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Divergence in Fitness and Evolution of Drug Resistance in Experimental Populations of *Candida albicans*

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The dissemination and persistence of drug-resistant organisms in nature depends on the relative fitness of sensitive and resistant genotypes. While resistant genotypes are expected to be at an advantage compared to less resistant genotypes in the presence of drug, resistance may incur a cost; resistant genotypes may be at a disadvantage in the absence of drug. We measured the fitness of replicate experimental populations of the pathogenic yeast *Candida albicans* founded from a single progenitor cell in a previous study (L. E. Cowen, D. Sanglard, D. Calabrese, C. Sirjusingh, J. B. Anderson, and L. M. Kohn, J. Bacteriol. 182:1515–1522, 2000) and evolved in the presence, and in the absence, of the antifungal agent fluconazole. Fitness was measured both in the presence and in the absence of fluconazole by placing each evolved population in direct competition with the drug-sensitive ancestor and measuring the reproductive output of each competitor in the mixture. Populations evolved in the presence of drug diverged in fitness. Any significant cost of resistance, indicated by reduced fitness in the absence of drug, was eliminated with further evolution. Populations evolved in the absence of drug showed more uniform increases in fitness under both conditions. Fitness in the competition assays was not predicted by measurements of the MICs, doubling times, or stationary-phase cell densities of the competitors in isolation, suggesting the importance of interactions between mixed genotypes in competitions.

The emergence and spread of drug resistance among viruses (8, 13, 30), bacteria (4, 24, 26, 32), and fungal pathogens (16, 42) has followed the widespread use of antimicrobial agents in recent years and now poses a growing public health problem (26). Whether drug-resistant organisms persist in nature depends on their fitness relative to drug-sensitive organisms. Correlations between resistance to fungicides and fitness traits such as latency, sporulation, and survival of plant-pathogenic fungi have provided mixed results: that there is no cost of resistance (34), that there is a cost for survival of resistant propagules but not somatic cells (35); and that there is a cost not correlated with any specific trait (15, 18). The evolutionary dynamics of drug resistance and its fitness costs have been studied in viruses (8, 31) and bacteria (2, 6, 7, 25, 38) but not in a comparable way in fungi, despite their increasing importance as opportunistic pathogens of humans (1, 14, 17). Experimental populations of the pathogenic fungus *Candida albicans* from a previous study (12) provided the opportunity here to measure relative fitness by comparing the reproductive rates of populations that had evolved resistance to the antifungal drug fluconazole with that of their drug-sensitive ancestor in a common environment with, and without, fluconazole. Our primary goal in this study was to determine whether replicate populations evolved in the presence of fluconazole diverged in fitness and then to measure any cost of resistance. Our expectation was that fluconazole resistance would carry a significant fitness cost in the absence of the drug.

In the presence of drug, a resistant genotype is expected to be at an advantage compared to less resistant genotypes. In the absence of drug, however, resistant genotypes may be at a disadvantage compared to their sensitive counterparts. Strategies to control the dissemination of drug resistance by restricting the use of antimicrobial agents (27) implicitly assume that resistant genotypes have reduced fitness relative to their sensitive counterparts in the absence of drug (38). A fitness cost of drug resistance has been demonstrated in a number of experiments when resistance genes are introduced into bacteria (20). The composite of different mutations conferring resistance among different genetic backgrounds may result in variation in the magnitude of the cost of resistance, as was the case with resistance to rifampin in *Bacillus subtilis* (11). Costs of resistance are anticipated to decline during subsequent evolution as natural selection continues to favor genotypes with a fitness advantage. For example, compensatory mutations reducing the cost of resistance have been identified in recent experimental studies with diverse microbes, including human immunodeficiency virus, *Salmonella enterica* serovar Typhimurium, and *Escherichia coli* during evolution both in the presence and in the absence of drug selection (reviewed by Levin et al. [25]). In several studies, the evolved, resistant microbes actually achieved superior fitness relative to that of the parental drug-sensitive strain even in the absence of drug (9, 31). Adaptations reducing the cost of resistance may create a genetic background where reversion to the ancestral drug-sensitive state is virtually precluded due to a selective disadvantage conferred on sensitive alleles in that background (38).

The focus of this study was the fungus *C. albicans*. The 12 initially identical experimental populations of *C. albicans* whose fitness we examine here were established in a previous study (12) in which we monitored adaptation to inhibitory concentrations of fluconazole over 330 generations. Each population propagated in the presence of drug evolved resistance as measured by an increase in the MIC of fluconazole during the course of the experiment. These increases in resistance fol-

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lowed strikingly different trajectories among populations. These populations also showed various patterns of overexpression of four genes known to be associated with azole resistance, accompanied in some cases by changes in the genome likely to have been selectively neutral. The different trajectories were attributed to the randomness of various kinds of resistance mutations occurring under the specified conditions of population size and culture regime, as well as the temporal sequence in which these mutations may have occurred.

The specific objectives of the present study were (i) to measure the change in fitness among the six experimental populations of *C. albicans* evolved in the presence of fluconazole and to determine any cost of resistance in the absence of fluconazole, (ii) to measure the change in fitness among the six control populations evolved in the absence of fluconazole, and (iii) to characterize the relationships among the level of drug resistance, as measured by standard laboratory MIC tests, standard growth parameters, and fitness in the presence of drug. We found that fitness diverged among the initially identical, replicate populations and that any apparent cost of resistance tended to decrease with further evolution. Furthermore, there were no simple, direct relationships among fitness in competition assays, MICs of fluconazole, and various cell growth parameters.

MATERIALS AND METHODS

Strains, culture conditions, and determination of MICs. The evolutionary history of the experimental populations of *C. albicans* characterized in this study has been previously described (12). Briefly, 12 populations of *C. albicans* were founded from a single colony and were serially propagated for 330 generations (~100 days) in RPMI 1640 medium (29). One milliliter from each overnight culture was serially transferred into 9 ml of fresh medium daily, and cultures were grown at 35°C with constant agitation. Six populations were grown in the absence of drug (N1 to N6) and six populations (D7 to D12) were grown in fluconazole (Roerig-Pfizer Inc., New York, N.Y.) at a concentration twice the most recently measured MIC for that population. Despite the drop in MIC that occurred with three populations (D7, D10, and D12) during the experiment, the drug concentrations were never reduced. MIC tests for populations D8 and D10 from generation 260 showed significant growth above the MIC to 64 μg/ml (this phenotype is referred to as a trailing endpoint [28]); these populations were grown with fluconazole at 128 μg/ml. Population samples were archived in 1 ml of 40% (vol/vol) glycerol containing 3% (wt/vol) trisodium citrate at −70°C. MICs were determined by the broth microdilution method using approved standard M27-A of the National Committee for Clinical Laboratory Standards (29).

Resistance to MPA. Resistance to mycophenolic acid (MPA) was used as a marker to distinguish the progenitor from the evolved populations in competition assays designed to measure relative fitness (see below). We encountered a spontaneous mutant that was resistant to MPA while attempting to transform the progenitor strain to resistance to MPA with a cloned putative inosine 5-monophosphate dehydrogenase (*IMH3*) allele (kindly provided by P. T. Magee and J. Beckerman, University of Minnesota; materials and conditions are available from us upon request), which has been demonstrated to confer resistance to MPA in *C. albicans* (5). This mutant satisfied our requirements for (i) stability during growth in the absence of MPA and (ii) the absence of any detectable impact on fitness, the MIC of fluconazole, doubling time, or stationary-phase cell density. While we do not know the nature of the MPA resistance mutation, we did not detect any differences between the MPA-resistant mutant and the progenitor in their nucleotide sequences, mRNA expression levels, or copy numbers of the *IMH3* gene (data not shown).

Measurement of relative fitness. The fitness of the 12 populations at generation 330 and of the generation associated with the MIC peak for the three populations that subsequently dropped in MIC was determined by placing each of the evolved populations in direct competition with the progenitor, genetically marked with resistance to MPA. Competition experiments were conducted in triplicate both in the presence and in the absence of fluconazole, under the same culture conditions used in the experimental evolution study. All competition experiments were conducted in RPMI 1640 at 35°C. For the competition experiments with populations evolved with drug that were conducted in the presence of drug, the concentration of fluconazole used was the concentration most recently experienced by the evolved competitor during the evolution experiment (see Fig. 1). For the competition experiments with populations evolved without drug and with the unmarked progenitor that were conducted in the presence of drug, the concentration of fluconazole used was twice the MIC for these populations (see Fig. 3). The competing populations were first conditioned by growing each competitor from the frozen archive separately for one complete growth cycle (24 h) in RPMI 1640 medium. Cell counts of the overnight cultures, performed with a hemocytometer, were used to prepare a competition mix containing approximately equal concentrations of the two competitors (~10^7 CFU/ml). One hundred microliters of the competition mix was used to inoculate 9.9 ml of fresh medium, and the competitors were allowed to grow together during one standard daily growth cycle. Initial and final densities of each replicate competition culture were determined by colony counts from dilution plates on minimal medium (0.667% yeast nitrogen base without amino acids, 2% D-glucose, 1.5% agar). The initial and final densities of each competitor were determined by transferring by means of applicators (Puritan; Hardwood Products Company L.P., Guilford, Maine) 200 colonies from the dilution plates from each replicate competition to minimal medium containing 10 μg of MPA (Sigma, St. Louis, Mo.) per ml in a grid pattern. Under these selective conditions, the genetically marked progenitor is able to grow while growth of the other competitor is inhibited.

Fitness was estimated as the difference in the numbers of doublings of the two competitors (evolved population minus the genetically marked progenitor), standardized by the total number of doublings in the competition assay. A more conventional measure of relative fitness uses the ratio of the number of doublings of the two competitors (for example, see reference 22). However, this ratio is very sensitive to sampling error if the two competitors are very different in the numbers of doublings achieved (39), as was the case in our study. There is also precedent for using the difference in the numbers of doublings of two competitors as a measure of relative fitness (39), and it is much less sensitive to sampling error; an important caveat in measuring relative fitness on the basis of this criterion is that the total numbers of doublings among competition experiments must be uniform for the fitness data to be compared. In our study, the total number of doublings achieved under conditions with drug was significantly lower than the total number achieved without drug. Standardization of the difference in the numbers of doublings of the two competitors by the total number of doublings was therefore necessary.

Control experiments were conducted in tandem with the competition assays for each population, both in the presence and in the absence of fluconazole. For the controls, the overnight culture of each competitor was diluted 100-fold in fresh medium, both at the same concentration of fluconazole used in the competition assay and in the absence of drug. One hundred colonies from dilution plates of the initial and final time points for each of the four controls were transferred in a grid pattern onto minimal medium containing 10 μg of MPA per ml in order to ensure the stability of the MPA-resistant phenotype in the genetically marked progenitor and to detect any spontaneous MPA resistance in the evolved population. No reversion to MPA sensitivity was detected for the marked progenitor in any of the experiments. Spontaneous resistance to MPA was identified in only one population sample, D11-330. D11-330 produced exclusively very small colonies on minimal medium which were reliably distinguishable from the much larger colonies of the marked progenitor. For the competitions with D11-330, colony size was used to confirm the identity of each competitor.

Measurement of growth parameters. Doubling times during the exponential growth phase and stationary-phase cell densities were determined for the progenitor, the 12 populations at generation 330, and the 3 drug-resistant populations at their MIC peak, with the same culture conditions used in the competition assays. These growth parameters were determined for the progenitor at all four concentrations of fluconazole used in the competition experiments (0.5, 16, 32, and 128 μg/ml). One hundred microliters of an overnight RPMI 1640 culture of each population sample recovered from the frozen archive was inoculated in triplicate into 9.9 ml of medium both in the absence of drug and in the presence of fluconazole at the same concentration used in the competition assays. The concentration of cells was monitored with a spectrophotometer at 530 nm (Du-64 spectrophotometer; Beckman Instruments, Inc.) at 0, 2, 4, 6, 8, 10, 12, and 24 h.

Statistics. Analyses of variance (SYSTAT 5.2.1) were performed on fitness, doubling time, and stationary-phase cell density data obtained both in the presence and in the absence of drug to test for the significance of variation among populations. The significance of differences between pairs of means was evaluated using Tukey tests (SYSTAT 5.2.1) in order to identify which populations

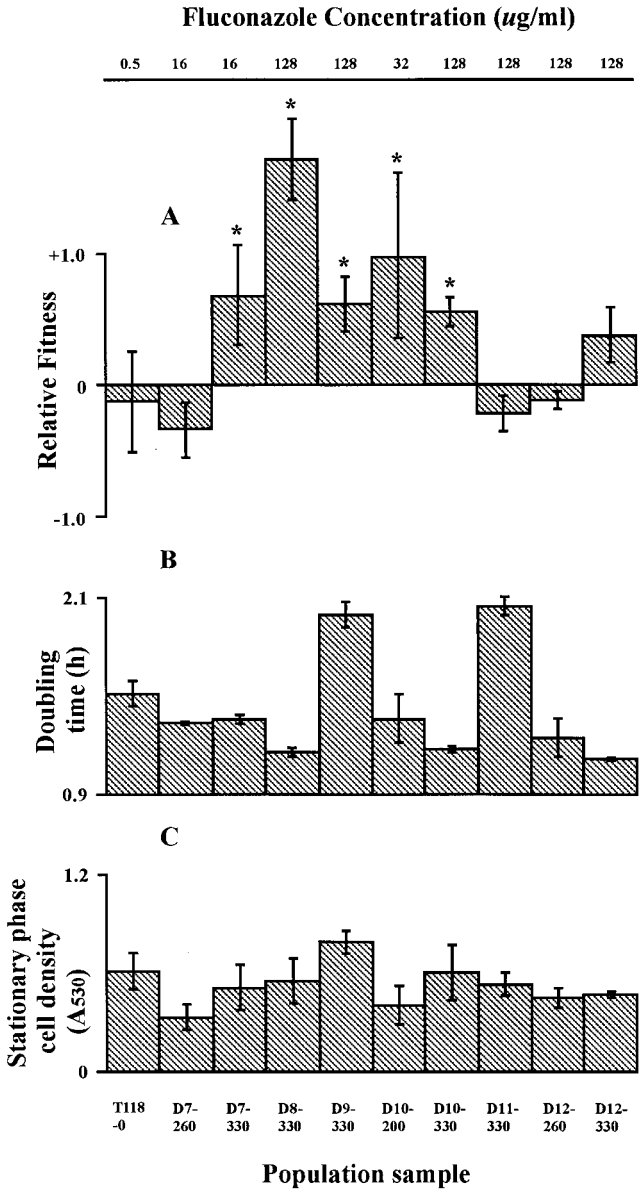

FIG. 1. (A) Fitness of the progenitor (T118-0) and the experimental populations evolved in the presence of drug (D7 to D12) relative to the genetically marked progenitor, determined with fluconazole at the concentrations indicated across the upper bar. Asterisks indicate that the numbers of doublings of the two competitors were significantly different ($P < 0.05$, paired t test). (B) Doubling time. (C) Stationary-phase cell density. Bars represent the 95% confidence interval for each sample ($n = 3$ replicate measurements).

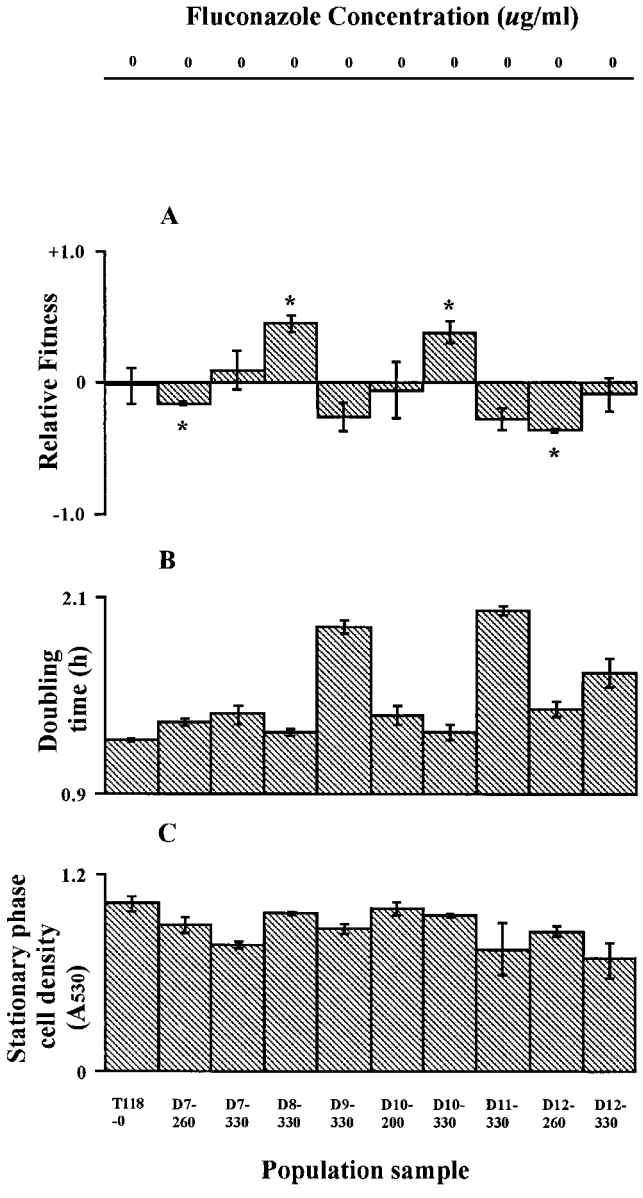

FIG. 2. (A) Fitness of the progenitor (T118-0) and the experimental populations evolved in the presence of drug (D7 to D12) relative to the genetically marked progenitor, determined without fluconazole. Asterisks indicate that the numbers of doublings of the two competitors were significantly different ($P < 0.05$, paired t test). (B) Doubling time. (C) Stationary-phase cell density. Bars represent the 95% confidence interval for each sample ($n = 3$ replicate measurements).

differed significantly from others. The strengths of associations between doubling time and fitness and between stationary-phase cell density and fitness were evaluated with Pearson correlation and Bonferroni-adjusted probabilities to account for multiple comparisons. Paired t tests on the number of doublings of each competitor were used to evaluate the significance of the outcome of each competition experiment.

RESULTS

Variation in fitness, doubling time, and stationary-phase cell density among the experimental populations of *C. albicans*. We measured fitness and growth parameters of the 12 replicate experimental populations of *C. albicans* founded from a single azole-susceptible cell and reared over 330 generations, as previously described by Cowen et al. (12). Fitness was measured by placing each population in direct competition with the progenitor (T118-0) genetically marked with resistance to MPA. The 16 population samples characterized included the unmarked progenitor, generation 330 of the 6 replicate populations evolved in the presence of inhibitory concentrations of fluconazole (D7 to D12), the generation of the MIC peak for the 3 drug populations that subsequently dropped in MIC (D7

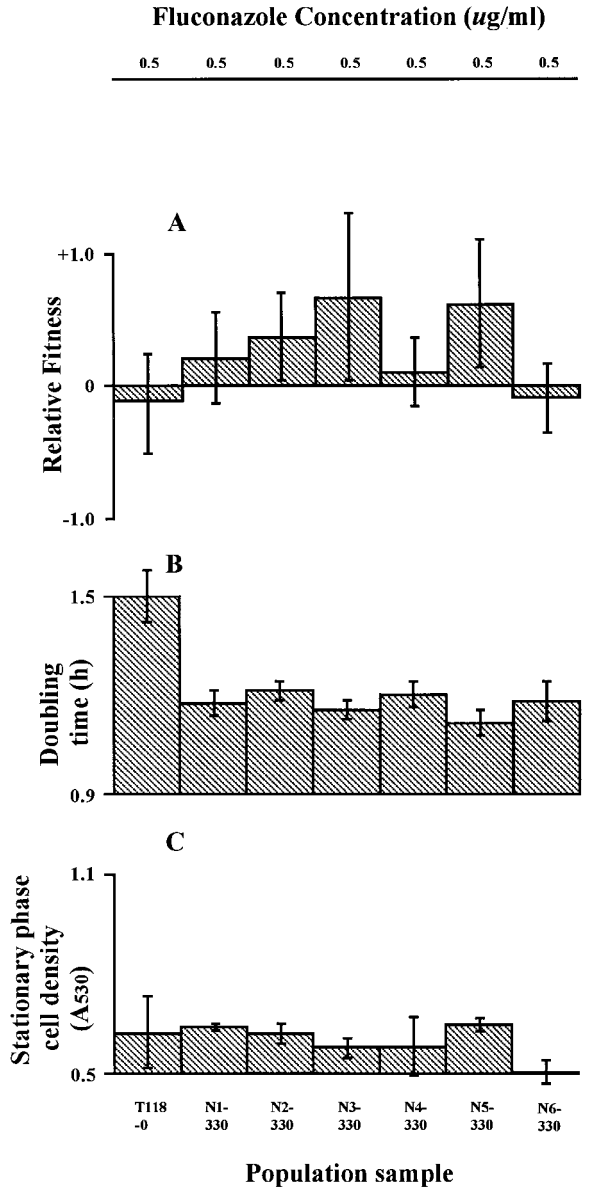

FIG. 3. (A) Fitness of the progenitor (T118-0) and the experimental populations evolved in the absence of drug (N1 to N6) relative to the genetically marked progenitor, determined with fluconazole at 0.5 μg/ml. Asterisks indicate that the numbers of doublings of the two competitors were significantly different ($P < 0.05$, paired t test). (B) Doubling time. (C) Stationary-phase cell density. Bars represent the 95% confidence interval for each sample ($n = 3$ replicate measurements).

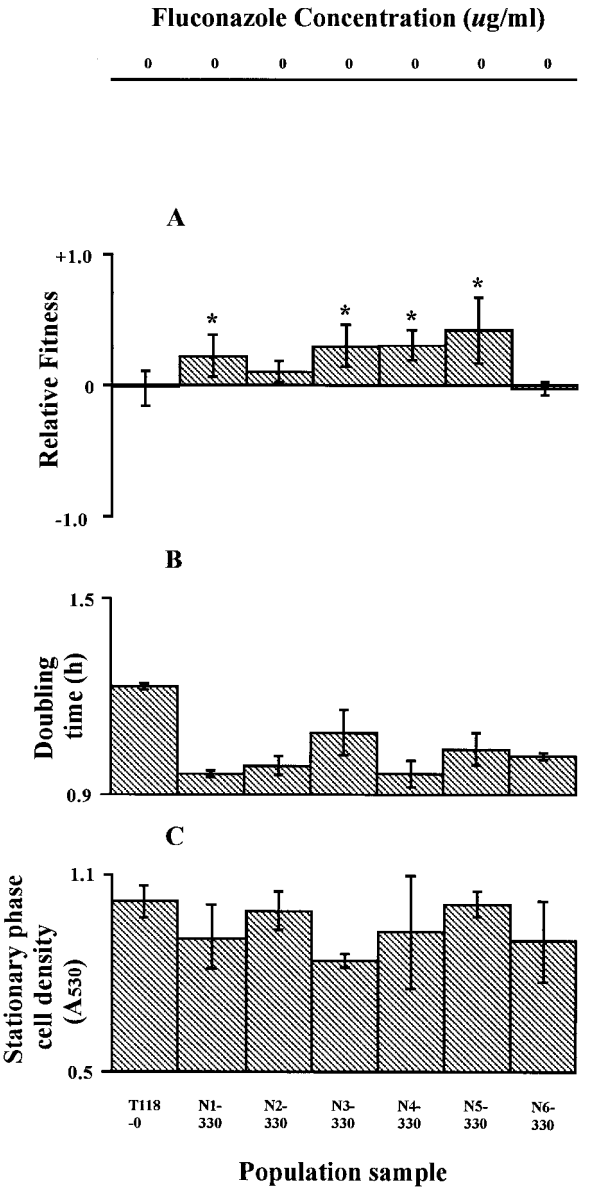

FIG. 4. (A) Fitness of the progenitor (T118-0) and the experimental populations evolved in the absence of drug (N1 to N6) relative to the genetically marked progenitor, determined without fluconazole. Asterisks indicate that the numbers of doublings of the two competitors were significantly different ($P < 0.05$, paired t test). (B) Doubling time. (C) Stationary-phase cell density. Bars represent the 95% confidence interval for each sample ($n = 3$ replicate measurements).

at generation 260, D10 at generation 200, and D12 at generation 260), and generation 330 of the 6 replicate populations evolved in the absence of drug (N1 to N6). The experimental populations diverged in fitness, measured as the difference in the numbers of doublings of the two competitors standardized by the total number of doublings in the competition assay (Fig. 1A, 2A, 3A, and 4A). In an analysis of variance, the variation in fitness among the 16 population samples was highly significant ($P < 0.0001$) when it was measured both in the presence of drug ($F_{15,32} = 8.64$ [the numbers associated with the value of the F distribution are the degrees of freedom associated with variances in the numerator and denominator, respectively]) and in the absence of drug ($F_{15,32} = 15.48$). Doubling times of 16 population samples were determined under the same conditions used for competition assays (Fig. 1B, 2B, 3B, and 4B). Doubling times for the progenitor were determined at all four concentrations of fluconazole used in the competition experiments (0.5, 16, 32, and 128 μg/ml), providing a total of 19 population samples for growth parameter estimates in the presence of drug. Variation in doubling time among the population samples was highly significant ($P < 0.0001$), both in the presence of drug ($F_{18,38} = 51.16$) and in the absence of drug

($F_{15,32} = 190.71$). Variation in the stationary-phase cell density among population samples (Fig. 1C, 2C, 3C, and 4C) was also highly significant ($P < 0.0001$), both in the presence of drug ($F_{18,38} = 5.65$) and in the absence of drug ($F_{15,32} = 5.65$). There was no significant correlation between doubling time and fitness or stationary-phase cell density and fitness for populations evolved in the presence of drug or for populations evolved in the absence of drug when these parameters where measured in either environment ($P > 0.25$ in all cases).

Selective neutrality of the genetic marker. Selective neutrality of the genetic marker was confirmed, as there was no significant fitness difference between the progenitor (T118-0) and the genetically marked progenitor either in the presence of fluconazole ($P > 0.6$) (Fig. 1A) or in the absence of drug ($P > 0.9$) (Fig. 2A). While doubling times of the progenitor were longer and stationary-phase cell densities were lower in the presence of drug (Fig. 1B and 1C), compared to those in the absence of drug (Fig. 2B and 2C), there was no significant difference among doubling times or stationary-phase cell densities measured at the different drug concentrations (data not shown) ($P > 0.2$, Tukey test).

Populations evolved with drug. The populations evolved in the presence of drug diverged in fitness relative to the ancestral drug-sensitive state (fluconazole MIC, 0.25 μg/ml). In population D7 at generation 260 (D7-260; MIC, 8 μg/ml), the lack of significant difference in fitness compared to the progenitor in the presence of drug at 16 μg/ml (Fig. 1A) coupled with the fitness disadvantage in the absence of drug (Fig. 2A) indicated a cost of drug resistance. By generation 330, however, compensatory changes in population D7 eliminated the cost of resistance; D7-330 increased in fitness in the presence of drug at 16 μg/ml (Fig. 1A), with no significant difference in fitness compared to the progenitor in the absence of drug (Fig. 2A). The significant improvement in fitness in the presence of drug in population D7 between generations 260 and 330 ($P < 0.05$, Tukey test) was accompanied by a drop in the fluconazole MIC from 8 to 0.5 μg/ml. Neither doubling times nor stationary-phase cell densities of D7-260 and D7-330 differed significantly from each other either in the presence (Fig. 1B and 1C) or in the absence (Fig. 2B and 2C) of drug.

Population sample D8-330 significantly increased in fitness in the presence of fluconazole at 128 μg/ml (Fig. 1A). The marked progenitor actually decreased in numbers during the competition experiment with drug. D8-330 also maintained a significant fitness advantage in the absence of drug (Fig. 2A). Due to the trailing endpoint of this population (i.e., consistent growth in the MIC test plate at increasing drug concentrations above the MIC), D8 was grown at 128 μg/ml during the evolution experiment from generation 260 rather than at twice the MIC of 4 μg/ml. D8-330 had a significantly shorter doubling time in the presence of drug (Fig. 1B) ($P < 0.001$, Tukey test), but there was no significant difference in doubling time between the progenitor and D8-330 in the absence of drug (Fig. 2B). There was no significant difference in stationary-phase cell density between D8-330 and the progenitor when they were grown either with or without drug (Fig. 1C and 2C).

D9-330 (MIC, 64 μg/ml) significantly increased in fitness at 128 μg of fluconazole per ml (Fig. 1A) but did not change in fitness compared to the progenitor in the absence of drug (Fig. 2A). D9-330 had a significantly longer doubling time than that of the progenitor ($P < 0.001$, Tukey test) both in the presence (Fig. 1B) and in the absence (Fig. 2B) of drug. There was no significant difference in stationary-phase cell density between the two population samples (Fig. 1C and 2C).

Population D10 at generation 200 (MIC, 16 μg/ml) significantly increased in fitness at 32 μg of fluconazole per ml (Fig. 1A) but did not increase in fitness in the absence of drug (Fig. 2A). By generation 330, the fitness of population D10 had improved significantly ($P < 0.005$, Tukey test) in the absence of drug. D10-330 maintained a significant fitness advantage relative to the progenitor both with 128 μg of fluconazole per ml (Fig. 1A) and without drug (Fig. 2A). Accompanying this improvement in fitness, the MIC of population D10 dropped to 1 μg/ml by generation 330, although this population had been growing at 128 μg of fluconazole per ml since generation 260. There were no significant differences in doubling time or stationary-phase cell density between D10-200 and D10-330 either in the presence or in the absence of drug (Fig. 1B and C and 2B and C).

Population sample D11-330 (MIC, 64 μg/ml) did not differ significantly in fitness from the progenitor either with 128 μg of fluconazole per ml (Fig. 1A) or without drug (Fig. 2A). D11-330 had a significantly longer doubling time than the progenitor ($P < 0.001$, Tukey test) both in the presence (Fig. 1B) and in the absence (Fig. 2B) of drug. The stationary-phase cell density of D11-330 was significantly lower than that of the progenitor only in the absence of drug (Fig. 2C) ($P < 0.005$, Tukey test).

D12-260 (MIC, 64 μg/ml) did not differ significantly in fitness from the progenitor at 128 μg of fluconazole per ml (Fig. 1A) and was less fit in the absence of drug (Fig. 2A). By generation 330, however, the cost of resistance was eliminated, accompanied by a decrease in MIC to 4 μg/ml. D12-330 did not differ in fitness from the progenitor either in the presence of drug at 128 μg/ml (Fig. 1A) or in the absence of drug (Fig. 2A). While D12-260 and D12-330 did not differ significantly in doubling time in the presence of drug (Fig. 1B), D12-330 had a significantly longer doubling time in the absence of drug (Fig. 2B) ($P < 0.001$, Tukey test). There were no significant differences in the stationary-phase cell densities of the two population samples (Fig. 1C and 2C).

Populations evolved without drug. In contrast to the divergence in fitness detected among the populations evolved under the selective pressure imposed by the presence of drug, the replicate populations evolved without drug showed a more uniform trend. In the presence of drug at 0.5 μg/ml (twice the fluconazole MIC for the progenitor and all of the populations evolved in the absence of drug), there was a trend toward increased fitness but the trend was not significant (Fig. 3A). In the absence of drug, four population samples (N1-330, N3-330, N4-330, and N5-330) increased in fitness (Fig. 4A) while the remaining two (N2-330 and N6-330) did not increase in fitness compared to the progenitor. There were no significant differences in doubling times or stationary-phase cell densities detected among populations N1 to N6 at generation 330, either in the presence (Fig. 3B and 3C) or in the absence (Fig. 4B and 4C) of drug. In contrast, at generation 330, N1 to N6 all had shorter doubling times relative to that of the progenitor, both in the presence of drug ($P < 0.005$ in all cases, Tukey test) and in the absence of drug ($P < 0.05$ in all cases, Tukey test).

DISCUSSION

This study was designed to identify changes in fitness accompanying the evolution of drug resistance in replicate experimental populations of the pathogenic yeast *C. albicans*. These changes were identified by placing evolved populations and a genetically marked version of the progenitor in a common environment and measuring the reproductive output of each competitor in the mixture. Among the populations evolved in the presence of drug, the change in fitness relative to the progenitor was highly divergent, ranging from a small decrease to a large increase, when measured both in the presence and in the absence of drug. In these populations, any detectable cost of resistance was mitigated with further evolution; by the end of the evolution experiment, none of the populations evolved with drug showed a significant cost of drug resistance. Among the populations evolved in the absence of drug, the change in fitness was more uniform in both environments, with increases in most. Fitness in the competition assays was not predicted by measurements of the MICs, doubling times, or stationary-phase cell densities of the competitors in isolation. Fitness could be explained only by complex interaction between competitors.

Fitness of populations evolved in the presence of drug. In the presence of drug, a resistant genotype is expected to be at an advantage. Of the nine population samples from the lineages evolved in the presence of drug, five (D7-330, D8-330, D9-330, D10-200, and D10-330) increased in fitness over the progenitor in the presence of drug (Fig. 1A) while four (D7-260, D11-330, D12-260, and D12-330) did not. None of the five population samples that achieved this fitness advantage incurred a cost of resistance, i.e., reduced fitness in the absence of drug (Fig. 2A). Two (D8-330 and D10-330) of the five population samples maintained a significant fitness advantage over the progenitor even in the absence of drug, while three (D7-330, D9-330, and D10-200) did not. In the two populations at generation 260 (D7 and D12) that showed a significant cost of resistance, the cost was eliminated by generation 330. The compensatory changes between generations 260 and 330 were mediated by selective sweeps, i.e., mutations conferring a selective advantage increased in frequency. Two selective sweeps in population D7, one associated with the MIC peak at generation 260 and one with the endpoint at generation 330, were identified by changes in markers with no known relation to drug resistance, including loss of heterozygosity and changes in DNA fingerprints (12).

The diversification in fitness of the populations evolved with inhibitory concentrations of fluconazole is consistent with our previous interpretation (12) that the populations gained altitude by taking different routes on the adaptive landscape (43, 44). While different molecular mechanisms were implicated in azole resistance among the replicate populations, there was no direct association between molecular mechanism and fitness consequence. For example, although overexpression of the gene *MDR1*, encoding an efflux pump of the major facilitator family (42), was detected in population samples D9-330, D11-330, D12-260, and D12-330 (12), these population samples differed significantly in fitness (Fig. 1A and 2A). Changes in addition to the change in the level of expression of *MDR1* must affect fitness in these evolved populations.

While most populations evolved with drug increased in fitness relative to the progenitor in the presence of drug, populations D11 and D12 did not. How could D11 and D12 adapt to the presence of drug without showing an increase in fitness by generation 330? The successive nature of selective sweeps in the experimental populations might offer an answer. For example, if genotype B has a fitness advantage over the ancestral genotype A and genotype C has a fitness advantage over genotype B, this does not necessarily imply that genotype C will have a fitness advantage over genotype A. Epistatic interactions between adaptive mutations were offered as an explanation of a comparable successive decrease in mean population fitness detected in asexual evolving populations of the yeast *Saccharomyces cerevisiae* (33). Alternatively, it is possible that the selective sweeps observed in the experimental populations were the consequence of competition between more than just two competitors, as new genetic variability is continually introduced by mutation.

Fitness of populations evolved without drug. In contrast to the divergence in fitness associated with the evolution of azole resistance, the increase in fitness among the populations evolved in the absence of drug was relatively uniform. In the absence of drug, adaptation to culture conditions was detected at generation 330 in four of the six populations (N1 to N6) evolved in the absence of drug (Fig. 4A). Adaptation to culture conditions may also be responsible for the nonsignificant trend towards improved fitness in five of the six populations determined in competition assays with drug (Fig. 3A). Adaptation to general environmental conditions has been well documented in a number of experimental studies with bacteria (19, 21–23, 40, 41).

The relationship between MIC and fitness. The relationship between drug resistance, as measured by standard laboratory MIC tests, and fitness in the presence of drug has important implications for predicting the response of a genotype to drug treatment. MICs determined according to the National Committee for Clinical Laboratory Standards protocol (29) have been used to establish breakpoints for clinical interpretation of antifungal susceptibility (36). The breakpoints for fluconazole MICs are as follows: <8 μg/ml, sensitive; 8 to 32 μg/ml, susceptible dose dependent; and ≥ 64 μg/ml, resistant. While correlation has been observed between fluconazole MIC and clinical outcome (reviewed by White et al. [42]), there have been failures in treating patients with susceptible strains and successes in treating patients with resistant strains (37). Our results, in a simple controlled laboratory experiment, demonstrate that MIC is not an adequate measure of fitness in the presence of drug. There were three clear examples of discordance between MIC and fitness. First, in population D7, the decrease in MIC from 8 μg/ml at generation 260 to 0.5 μg/ml at generation 330 was accompanied by a significant increase in fitness measured with 16 μg of fluconazole, per ml. Second, in population D10, the decrease in MIC from 16 μg/ml at generation 200 to 1 μg/ml at generation 330 was accompanied by no significant change in fitness (even though the competition assay for generation 200 was in 32 μg of fluconazole per ml and the competition assay for generation 330 was in 128 μg/ml of fluconazole). Third, in population D12, the decrease in MIC from 64 μg/ml at generation 260 to 4 μg/ml at generation 330 was accompanied by no significant change in fitness compared to the progenitor measured in 128 μg of fluconazole per ml.

The drop in MIC that occurred repeatedly in the experimental populations without an accompanying reduction in fitness in the presence of a high drug concentration may reflect differences between conditions of the MIC test and the experimental liquid culture. Alternatively, the drop in MIC may be a function of interactions between mixed genotypes (evolved versus progenitor), which occur in competitions but not in MIC tests.

Another striking example of discordance between the MIC of fluconazole and fitness was the interaction between population sample D8-330 and the progenitor in competition assays done in the highest concentration of drug used, 128 μg/ml. The low MIC of 4 μg/ml for D8-330 ranks this isolate as sensitive. Despite this, population D8 predominated at the end of the competition, while the numbers of the marked progenitor actually decreased in the triplicate competition assays conducted in the presence of drug. The nature of the interaction between D8-330 and the progenitor remains unknown. However, D8-330 was the only population sample with major overexpression of *CDR2* (12), a gene encoding an efflux pump of the ABC transporter family and implicated in azole resistance. No inhibitory effect on the growth of either the progenitor or D8-330 was observed with cell-free culture filtrates of either population prepared either with or without drug (data not shown).

Is the trailing endpoint in MIC tests implicated in the discordance between fitness and MIC? Continued growth at drug concentrations above the MIC can complicate the interpretation of endpoints. For example, population D8 was grown at the high drug concentration of 128 μg/ml from generation 260, as was D10, due to the strong trailing endpoint in the MIC test plates for which the actual MICs were scored as much lower. Trailing may account for the elevated level of fitness in the presence of drug relative to expectations based on MIC for the population samples D8-330 and D10-330. But trailing endpoints of lower magnitudes were also shown by the progenitor, all populations grown without drug, D7-330, and D12-330. A trailing endpoint is therefore not sufficient as the sole explanation for the discordance between MIC and fitness.

The relationship between cell growth parameters and fitness. In addition to the discordance between MIC and fitness, there was no consistent relationship between cell doubling time or stationary-phase cell density and fitness relative to the progenitor for populations reared in the presence of drug. For example, population samples that did not differ from each other in either of these parameters, such as D7-260 and D7-330 or D10-200 and D10-330, differed significantly from each other in fitness (Fig. 1 and 2). These parameters do not account for the fitness advantage of D8-330 over the progenitor in the absence of drug (Fig. 2). In D9-330, fitness increased significantly in the presence of drug (Fig. 1A) despite a longer doubling time (Fig. 1B) and a stationary-phase cell density that did not differ significantly from that of the progenitor (Fig. 1C). A more consistent relationship was detected in some cases, such as with D12-260, with a decrease in fitness in the absence of drug (Fig. 2A) coupled with a significantly longer doubling time than that of the progenitor in the absence of drug (Fig. 2B) and no difference in stationary-phase cell density (Fig. 2C).

In contrast to the populations evolved with drug, the relationship between growth parameters and fitness was more predictable for the populations evolved in the absence of drug. At generation 330, among populations N1, N2, N3, N4, N5, and N6 in competitions without drug, four of the six had a fitness advantage (Fig. 4A) and all had shorter doubling times (Fig. 4B) as well as stationary-phase cell densities that did not differ significantly from that of the progenitor (Fig. 4C). In the presence of drug, the larger variances in fitness may have obscured very small increases in fitness over the progenitor (Fig. 3A), but all had shorter doubling times (Fig. 3B) and stationary-phase cell densities that were not significantly different from that of the progenitor (Fig. 3C). Despite the trend toward a relationship between growth parameters and fitness of the populations reared without drug, the correlation between doubling time and fitness or stationary-phase cell density and fitness for populations evolved with drug or evolved without drug was not significant when measured in either environment ($P > 0.25$ in all cases). The lack of correlation between growth parameters and fitness provides further support for the importance of interactions between genotypes in determining the outcome of competitions.

Implications for the emergence and spread of drug resistance. The divergence in fitness associated with the evolution of drug resistance in experimental populations of *C. albicans* has important implications for the emergence and spread of drug resistance. Some resistant isolates may incur a fitness cost and consequently may decline in frequency with suspension of the use of an antimicrobial agent. Other isolates may evolve resistance, with a decrease in the frequency of the resistant type precluded by a fitness advantage maintained both in the presence and in the absence of drug. Our previous results (12) confirmed that drug resistance was remarkably stable relative to the progenitor after 50 generations of further evolution in the absence of drug. Another study (3) suggests that the frequency of drug-resistant types increases faster under constant selection than it decreases in the absence of selection. While a cost of resistance will ensure that the frequency of resistant bacteria will decline following cessation of use of a given antibiotic, they will rapidly increase in frequency upon reintroduction of the drug if even a low frequency of resistant bacteria remains (24). The paucity of fitness cost associated with the evolution of drug resistance in the experimental populations of *C. albicans* raises the possibility that once resistant genotypes emerge, it will be very difficult to control their spread, even with a severe reduction in drug use.

Identifying the attributes that make one genotype or species a better competitor than another in a given environment remains an important challenge in microbial ecology. The difficulty is at least partly attributable to the integration of component genes and pathways in the composite phenotype of competitive fitness (21). Our results suggest that fitness is the result of complex interactions between mixed genotypes in competition assays. Fitness in competition assays is not predicted when the competitors are evaluated separately for MIC or growth parameters such as doubling time and stationary-phase cell density.

While evolution of microbial populations in an infected host may be different from that occurring in a laboratory environment (7, 10), our results are consistent with evidence from natural populations. The most common mechanisms of azole resistance identified among resistant isolates of *C. albicans* recovered from patients (42) were also detected in the experimental populations (12). In addition, the clonal spread of

fluconazole-resistant isolates of *C. albicans* to patients who had never received treatment with the drug (45) might be expected given the negligible cost of resistance in the experimental populations. Even in this simple laboratory system, however, we found complex interactions between mixed genotypes in competition. In the host, the potential for interactions among genotypes of *C. albicans* is far greater due to the additional complexities of the host immune system, other microbes, and heterogeneity among different anatomical sites.

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