.4	0.7	0.0125	0.31
A10	P76055[a]	2	**a**/α, bloodstream, USA	0.8	1.5	0.0125	0.81
A11	P78042[a]	3	**a**/α, bloodstream, USA	0.3	0.6	0.0125	0.30
A12	T101[b]	3	**a**/**a**, oropharynx, Canada	1.2	1.7	32	0.49
A13	OKP90[c]	2	**a**/**a**, healthy, oral, South Africa	0.1	1.2	0.0125	0.44
A14	AM2003.089[b]	2	**a**/α, oropharynx, UK	0.5	1.2	0.0125	0.58
A15	AM2003.0165[b]	2	α/α, bloodstream, UK	0.3	1.2	0.0125	0.35
A16	AM2003.0069[b]	3	**a**/α, vagina, UK	0.6	1.4	0.0125	0.5
A17	SC5314[d]	1	**a**/α, bloodstream, USA	0.2	0.5	0.0125	0.18
A18	FH1[e]	3	**a**/α, marrow transplant, rectal, USA	1.6	1.7	4	0.65
A19	DSY294[f]	11	**a**/α, HIV, oral, France	0.7	1.4	0.0125	0.37
A20	T118[g]	1	**a**/α, HIV, oral, Canada	0.9	1.7	4	0.41

[a]Original reference, 79.
[b]Original reference, 73.
[c]Original reference, 80.
[d]Original reference, 81.
[e]Original reference, 82.
[f]Original reference, 83.
[g]Original reference, 84.
[h]Fitness was measured as the optical density (A_{600}) in YPD + 1 μg/ml FLC, the evolutionary drug environment. Drug resistance was measured as MIC_{50} in μg/ml at 30°C by broth microdilution assay. Tolerance was measured as the average optical density at 72 h in the measured drug concentrations of drug above the MIC divided by optical density in the lowest measured drug level.

trajectories of replicates from 20 diverse *C. albicans* strains for 100 generations of evolution in 1 μg/ml of fluconazole. We found that the majority of replicates acquired the ability to grow more rapidly in the evolutionary level of drug, with the degree of improvement inversely correlated with parental strain fitness. While very few replicates from any background acquired clinical levels of drug resistance, changes in tolerance and ploidy were prevalent, especially in strains with low parental fitness. We find that drug tolerance is an evolvable phenotype and one that changed in the majority of strains. Importantly, evolved variation in fitness, tolerance, and genome size among replicate evolved lines is inversely correlated with parental strain fitness. Thus, initial strain fitness provides a link between strain genetic background and the acquisition of phenotypic and genotypic diversity among replicate populations adapting to fluconazole.

RESULTS

We evolved replicate lines from 20 clinical strains of *C. albicans* that span the phylogenetic diversity of the species (47) and vary in mating-type zygosity, geographic origin, and site of isolation (Table 1). Twelve replicates of each of the 20 strains were evolved in parallel in fluconazole (yeast extract-peptone-dextrose [YPD] + 1 μg/ml FLC) for 100 generations (20 strains × 12 replicates per strain = 240 replicates), by serial passaging at 1:1,000 dilution every 72 h for 10 passages. We use the term "evolved" to indicate these 240 replicates and "parental" to refer to the clinical strains prior to the evolution experiment we conducted. Fitness was measured as optical density (OD) in the evolutionary level of fluconazole (1 μg/ml FLC) for parental and evolved populations at 24 and 72 h. We also measured drug response phenotypes for parental and evolved strains from broth microdilution assays. Resistance to fluconazole was measured as MIC_{50}, the concentration of drug at which growth is inhibited by at least 50% relative to growth in a drug-free environment, after 24 h of growth. Tolerance to
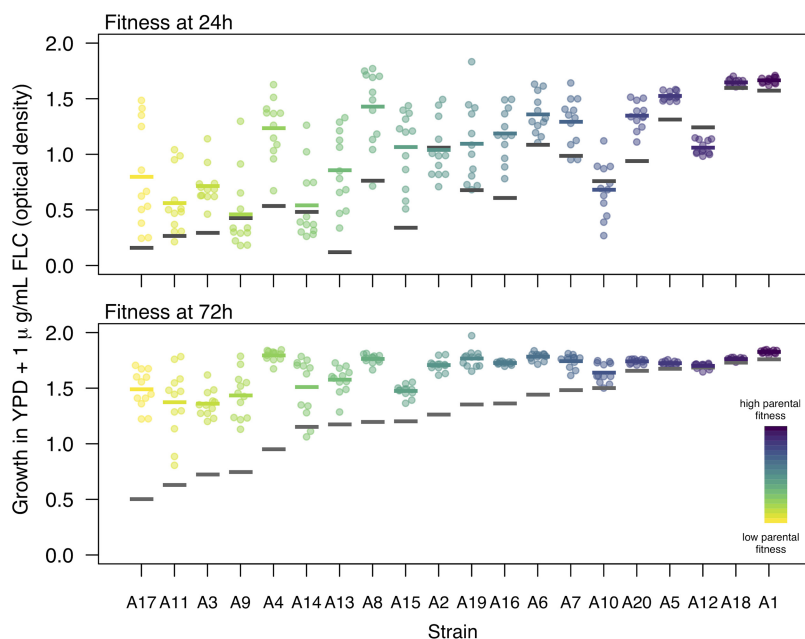
FIG 1 The majority of evolved replicates improved growth in the evolutionary environment after 100 generations of evolution. Fitness was measured as growth (optical density) in YPD + 1 μg/ml fluconazole, the evolutionary environment after 24 h (top) and 72 h (bottom). Strains are ordered by parental fitness in the evolutionary environment at 72 h. Parental fitness (the median growth among 12 parental replicates) is indicated for each strain by a gray bar. Each colored point represents one of 12 evolved replicates, while the colored bars indicate median evolved growth for visual comparison to parental growth.

fluconazole was calculated as the ratio of growth at drug concentrations above the MIC relative to growth in a very low level of drug after 72 h (the transfer time). Although previous work measured tolerance at 48 h, we found that the two time points gave very similar results. Parental optical densities at 24 h and 72 h were correlated (Spearman's rank correlation, $S = 158$, P value $< 2.2e-16$, rho $= 0.88$), while parental resistance and tolerance were not (Spearman's rank correlation, $S = 1141.8$, P value $= 0.55$) (Table 1).

Adaptation is influenced by strain background. The majority of replicates evolved significantly higher fitness in the evolutionary drug environment after only 100 generations of adaptation (fitness measured at either 24 or 72 h) (Fig. 1). The major exception was replicates from A12, the strain with the highest parental MIC_{50}, which evolved significantly lower 24-h fitness and had no change in 72-h fitness. Four strains with initial MICs of ≤1 (A2, A9, A10, and A14) also did not acquire improved average fitness at 24 h, though all had significantly increased fitness at 72 h, on average (significance determined as appropriate by parametric or nonparametric t tests; methods and detailed results in Table S1 in the supplemental material).

Parental strain fitness significantly influenced both the mean fitness improvement after 100 generations and the variability in fitness improvement among the replicates; neither mating type nor clade had a significant effect on these parameters (Fig. 2) (analysis of variance [ANOVA]; mean improvement at 24 h: $F_{1,13} = 7.99$, $P = 0.014$; clade, $F_{4,13} = 0.64$, $P = 0.64$; MAT zygosity, $F_{1,13} = 0.27$, $P = 0.61$; variability at 24 h: $F_{1,13} = 40.94$, $P < 0.0001$; clade, $F_{4,13} = 0.82$, $P = 0.54$; MAT zygosity, $F_{1,13} = 2.22$, $P = 0.16$; mean improvement at 72 h: $F_{1,13} = 158.73$, $P < 0.0001$; clade, $F_{4,13} = 1.97$, $P = 0.16$; MAT zygosity, $F_{1,13} = 0.25$, $P = 0.63$; variability at 72 h: $F_{1,13} = 6.353$, $P < 0.026$; clade, $F_{4,13} = 1.01$, $P = 0.44$; MAT zygosity, $F_{1,13} = 1.13$, $P = 0.31$). Additional aspects of strain background that are not accounted for in these models also contributed to evolved fitness, as can be visualized from the deviation of points from the correlation line of fit in Fig. 2. The variance among evolved replicates from each strain reflects stochasticity in the evolutionary process. Variance in evolved fitness and the degree of fitness
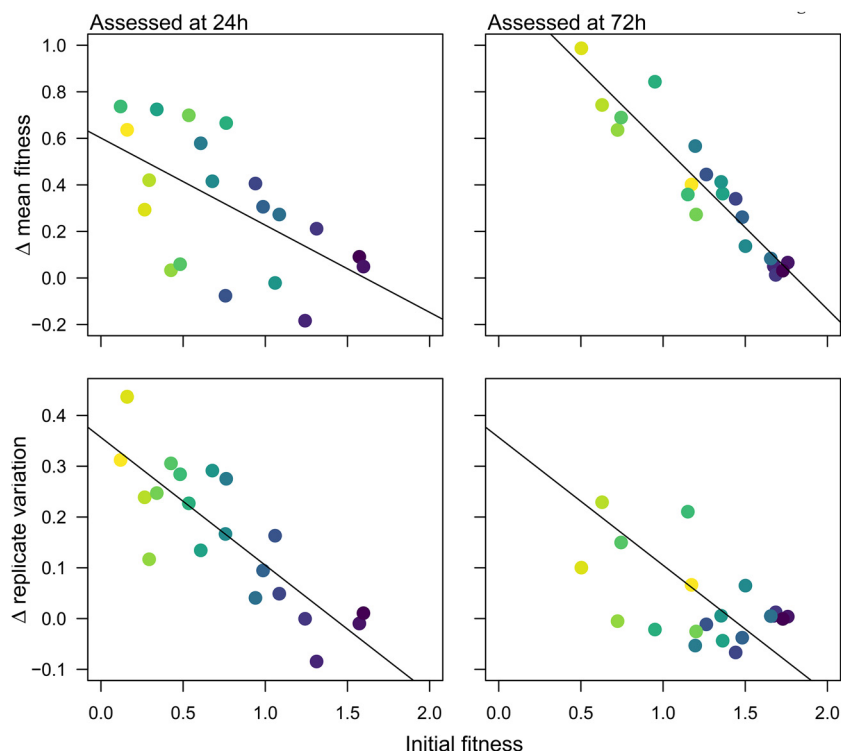
FIG 2 Significant association between parental fitness and change in mean fitness (upper graphs) and replicate variability (lower graphs). Fitness was measured as optical density in YPD + 1 μg/ml fluconazole after 24 h (left panels) and 72 h (right panels). The colors are as in Fig. 1, based on parental fitness at 72 h (low parental fitness = yellow, high parental fitness = purple). The regression line is for visualization purposes to illustrate the one-way relationship between parental fitness and change in fitness or change in variability, which was significant ($P < 0.05$) in multiway models that also take into account clade and mating locus ($P > 0.05$ in all cases; see text for details).

improvement were inversely correlated: strains that were least fit initially and that increased in growth the most on average also had the most variability among replicates from the same strain background (Spearman's rank correlation test; 24 h, $S = 694$, $P = 0.035$, rho = 0.48; 72 h, $S = 476$, $P = 0.003$, rho = 0.64). Overall, strain background influenced both the degree of growth improvement and the variability in improvement among replicates, both mediated in part (but not entirely) by the parental growth ability of the strain in the evolutionary environment.

Increases in drug resistance to 1 μg/ml were common but beyond 1 μg/ml were rare. One hundred ten replicates (76%) derived from the 12 backgrounds that had a parental MIC_{50} below 1 μg/ml evolved to have an MIC_{50} of 1 μg/ml within the 100-generation experiment (Fig. 3A, left panel). Selection for increased resistance was predominantly limited to the evolutionary drug level: only six replicates (4%) from four of these strains evolved a higher MIC_{50}, and none of the replicates from the three strains with parental MIC_{50}s equal to 1 μg/ml changed in MIC_{50} (Fig. 3A, middle). Replicates from the five strains with parental MIC_{50} above 1 μg/ml exhibited diverse responses (Fig. 3A, right panel): in three strain backgrounds (A1, A18, and A12), all replicates retained the parental MIC_{50}; for a single strain (A20), all replicates decreased to a MIC_{50} of 1 μg/ml; replicates from the fifth strain (A5) exhibited variable outcomes (four increased, four decreased, and four were unchanged in their MIC_{50}). In total, only 10 replicates from five strain backgrounds increased in MIC_{50} beyond the evolutionary level of the drug (Fig. 3A and Fig. S1). No clear factor was associated with the appearance of resistance to drug above the evolutionary level: these events were spread across the major clades and were not associated with mating locus (three have a homozygous mating locus, two are heterozygous). Combined, this suggests that
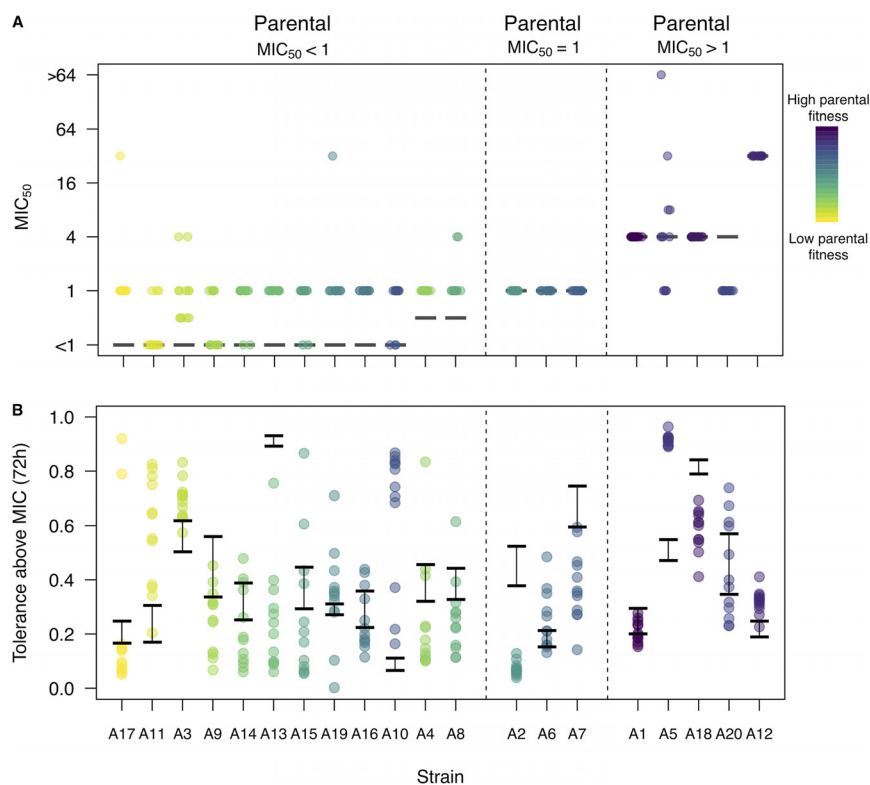
FIG 3 Evolved variation in clinical resistance and tolerance. Strains are arranged on the x axis by parental MIC, with each panel separating the three MIC-based classes of strains. (A) The majority of evolved replicates did not acquire clinical resistance. Clinical drug resistance was measured as MIC_{50} using broth microdilution assays (1). The black lines indicate parental MIC_{50}s. (B) Tolerance was variable among the replicates. Tolerance was measured as the average growth observed in supra-MIC levels of fluconazole normalized to the growth in a very low level of drug after 72 h. The black lines indicate the range of tolerance values measured among parental replicates. Each point represents an individually evolved replicate line, colored as in Fig. 1, based on fitness in the evolutionary environment.

the majority of selected mutations confer a narrow benefit up to, but not exceeding, the evolutionary level of the drug.

Changes in drug tolerance were common. Changes in tolerance were prevalent among evolved replicates (Fig. 3B; Fig. S2). The overall picture is similar regardless of the time at which tolerance was measured (48 or 72 h; Fig. S2). Consistent with a previous study examination (3), the magnitude of tolerance change increased sharply between 24 and 48 h of drug exposure (Fig. S2).

Approximately 75% of the evolved replicates changed tolerance level: 55 replicates from 15 strain backgrounds increased, 122 replicates from 19 strain backgrounds decreased. The proportion of replicates within a strain that increased or decreased in tolerance varied considerably among backgrounds (Fig. 3B). Parental strain fitness influenced the degree of stochasticity of tolerance among evolved replicates, similar to the variation in evolved fitness, variation in evolved tolerance among replicates was significantly and negatively correlated with initial fitness (Spearman's rank correlation, fitness measured at 24 h, $S = 2,226$, P value $= 0.002$, rho $= -0.67$; 72 h, $S = 1,986$, P value $= 0.029$, rho $= -0.49$). These results are consistent with the idea that genetic background, mediated in part by parental fitness, acts not only on evolved trait means but also on evolved trait variance.

If we consider each replicate independently, there was no association between change in tolerance and change in fitness (linear mixed-effect model; change in fitness, $F_{1,132.5} = 0.03$, $P = 0.87$; MAT zygosity, $F_{1,18.8} = 0.65$, $P = 0.43$; clade, $F_{4,19.1} = 1.99$, $P = 0.13$). Looking at evolved replicates within each strain, a significant negative correlation between change in tolerance and change in fitness was found only for three strain backgrounds (A13, A14, and A19) (Fig. S4).
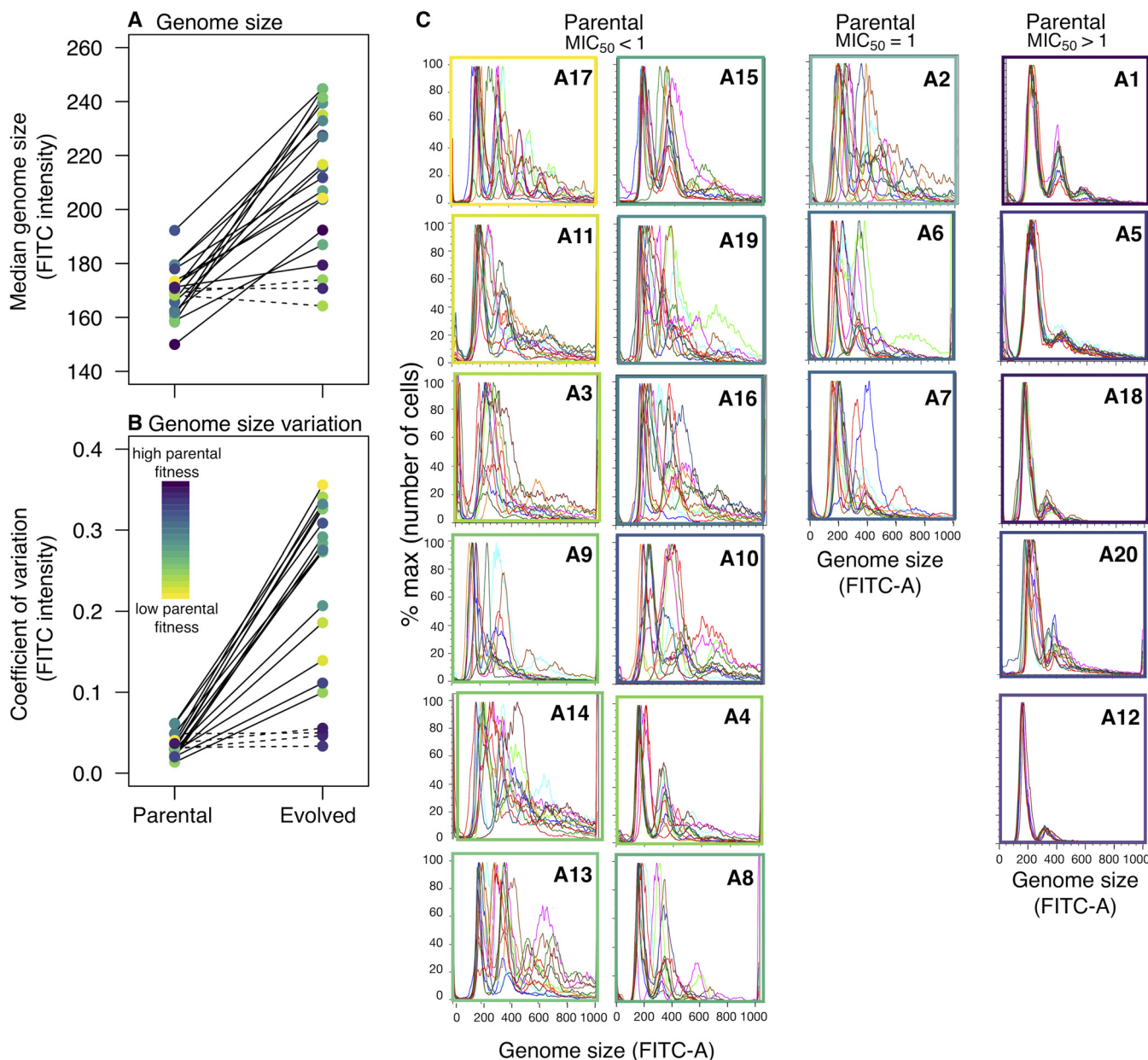
FIG 4 Genome size and variation in genome size increased after evolution to low fluconazole. (a and b) Median genome size (a) and coefficient of variation (CV; i.e., variability among replicates) (b) in each strain background. A dashed line indicates a nonsignificant change between parental and evolved replicates. (c) Flow cytometry traces of each replicate evolved line, ordered and grouped by parental MIC_{50}. Box color indicates parental fitness in the evolutionary environment.

Genome size changes are pervasive. Exposure to fluconazole is known to induce the formation of tetraploid and, subsequently, aneuploid cells in *Candida albicans* (24); specific aneuploidies can provide a selective advantage under drug stress (22, 25, 48). Here, we estimated genome size by flow cytometry and found that evolved replicates underwent a significant increase in median genome size across the majority of strain backgrounds (Fig. 4a; Table 2). The three exceptions were A12, the strain that had the highest initial MIC_{50}, and A4 and A9, strains with initial MIC_{50} values of <1 μg/ml. In some cases, multiple subpopulations with different genome sizes were observed (see Materials and Methods); for analysis, only values from the largest subpopulations were used (i.e., the most prominent peak in the flow trace), which underestimates the degree of genome size diversity in the population. Looking at A4 and A9, nondiploid sub-

TABLE 2 Welch two-sample t tests to compare parental and evolved genome size

Strain	Statistic
A1	$t_{20.6} = 11.70$, $P < 0.0001$
A2	$t_{11.2} = 5.34$, $P = 0.002$
A3	$t_{11.1} = 5.69$, $P = 0.0001$
A4	$t_{11.4} = 1.91$, $P = 0.08$
A5	$t_{20.14} = 18.07$, $P < 0.0001$
A6	$t_{11.1} = 3.82$, $P = 0.003$
A7	$t_{11.1} = 2.80$, $P = 0.017$
A8	$t_{11.1} = 2.80$, $P = 0.017$
A9	$t_{11.2} = 0.88$, $P = 0.40$
A10	$t_{11.4} = 3.31$, $P = 0.007$
A11	$t_{11.5} = 5.24$, $P = 0.0002$
A12	$t_{18.8} = 0.71$, $P = 0.49$
A13	$t_{11.1} = 5.46$, $P = 0.0002$
A14	$t_{11.1} = 4.10$, $P = 0.002$
A15	$t_{11.1} = 2.79$, $P = 0.018$
A16	$t_{11.3} = 4.17$, $P = 0.0015$
A17	$t_{11.1} = 2.94$, $P = 0.013$
A18	$t_{18.4} = 2.98$, $P = 0.008$
A19	$t_{11.4} = 2.93$, $P = 0.013$
A20	$t_{11.5} = 5.91$, $P < 0.0001$

populations are observed for some replicates (Fig. 4c; Fig. S5); thus, only one strain background (A12, the strain with the highest parental MIC) had no replicates with a significant deviation from diploidy.

The coefficient of variance for ploidy level, a measure of variability among replicates, also increased for the majority of strains (Fig. 4b). Thus, genome size variation among replicates increased over time in drug, as expected if the drug induced ploidy shifts and if evolved replicates acquired different final numbers of chromosomes. Importantly, the least ploidy variation was seen with replicates evolved from the five parental strain backgrounds that had parental MIC_{50} levels above the evolutionary drug concentration (Fig. 4c, right panels) and thus would be expected to be less sensitive to the drug stress used. When these five strains were removed from the analysis, median evolved genome size was not correlated with parental fitness in fluconazole (Pearson's correlation, 24 h, $t_{13} = -0.30$, $P = 0.84$; 72 h, $t_{13} = -0.22$, $P = 0.83$), nor with the mean fitness change in low drug (Spearman's rank correlation, fitness at 24 h, $S = 541.19$, $P = 0.45$; 72 h, $S = 953.4$, $P = 0.12$). Genome size variation was thus extremely prevalent and equally likely among evolved replicates from strain backgrounds with parental MICs at or below the evolutionary level of drug.

These median numbers, however, obscure the tremendous variation in genome size observed among evolved replicates (Fig. S5). Looking at replicates from the 15 strains with parental MIC_{50}s of ≤ 1, final genome size was not significantly correlated with any of the predictor variables that we tested (change in fitness, $F_{1,46.4} = 0.005$, $P = 0.95$; clade, $F_{4,9.2} = 0.94$, $P = 0.48$; MAT zygosity, $F_{1,8.7} = 0.002$, $P = 0.97$). There was similarly no correlation between evolved genome size and change in fitness within replicates from any strain background (Fig. S6). A14 was the only strain background with a significant negative correlation between change in tolerance and evolved genome size (Fig. S7).

The variance in evolved genome size followed a similar pattern as variance in evolved tolerance: a significant negative correlation with parental fitness at 24 h but not at 72 h (Spearman rank correlation, 24-h fitness, $S = 2,076$, $P = 0.01$, rho $= -0.56$; 72-h fitness, $S = 1,908$, $P = 0.05692$, rho $= -0.43$) and a positive correlation with the variance in fitness improvement (24 h, $S = 362$, $P = 0.0004$, rho $= 0.73$; 72 h, $S = 440$, $P = 0.002$, rho $= 0.67$). Accordingly, variance in evolved genome size was also significantly correlated with variance in evolved tolerance ($S = 538$, P value $= 0.007$, rho $= 0.60$). There was no direct link between evolved genome size and either fitness improvement or change in tolerance among replicates or strain backgrounds. However,

the variability among evolved replicates is consistently larger in some strain backgrounds than others, regardless of the trait we measured (evolved fitness, drug tolerance, genome size), and this was mediated, in part, by parental strain fitness in the evolutionary environment.

DISCUSSION

The adaptation to antimicrobial drugs by microbial pathogens is inherently an evolutionary process that relies upon beneficial genetic mutations. To examine the influence of genetic background on adaptation in drug, we evolved 20 diverse *C. albicans* isolates (240 replicates in total) for 100 generations in 1 μg/ml fluconazole. The majority of replicates rapidly increased in fitness in the evolutionary environment, in a manner that correlated inversely with parental fitness. Accordingly, the majority of replicates from strains with parental drug susceptibility (measured as MIC_{50}) below 1 μg/ml fluconazole increased MIC_{50} to the evolutionary drug level of 1 μg/ml. Only 10 replicates from five backgrounds increased in drug resistance beyond the evolutionary drug level, however. In contrast, changes in drug tolerance were much more common with replicates both increasing and decreasing in tolerance. These changes in tolerance may be due to pleiotropic effects from genetic changes that improve growth in the evolutionary environment or by virtue of physiological or possible epigenetic changes. Overall, there was little or no correlation between change in tolerance and parental fitness or change in fitness. However, parental fitness was negatively correlated with the variance in tolerance among evolved replicates, and the variance in evolved fitness was also negatively correlated with the variance in evolved genome size.

The negative correlation between parental fitness and the improvement in fitness and that between parental fitness and the variation among replicates in fitness, drug tolerance, and genome size are both consistent with predictions from Fisher's abstract geometric model (49, 50). These results among very diverse parental strains are similar to the pattern of diminishing returns for fitness seen in bacteriophage (51) and *Escherichia coli* (52) and are consistent with the negative correlation between parental fitness and the rate of adaptation in strains that differ by many mutations (53) and in strains that differ by only one or a few beneficial (54, 55) or deleterious (56) mutations.

Only a small number of replicates increased MIC_{50} to a drug level above the evolutionary environment. From the raw (optical density) data for specific replicates from A3, A8, A17, and A19 (parental MIC_{50} of all four is <1 μg/ml), MIC_{50} clearly increased beyond 1 μg/ml (see Fig. S1 in the supplemental material), indicating that these isolates acquire resistance to a higher drug concentration. Conversely, replicates from strain A20 (parental MIC_{50} = 4 μg/ml) uniformly improved their fitness at 1 μg/ml fluconazole, yet had reduced fitness at 4 μg/ml fluconazole, indicative of a cost-benefit tradeoff for fitness in lower versus higher drug concentrations of fluconazole. Other MIC_{50} results require a more cautious biological interpretation and may be due to a technical issue, rather than biological improvement. MIC_{50} is calculated as the drug concentration where growth is reduced 50% relative to growth in no drug; hence, MIC_{50} could increase arithmetically if growth is reduced in the absence of drug even if growth in the presence of drug has not changed. This mathematical quirk is also why we use growth in the lowest drug level in the denominator of the tolerance calculation: in parental strains, we observed equivalent growth in no drug and growth in the lowest drug level, while some evolved strains exhibited lower growth in no drug compared to low drug. This arithmetic issue appears to have occurred for some replicates, primarily from initially low-MIC_{50} parental strains (i.e., single replicates in A3 and A8 [Fig. S1]). Replicates from strain A5 highlight how small differences in OD can influence the calculation of MIC_{50}: four strains have numerically increased and four have numerically decreased MIC_{50}, yet the actual numbers reveal only a minor separation among replicates (Fig. S1). Visual examination of both the assay plates and graphic display of the raw optical density values is thus required to properly interpret numerical differences in MIC_{50}.

In bacteria, exposure to subinhibitory concentrations of antibiotics can select for *de*

novo mutations that confer resistance (57–62) or provide a pleiotropic benefit alongside existing resistance mutations (e.g., references 57 and 63 to 66). Here, consistent patterns among strains are lacking: of the five strain backgrounds that were exposed to a subinhibitory level of fluconazole, no significant changes were observed among replicates from three of them (A1, A12, and A18), MIC_{50} decreased in all replicates from one strain (A20), and variable, yet numerically significant, changes occurred in replicates of another strain (A5). This variability in phenotypic outcomes is reminiscent of the case in *Sclerotinia sclerotiorum*, a plant-pathogenic fungus, where both MIC increases and decreases (and no change) were observed and no consistent relationship was found between the change in resistance and exposure to sublethal concentrations of five different antifungal drugs (67). Fungal strains exposed to subinhibitory drug levels thus seem less likely than bacteria to gain an advantage at higher drug levels. Given the possible role of aneuploidy in this process, we speculate that differences in chromosome geometry (circular versus linear) and mechanisms that affect chromosome segregation may underlie these differences in the dynamics of antimicrobial responses.

The majority of replicates from strains with parental MICs at or below the evolutionary drug concentration had increased DNA content, interpreted as larger genome size. Furthermore, changes in DNA content and changes in fitness, MIC_{50}, or tolerance did not correlate, consistent with the idea that if the fluconazole-exposed isolates carry several aneuploid chromosomes, not all of them are necessarily causative of the observed fitness increases. Exposure to fluconazole at 10 μg/ml is known to induce the formation of "trimeras" (24), cells indicative of mitotic defects that result in aneuploidy at higher fluconazole concentrations; trimeras also were evident when lab strain SC5324 was exposed to 1 μg/ml fluconazole (M. Bibi and J. Berman, unpublished data). Growth in fluconazole is expected to exert selection pressure for some aneuploids more than others; specific genes within an aneuploid chromosome that are responsible for increased drug resistance (26) and drug tolerance (27) also have been identified. In many other cases, more than one gene may contribute to the phenotype (22, 27, 48, 68, 69). While we assume that changes in DNA content of a given isolate are largely due to chromosomal aneuploidy, it is important to note that increased levels of mitochondrial and/or ribosomal DNA can also contribute to differences in DNA content level detected by flow cytometry, as was recently shown to occur in different deletion mutants within the "isogenic" collection of *Saccharomyces cerevisiae* deletion mutants (70).

Given that some gene products, and the allelic ratios of the genes encoding them, may be more limiting than others in the face of a specific stress, it is quite clear that the degree to which a given aneuploid chromosome (or other copy number change) may contribute to stress tolerance or resistance is likely to be affected by the genetic background. The lack of a correlation between evolved genome size and fitness also suggests that there is a low cost to aneuploidy in this environment (i.e., there is no clear benefit to increased size, but there is also no clear cost). We observed lower genome size variation in some strain backgrounds than others of a similar parental fitness (i.e., A4 and A9), suggesting that these strain backgrounds may be less tolerant of aneuploidy than others (15). Variability in DNA content among evolved replicates was also very low for strains with parental MIC_{50}s of >1 μg/ml, presumably because these cells were under little stress in the evolutionary environment (Fig. S5). Thus, we posit that the observed variation in evolved ploidy may be integrally connected to the rapid appearance of altered drug responses. This observation may have clinical relevance, given that aneuploidy was common, albeit transient, in a study of sequential clinical isolates of *C. albicans*. Importantly, aneuploidy appeared concomitant with major shifts in drug resistance and yet was not retained in strains that acquired bona fide drug resistance (13). The DNA content measurements reported here were captured directly from populations of cells in the drug environment, and it is important to note that aneuploid chromosomes can be lost extremely rapidly in the absence of selective pressure (e.g., following a single overnight growth cycle in permissive medium). This

highlights the important idea that aneuploidy may provide a rapid, highly frequent, yet transient and suboptimal, genome change that facilitates adaptation until more robust and stable genome changes (e.g., point mutations) can be acquired (71, 72).

Conclusion. Experimental evolution studies can isolate important factors that influence adaptation. Here, neither clade nor mating type zygosity significantly influenced the observed evolutionary dynamics. However, genetic background did have a significant influence on the rate and variability of adaptation, mediated in part through parental fitness relative to the selective conditions used. Evolved changes in DNA content were prevalent among strains with parental MICs of <1 and largely absent from those with parental MICs above the drug level used for evolution, highlighting the context dependence (relative to strain MIC) of drug stress. Importantly, parental fitness was correlated with evolved variation among replicates for fitness, drug tolerance, and genome size, thereby emphasizing that strain background can influence both the magnitude and variation in adaptive responses to drug.

MATERIALS AND METHODS

Strains. Twenty clinical strains of *Candida albicans* were selected to represent the phylogenetic diversity of the species. The strain set includes at least four strains from each of the four major clades that encapsulate ~70% of the typed *C. albicans* strains and spans nearly the entire known phylogenetic diversity of the species (47, 73) as well as four commonly studied laboratory strains (SC5314, FH1, DSY294, and T188) (Table 1). For each clade, strains with both heterozygous (*MAT**a**/MATα*) and homozygous (*MAT**a**/MAT**a**, MATα/MATα*) mating loci were chosen. All 20 strains were initially diploid, though strain A1 was trisomic for chr7 and A11 was trisomic for chr4. Strains were chosen blind with respect to parental fitness or drug resistance, and we are agnostic to their potential histories of clinical drug exposure. Full strain information including clade designation, country of origin, and infection niche was obtained from the original manuscripts (Table 1) (74). Mating type genotype was confirmed by PCR with *MAT**a*** - and *MATα*-specific primers (*MAT**a*** F-TTGAAGCGTGAGAGGCAGGAG, *MAT**a*** R-GTTTGGGTTCCTTCTT TCTCATTC, *MATα* F-TTCGAGTACATTCTGGTCGCG, *MATα* R-TGTAAACATCCTCAATTGTACCCG). All strains were initially streaked onto YPD and grown for 48 h at 30°C. A single colony was frozen down in 15% glycerol and stored at −80°C. Thus, minimal genetic variation should be present in the initial freezer stock, which we refer to throughout as the "parental strains."

Evolution experiment. Strains were evolved in 1 μg/ml fluconazole, the epidemiological cutoff value that denotes the upper limit of drug susceptibility (MIC_{50}) in the wild-type *C. albicans* population (5). This concentration of drug was equivalent to the parental MIC_{50} of three strains, above the MIC_{50} for 12 strains, and below the MIC_{50} for one highly resistant strain ($MIC_{50} = 32$ μg/ml) and four additional strains ($MIC_{50} = 4$ μg/ml) (Table 1).

To initiate the evolution experiment, we generated 12 independent replicates from each parental strain. Cultures were struck from frozen parental stocks onto YPD plates (1% yeast extract, 1% peptone, 2% dextrose, 1% agar; the standard lab rich medium) and incubated at 30°C overnight. For each strain, colonies were randomly chosen by spotting each plate 12 times and picking the closest colony to each dot. Colonies were separately inoculated into 1 ml YPD in a 96-well (3-ml) deep culture box and grown shaking overnight at 30°C.

From the overnight cultures, we froze 100 μl from each replicate in duplicate in 50% glycerol as the parental (t0) replicates. Overnight cultures were diluted 1:1,000 into YPD + fluconazole in two steps: first, 10 μl of the overnight culture was transferred into 990 μl YPD (a 1:100 dilution), followed by a transfer of 20 μl diluted culture in YPD into 180 μl of YPD + 1.11 μg/ml FLC (for a final concentration of 1 μg/ml FLC) in round-bottom microtiter plates. To minimize the likelihood of contamination and keep environmental conditions similar, culture from replicates from one strain was inoculated into row A while culture from replicates from a second strain was inoculated into row H. Plates were sealed with Breathe-Easy sealing membranes (Sigma Z380058) and incubated statically at 30°C to mimic the static growth used for clinical resistance assays. Plates were contained within small sealed Rubbermaid containers with wet paper towels inside to minimize evaporation.

After 72 h, wells were mixed by pipetting, and another two-step 1:1,000 transfer was conducted into fresh YPD + fluconazole medium. In total, 10 transfers were conducted, yielding 100 generations of evolution [9.97 generations between transfers, $\log_2(1,000) = 9.97 \times 10$ transfers = 99.7 generations]. Fifty microliters of the evolved replicate cultures was frozen in duplicate in 50% glycerol after the 10th transfer (t10) and maintained at −80°C.

Growth in the evolutionary environment. We measured fitness in the evolutionary drug environment as optical density (A_{600}, OD) at both 24 and 72 h. OD reflects the ability to convert nutrients from the environment into cellular biomass. Fitness at 24 h can be thought of as a composite parameter reflecting both lag phase and the exponential growth rate (strains that have either a lower lag or a higher growth rate will have higher 24-h fitness) (75). Fitness at 72 h reflects growth at the time of transfer and the amount of biomass present at stationary phase. OD at 24 h is also consistent with the clinical assessment of drug resistance.

Clinical resistance and tolerance. The initial susceptibility and tolerance of all strains were tested using broth microdilution liquid assays to measure the MIC (MIC_{50}) and tolerance as supra-MIC growth

(SMG), respectively. The liquid assay experiments followed the initial cell dilution regulations from the clinical CLSI M27-A guidelines (1), except with YPD incubated at 30°C as the base medium and optical density at A_{600} (OD) instead of a McFarland 0.5 standard to determine the initial density of cells. OD readings were taken at 24 h after inoculation. From these data, MIC_{50} was numerically calculated following the guidelines for azole drugs (1), where the MIC is defined as the lowest concentration in which a score of 2 (prominent decrease in turbidity; approximately 50% as determined visually or spectrophotometrically) is observed.

Four liquid broth microdilution assays were conducted on both the parental and evolved replicates, and an additional two assays were conducted on the parental replicates. We were not able to assay all drug concentrations in each assay due to capacity (2 time points $\times$ 20 strain backgrounds $\times$ 12 replicates). Each assay was separately initiated from culture maintained at $-80°C$ and grown overnight in YPD. A single measurement was taken for each replicate at each concentration of drug measured in a given experiment (see Table S2). The median OD among experiments was determined at each concentration of drug for each replicate. Following guidelines, the MIC_{50} was then calculated as the highest concentration of drug with an OD greater than 50% of the measured OD in YPD (i.e., optical density in medium without drug).

Tolerance was measured from the liquid assay results for each parental and evolved replicate. As in reference 3, average growth (measured as optical density, A_{600}) above MIC_{50} was divided by the measured growth in the lowest drug level (0.06 μg/ml fluconazole) so that tolerance reflects the fraction of realized growth and is between 0 and 1.

$$\text{Tolerance} = \frac{\text{average growth above } MIC_{50}}{\text{growth at 0.06 μg/ml fluconazole}}$$

Tolerance was assessed at 48 and 72 h. We consider evolved replicates to have increased or decreased in tolerance when their measured tolerance was respectively above or below the range of parental tolerance levels for t0 replicates from that strain.

Ploidy variation. Flow cytometry was performed on a BD Biosciences BD LSR II. In all cases, all replicates from the same time point were fixed, stained, and measured in parallel. Parental replicates were measured twice independently from the freezer stocks maintained at $-80°C$. Freezer cultures were thawed and mixed, and 10 μl was added to 500 μl YPD in deep 96-well boxes, covered with a Breathe-Easy membrane, and shaken at 200 rpm and 30°C overnight. After ~16 h of growth, 20 μl of culture was washed in 180 μl of Tris-EDTA (TE), in a round-bottom microtiter plate, pelleted, resuspended in 20 μl TE, and fixed by adding 180 μl 95% cold ethanol.

Samples from parental (t0) replicates were grown up overnight in YPD. Samples from evolved (t10) replicates were fixed in ethanol at the end of the last growth cycle; 50 μl of 72-h culture from t10 was washed in 150 μl TE in a round-bottom microtiter plate, pelleted, and resuspended in 20 μl TE and 180 μl 95% cold ethanol. Ethanol-fixed cultures were stored at $-20°C$ for up to 4 weeks. The remainder of the protocol was identical for both time points, following the work of Gerstein et al. (76). As described in detail in the work of Gerstein et al. (76), we used the cell cycle analysis in Flow-Jo (TreeStar) to determine the mean G_1 peak for each replicate; when more than one peak was evident, we recorded both the major and minor G_1 peaks.

Although we always used the same machine settings, subtle but significant variation is always observed in flow cytometry data. To better compare t0 and t10 data, we performed a day-correction based on the median G_1 intensity of the A12 parental and evolved replicates, which always measured cleanly as diploids.

Statistical methods. All analysis was conducted in the R programming language (77). To maximize statistical power when testing the influence of mating type, we examined the effect of a heterozygous mating type versus homozygous mating type, i.e., we combined *MAT***a**/**a** and *MAT*α/α strains.

MIC_{50} values were log transformed prior to statistical analysis. When parametric tests were used, all assumptions were tested and met. When data transformations were insufficient to meet the test assumptions, nonparametric tests were used. Spearman's rank correlation was used when comparing the mean responses among replicates. This correlation method uses a rank-based measure which does not require the replicate data to come from a bivariate normal distribution. In all cases, the specific test is indicated inline.

We used linear mixed-effect models to determine which factors influenced evolved tolerance and genome size. Since there was significant variation in parental tolerance, the response variable in the tolerance model was evolved − parental tolerance. In both models, the predictor variables were change in fitness (OD at 72 h, evolved − parental), zygosity, and clade, with strain as a random effect. The models were implemented with the lmer package (78) in the R programming language (77). Significance was determined from the type III ANOVA table with Satterthwaite's approximation to degrees of freedom.

Data accessibility. All data and the R code required to run the analyses and create the visualizations are available on GitHub (https://github.com/acgerstein/C_albicans-LDE).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.01 MB.

FIG S2, PDF file, 0.05 MB.

FIG S3, PDF file, 0.01 MB.

FIG S4, PDF file, 0.01 MB.

FIG S5, PDF file, 0.02 MB.
FIG S6, PDF file, 0.01 MB.
FIG S7, PDF file, 0.01 MB.
TABLE S1, PDF file, 0.5 MB.
TABLE S2, PDF file, 0.5 MB.

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