Mode of Selection and Experimental Evolution of Antifungal Drug Resistance in *Saccharomyces cerevisiae*

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ABSTRACT

We show that mode of selection, degree of dominance of mutations, and ploidy are determining factors in the evolution of resistance to the antifungal drug fluconazole in yeast. In experiment 1, yeast populations were subjected to a stepwise increase in fluconazole concentration over 400 generations. Under this regimen, two mutations in the same two chromosomal regions rose to high frequency in parallel in three replicate populations. These mutations were semidominant and additive in their effect on resistance. The first of these mutations mapped to *PDR1* and resulted in the overexpression of the ABC transporter genes *PDR5* and *SNQ2*. These mutations had an unexpected pleiotropic effect of reducing the residual ability of the wild type to reproduce at the highest concentrations of fluconazole. In experiment 2, yeast populations were subjected to a single high concentration of fluconazole. Under this regimen, a single recessive mutation appeared in each of three replicate populations. In a genome-wide screen of ~4700 viable deletion strains, 13 were classified as resistant to fluconazole (*ERG3*, *ERG6*, *YMR102C*, *YMR099C*, *YPL056C*, *ERG28*, *OSH1*, *SCS2*, *CKA2*, *SML1*, *YBR147W*, *YGR283C*, and *YLR407W*). The mutations in experiment 2 all mapped to *ERG3* and resulted in the overexpression of the gene encoding the drug target *ERG11*, but not *PDR5* and *SNQ2*. Diploid hybrids from experiments 1 and 2 were less fit than the parents in the presence of fluconazole. In a variation of experiment 2, haploids showed a higher frequency of resistance than diploids, suggesting that degree of dominance and ploidy are important factors in the evolution of antifungal drug resistance.

RESISTANCE to antimicrobial agents enters microbial populations through mutation or immigration. Resistant genotypes then increase in frequency in response to the natural selection imposed by the presence of the drug. The key determinant of whether resistance spreads and persists in a population is the fitness of these resistant genotypes in the presence and in the absence of an antimicrobial agent (Andersson and Levin 1999; Levin *et al.* 2000).

For controlling pathogenic fungi, only a small number of different kinds of antifungal drugs are available (Georgopapadakou and Walsh 1994; Cowen *et al.* 2002b). Of the antifungal drugs that are commonly used, most target the ergosterol biosynthesis pathway. Ergosterol is the major sterol in fungal cell membranes and is not present in animal cell membranes. In addition to its effect on fungal membrane fluidity and permeability, ergosterol has a role in regulating cell division. The widely used azole drugs target the cytochrome p450 enzyme, lanosterol demethylase, which is encoded by *ERG11*, a gene essential for ergosterol biosynthesis. Resistance to azole drugs can occur through diverse mechanisms, including: (i) alteration in sterol biosynthesis that results in the substitution of other sterols for ergosterol, (ii) overexpression of the target protein so that sufficient enzyme activity remains even in the presence of the drug, (iii) overexpression of various membrane efflux pumps that reduce intracellular drug concentration, and (iv) alteration in the aminoacid sequence of the target protein that reduces its binding affinity for azoles (Sanglard *et al.* 1998; Lupetti *et al.* 2002). Additional mechanisms of resistance, not yet described, may exist. Because of the different possible mechanisms of azole resistance, each with different possible fitness effects, we hypothesized that the particular mode of selection could determine the mechanisms of resistance that ultimately become established in a fungal population. For example, selection with different drug concentrations may favor different mechanisms of resistance to the same agent.

Much of what is known about antifungal drug resistance comes from studies of the yeast *Candida albicans*, a widespread commensal and important pathogen of humans. In *C. albicans*, Cowen *et al.* (2000) provided evidence for the evolution of divergent mechanisms of resistance to the antifungal drug fluconazole (FLC) in experimental populations. The divergent response of

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the *C. albicans* populations in that study may be attributed in part to population size, which was reduced to $\sim 10^6$ cells per daily batch transfer, and to the random nature of mutation availability. In smaller populations, the random nature of the various possible mutations for resistance is expected to lend more of an element of chance to the outcome than it does in larger populations in which mutations are more available and selection is more efficient, resulting in more frequent fixation of the fittest genotypes.

In recent studies, we characterized the two divergent programs of adaptation to the presence of FLC in *C. albicans* (Cowen *et al.* 2000, 2002a). The first program of adaptation was observed only once among six experimental populations and included constitutive overexpression of *CDR1* and *CDR2*, which encode efflux pumps (ABC transporters) known to play a role in azole resistance, and altered expression of eight additional genes. The other program of adaptation included constitutive overexpression of *MDR1* (Cowen *et al.* 2000, 2002a), which encodes another kind of efflux pump (major facilitator) also known to play a role in FLC resistance. This second program of adaptation arose in parallel in three different populations and was accompanied by altered expression of >100 other genes. This second program was further characterized by a succession of early and late patterns of gene expression. Although the early and late expression profiles were highly similar among populations, these patterns, except for the consistent overexpression of *MDR1*, were almost completely dissimilar from one another. The patterns of gene expression associated with the first and second programs of adaptation are not unique to experimental populations. Essentially the same patterns of gene expression also occur in clinical isolates of *C. albicans* that are resistant to FLC (Cowen *et al.* 2002a).

In addition to their different patterns of gene expression, the two programs of adaptation in *C. albicans* had markedly different fitness profiles. The first program, which included overexpression of *CDR1* and *CRD2*, was associated with extremely high fitness both in the presence and in the absence of FLC (Cowen *et al.* 2001). The second program of adaptation, which included overexpression of *MDR1*, was associated with various levels of fitness, all lower than that associated with the first program of adaptation (Cowen *et al.* 2001).

Although the phenotypic attributes of drug resistance in *C. albicans* are well studied, genetic analysis is hampered by the inability to make meiotic crosses. In this study, we turned to *Saccharomyces cerevisiae*, a yeast relatively closely related to *C. albicans*, but with well-established methods for molecular genetics and functional genomics. Our goal was to test the hypothesis that the mode of selection to which populations are exposed determines the evolution of resistance in experimental populations. Two modes of selection were used in this study: in experiment 1, yeast populations were subjected to a stepwise increase in FLC concentration from low to high over 400 generations; in experiment 2, yeast populations were subjected at the outset to a high concentration of FLC.

TABLE 1

PCR primers for tagged replacements at *URA3*

First PCR from pFA6KanMX4
Forward
GCAGGAAACGAAGATAAATC-tag-CGTACG CTGCAGGTCGAC
Reverse
TTTACTTATAATACAGTTTT-tag-ATCGAT GAATTCGAGCTCG
Tags
1 ATCTTACAAAATTTGGTTTA
2 TCTGACATGACTAAGTTCAC
3 GTCTTAGGTATCGACGGCAT
4 CGAACATAGTTGCTAATGCT
5 CATCTGGAAGTGAAATCCAT
Invariant Probe
GCCATCAAAATGTATGGATGC
Second PCR
Forward
TCTTAACCCAACTGCACAGAACAAAAACCTGCAG GAAACGAAGATAAATC
Reverse
GCTCTAATTTGTGAGTTTAGTATACATGCATTTAC TTATAATACAGTTTT

MATERIALS AND METHODS

Strains: The progenitor of all experiments was a prototrophic *MAT***a** strain derived from a cross of two strains ("FY69," *MAT***a** *leu2*Δ *GAL2*, ATCC 90842 and "S288C-ura3," *MAT*α *ura3*, ATCC 90842) closely related to the laboratory standard S288C. The entire *URA3* open reading frame (ORF) of this strain was replaced with the KanMX4 cassette flanked by two unique 20-nucleotide bar codes to create five uniquely tagged strains. The PCR primers used to prepare the transforming DNA from plasmid pFA6a-KanMX4 (Wach *et al.* 1994) are listed in Table 1; transformation was by a multi-well procedure (http://sequence-www.stanford.edu/group/yeast_deletion_project/protocols.html; Winzeler *et al.* 1999). Each transformant was auxotrophic for uracil and resistant to 5-fluoroorotic acid (5-FOA). One of the two tags from each of the five strains was used throughout this study; these tags are designated 1–5.

Tests of minimum inhibitory concentration of FLC: These tests were constructed and interpreted according to standard protocol (National Committee for Clinical Laboratory Standards 1997) except that 0.5× yeast peptone dextrose (YPD; Adams *et al.* 1997) was the test medium used throughout this study. FLC was obtained as a gift from Pfizer Canada. All tagged strains had an initial minimun inhibitory concentration (MIC) of 16 μg/ml of FLC. Although the majority of strains had identical MICs among replicate tests, the resistant strains from experiment 1 occasionally varied twofold in MIC of FLC. This level of variation is within the range of variation commonly observed in MIC tests (National Committee for Clinical Laboratory Standards 1997).

Contamination checks and fitness assays: With the possibility of uniform evolutionary trajectories of adaptation, it was important to rule out cross-contamination among the replicate populations. The unique tags were used to confirm identity throughout each experiment; no contamination was observed and each tag appeared where expected without exception. The tags were also used as a means for quantifying fitness, or reproductive output, by measuring change in the proportion of strains in mixed cultures over time. To measure fitness, a segment of the *URA3* region in the replacement strains was amplified from genomic DNA from mixed cultures with primers AGAAGGTTAATGTGGCTGTGG and GCCATCAAAATG TATGGATGC. Amplicons of 525 bp, which included 279 bp of the remaining *URA3* region and 246 bp of KanMX4, were transferred to nylon membranes by capillary blotting and were then probed in succession with ^{32}P-end-labeled oligonucleotides complementary to the tagged regions, as well as with one oligonucleotide complementary to an invariant region within the KanMX4 replacement. There was no detectable cross-hybridization among the tag sequences. Signal intensity was measured with a phosphorimager with appropriate background subtraction (background was usually <1% of the signal). Exposures were timed to yield signals <5% of the saturation capacity of the phosphor screen.

For each strain in a mixed culture, the number of doublings was calculated as $\log_2(R_f \times D_f/R_i \times D_i)$, where R_f is the ratio of the signal for the tag to the signal for the invariant probe at the end of the incubation and R_i is the corresponding ratio at the beginning of a batch culture. D_f is the optical density (530 nm) at the end of the incubation and D_i is the optical density at the beginning. For pairs of tagged strains, calibrations were constructed by diluting one stationary-phase culture in six twofold increments into another culture and vice versa. For each tagged strain, the ratio of the signal for the tag to the invariant probe was plotted as a function of the proportion of the strain in the mixed culture. The mean R^2 for all calibrations was 0.99.

At each time point of experiment 1, cells from the −80° archive were streaked out on YPD agar and a single colony was used to establish a 10-ml overnight culture. The rationale for choosing single colonies, rather than mass cultures, for fitness assays was that resistance evolved rapidly in experiment 1, most likely resulting in near fixation of the fittest genotype. The fitness of each strain was measured relative to the progenitor at 0, 16, 32, 64, and 128 μg/ml FLC by mixing stationary cultures of P1 ("P" means progenitor; the number indicates the tag), D2, D3, and D4 ("D" means propagated in increasing concentrations of the drug; the number indicates the tag) in the proportions 0.50, 0.17, 0.17, and 0.17, respectively. These mixtures were propagated in two successive batch cultures of 0.5× YPD with 100-fold dilution at the time of transfer. The number of cell doublings over each daily increment was summed for the total period. The other fitness tests were done in exactly the same way except that strains were mixed in equal proportions in pairs. In some of the tests, the progenitor strain was P5 ("P" means progenitor; the number indicates the tag).

Construction of hybrid diploids and isolation of tetrads: The progenitors P1 and P5 and the evolved lines D2, D3, and D4 at generation 400 were diploidized by transforming with *URA3*-based plasmid pCY709, which contains the *HO* gene under its own promoter. Transformants were cultured overnight in medium without uracil and then colonies that had lost the plasmid were identified on medium with 5-FOA (Adams *et al.* 1997). *MAT***a**/α diploidy was confirmed by the criteria of lack of ability to mate, ability to sporulate, and increased cell size relative to haploids. Diploid strains were allowed to sporulate and tetrads of spores were isolated by the standard method (Sherman and Hicks 1991). Hybrid diploids were constructed by mixing overnight cultures of haploid strains with compatible mating types in liquid 0.5× YPD medium overnight. Zygotes were isolated by micromanipulation.

Quantification of mRNA transcripts: Northern hybridizations were used to quantify mRNA levels corresponding to *PDR5*, *SNQ2*, *FLR1*, and *ERG11* relative to a standard, *YEF3*, and were performed exactly as described by Cowen *et al.* (2000), except that the reference for basal expression was the progenitor, P1.

Genome-wide screen for fluconazole resistance: Approximately 4700 *MAT***a** haploid deletion strains from the *S. cerevisiae* deletion consortium were maintained in an ordered array on 16 single-well agar plates of 86 × 128 mm at a density of 768 strains per plate (384 individual strains in duplicate). Strains were robotically pinned onto 0.5× YPD + adenine medium containing 64 μg/ml FLC and incubated at 30° for 2 days. Strains viable at this drug concentration were identified as putative suppressors of fluconazole sensitivity. Resistant strains were individually confirmed with spot assays; four 10-fold serial dilutions starting at OD = 1 were spotted onto media containing 64 μg/ml FLC and growth was compared to a wild-type control after 2 days at 30°. Strains that grew on the FLC plates at all dilutions were classified as "strongly resistant," strains that grew only when OD > 0.01 were classified as "moderately resistant," and strains that grew only at OD = 1 were classified as "mildly resistant." Strains not growing at any dilutions were classified as "not resistant."

Construction of isogenic *MAT*a/a and *MAT*α/α diploids: Recovery of diploids homozygous for mating type followed the procedure of Reynolds and Fink (2001). A *MAT***a**/α diploid (P1) was transformed with p*GAL1-HO* and transformants were grown overnight in rich medium containing galactose as a carbon source. Colonies that had lost p*GAL1-HO* were identified on medium with 5-FOA. Colonies that secreted either pheromone were located by replica plating to medium containing cells highly sensitive to one or the other pheromone (strains SY2014, *MAT*α *ste3*Δ*306::LEU2 sst2*Δ and SY2625, *MAT***a** *bar1*Δ). Colonies secreting pheromone were identified by the presence of a halo of growth inhibition in the background cells. The putative homozygous diploids did not sporulate and had cell volumes greater than those of the haploids.

RESULTS

Experiment 1: *Mutation and fitness:* Three haploid *MAT***a**-tagged strains, D2, D3, and D4, were propagated separately in 10-ml batch cultures of 0.5× YPD, with daily transfer of 0.1 ml of the stationary-phase culture to 9.9 ml of fresh medium for an average of 6.6 generations per day. The concentration of FLC was 16 μg/ml for the first 100 generations, 32 μg/ml for the second 100 generations, 64 μg/ml for the third 100 generations, and 128 μg/ml for the fourth 100 generations, for a total of 400 generations. Samples of populations were archived at −80° in 15% glycerol at 0, 100, 200, 300, and 400 generations.

The entire fitness surface for experiment 1 is shown in Figure 1. At generation 0, none of the tagged strains had been subjected to FLC and all responded similarly to the various concentrations of FLC. All populations underwent >13 doublings in the absence of FLC and 7–8 doublings even at 128 μg/ml of FLC. By generation

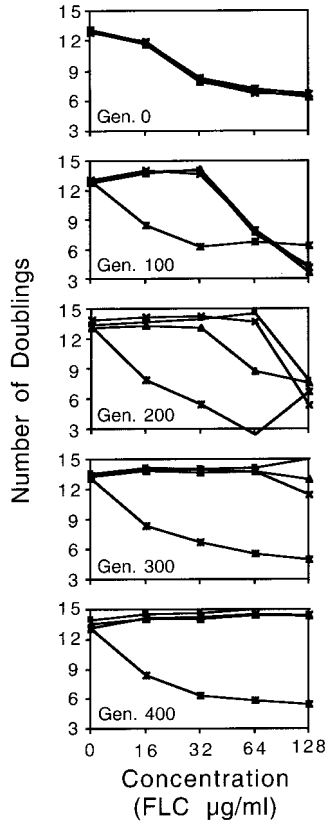

FIGURE 1.—Fitness of the progenitor P1 (*) and evolved populations D2 (▲), D3 (■), and D4 (×) from experiment 1 in 0–128 μg/ml FLC.

100, populations D2, D3, and D4 all showed enhanced fitness at 16 and 32 μg/ml FLC, but not at higher concentrations of the drug. Unexpectedly, the fitness of the progenitor was significantly higher than that of the evolved populations D2, D3, and D4 at generation 100 when measured in 64 and 128 μg/ml FLC. This effect was reproducible on agar medium with 128 μg/ml FLC (Figure 2); the progenitor produced small colonies that stopped growing after 1 day, but the evolved populations produced even smaller colonies indicating fewer cell divisions, a result consistent with the fitness assays. At generation 200, populations D2, D3, and D4 had higher fitness at all concentrations of FLC, except at 128 μg/ml, in which the fitness of the evolved and progenitor populations was approximately equal. By generations 300 and 400, populations D2, D3, and D4 showed higher fitness than the progenitor in all concentrations of FLC. At no time in the experiment was the fitness of D2, D3, and D4 less than that of the progenitor P1 in the absence of FLC; no fitness cost of resistance was evident.

Genetic analysis: The following observations showed that D2, D3, and D4 each accumulated two semidominant mutations in the same two unlinked chromosomal regions in the same order during experiment 1 and that the two mutations were approximately additive in their effect on MIC of FLC.

To measure the degree of dominance, six hybrid diploids were constructed by mating *MAT***a** and *MAT*α meiotic offspring of diploid D2, D3, and D4, which had MICs of 256 μg/ml, with those of the progenitor diploid P1, which had a MIC of 16 μg/ml. The six hybrid diploids had intermediate MIC values of 64 or 128 μg/ml (Table 2), indicating semidominance. These same six hybrid diploids had levels of fitness intermediate between the P1 and the D2, D3, and D4 homozygous, nonhybrid diploids in 128 μg/ml FLC, again indicating semidominance. All diploids showed approximately equal fitness in the absence of FLC (Table 2).

To examine segregation of mutations, the six hybrid diploids representing crosses between P1 and D2, D3, and D4 were allowed to sporulate and meiotic tetrads were analyzed. A total of 8 tetrads were interpreted as parental ditypes, 7 as nonparental ditypes, and 30 as tetratypes (see Figure 3 for examples of each). This ratio closely approximates the expected 1:1:4 ratio of parental ditypes, nonparental ditypes, and tetratypes expected with segregation of alleles at two unlinked loci with a large combined gene-to-centromere distance. Our interpretation of these segregation patterns is that one mutant gene determines a greater level of resistance (MIC 64 μg/ml) than the other (MIC 32 μg/ml) and that both mutant genes together confer an even higher level of resistance. There was no ambiguity about which mutations came first in experiment 1: the larger jumps in MIC, which were accompanied by point mutations in *PDR1* (see below) and elevated expression of *PDR5* and *SNQ2* (see below), all occurred in the first 100

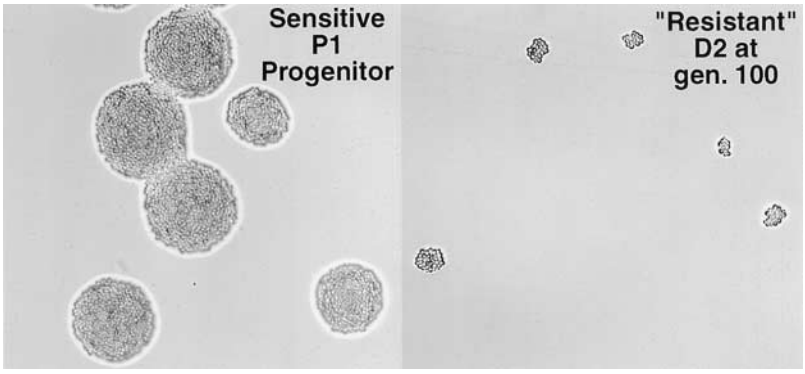

FIGURE 2.—Unexpected fitness deficit of D2 from generation 100 on 0.5× YPD agar with 128 μg/ml FLC. The progenitor P1 (left) underwent more cell divisions, resulting in larger colonies, than did the evolved, "resistant" (MIC, 64 μg/ml) strain D2 (right) during 2 days of incubation.

TABLE 2

Fitness of diploids constructed from the progenitor haploid and FLC-resistant haploids from experiment 1

Diploids/ progenitor[a]	MIC FLC	No. of doublings ±SD (n = 3 replicates)		
		Tag X[b]	Tag Y[b]	Tag 5 (all P5)
		Parent diploids[c]		
P1/P5	16	6.2 ± 0.1		6.2 ± 0.2
D2/P5	256	14.7 ± 0.0		5.5 ± 0.4
D3/P5	256	14.1 ± 0.1		6.6 ± 0.8
D4/P5	256	14.4 ± 0.0		7.2 ± 1.0
		Reconstructed diploids		
P1 × P1/P5	16	7.2 ± 0.1		6.9 ± 0.2
D2 × D2/P5	256	14.5 ± 0.2		4.3 ± 2.8
D3 × D3/P5	256	14.4 ± 0.2		7.3 ± 1.5
D4 × D4/P5	256	14.0 ± 0.1		7.1 ± 1.9
		Hybrid diploids (D × D)		
D2 × D3/P5	256	14.5 ± 0.1	14.4 ± 0.1	8.8[e]
D2 × D4/P5	256	14.4 ± 0.1	14.0 ± 0.2	6.4 ± 1.5
D3 × D2/P5	256	14.4 ± 0.2	14.5 ± 0.2	6.7 ± 1.8
D3 × D4/P5	256	14.2 ± 0.1	14.2 ± 0.1	7.1 ± 1.0
D4 × D2/P5	256	14.7 ± 0.1	14.5 ± 0.2	6.5 ± 1.0
D4 × D3/P5	256	14.4 ± 0.1	14.4 ± 0.0	7.2 ± 1.1
		Hybrid diploids (P × D)[d]		
P1 × D2/P5	64	8.4 ± 0.1	8.5 ± 0.1	7.1 ± 0.1
P1 × D3/P5	128	7.4 ± 0.2	7.6 ± 0.2	7.3 ± 0.2
P1 × D4/P5	64	9.2 ± 0.4	9.2 ± 0.3	7.5 ± 0.3
D2 × P1/P5	64	8.4 ± 0.3	8.3 ± 0.2	7.0 ± 0.6
D3 × P1/P5	128	11.8 ± 0.2	11.3 ± 0.2	6.8 ± 0.5
D4 × P1/P5	64	8.8 ± 0.2	8.9 ± 0.0	7.1 ± 0.2

The mean number of doublings for all diploid strains in 0.5× YPD with no FLC was 13.5 ± 0.2 (data not shown).

[a] Diploids are listed with *MAT*α strain first and the *MAT***a** strain second. Progenitor diploid P5 after the slash (/) was the reference strain in each fitness assay.

[b] Fitness of each diploid carrying two tags (*e.g.*, D2 × D3) was measured with both tags (*e.g.*, tag 2 and tag 3).

[c] Parent diploids were constructed from the haploid strains by transient transformation with a plasmid carrying the *HO* gene.

[d] Because these assays were done at a concentration of FLC that was higher than the measured MIC, the overall density at the end of these competition assays remained low and these measures of fitness were therefore more variable than those done under conditions in which the mixed cultures reached high density at the end of the assay period.

[e] No standard deviation available; only one measurement was made.

generations. The mutations of lesser effect on MIC became apparent only in subsequent generations.

In addition to the six hybrid diploids of P1 with D2, D3, and D4 at generation 400, six hybrid diploids were also constructed among the fluconazole-resistant D2, D3, and D4 at generation 400. Each of these hybrids had the same MIC of 256 μg/ml FLC as the parent diploid versions of D2, D3, and D4 (Table 2) and had the same high levels of fitness in 128 μg/ml FLC as the nonhybrid diploids and their predecessor haploids. Meiotic offspring of hybrids among D2, D3, and D4 showed no segregation for resistance to fluconazole; in a total of 34 tetrads from these hybrids, all spores had MICs of 256 μg/ml. This result showed that the two determinants of resistance in D2, D3, and D4 were located in the same chromosomal regions.

As a control for the genetic analyses above, homozygous, nonhybrid diploid versions of P1 and D2, D3, and D4 at generation 400 were also allowed to sporulate and 5–10 tetrads were analyzed from each. No segregation in the levels of MIC was observed in any of these tetrads. The spore progeny of diploid D2, D3, and D4 were all highly resistant (MIC 256 μg/ml) and those of diploid P1 had the basal level of resistance (MIC 16 μg/ml).

To further characterize the FLC-resistant phenotypes, the expression of four genes known to play a role in resistance to FLC was measured. From generation 100 on, populations D2, D3, and D4 all overexpressed the

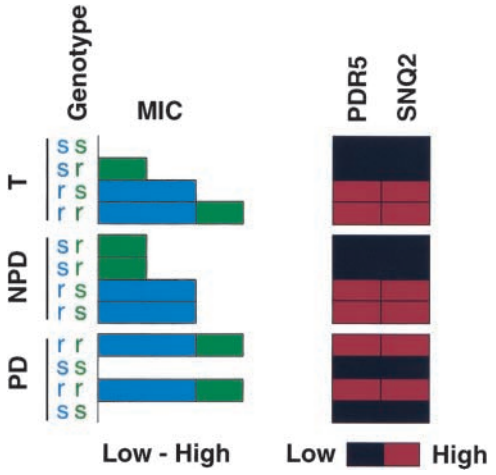

FIGURE 3.—MIC of FLC and expression of *PDR5* and *SNQ2* in three meiotic tetrads from the cross P1 × D2 (generation 400, experiment 1). Genotypes are for the first and second mutations in experiment 1: r, the mutant allele conferring resistance; s, the wild-type allele; T, tetratype; NPD, nonparental ditype; PD, parental ditype. For MICs, the blue bars indicate the greater contribution of the first (*PDR1*) mutations (64 μg/ml) and the green bars indicate the contribution of the second mutations (32 μg/ml). For *PDR5* and *SNQ2*, black indicates the basal level of expression of the progenitor and red indicates an increase in expression comparable to those in Table 3.

ABC transporter genes *PDR5* and *SNQ2*, three- to fourfold with respect to P1 and all of its derivatives (Table 3). None of these same strains overexpressed the major facilitator gene *FLR1*, the expression of which was barely detectable in any of the strains assayed in this study (data not shown). The expression of *ERG11* was more variable in D2, D3, and D4, with no consistent trend (Table 3). The hybrid diploids between P1 and D2, D3, and D4 expressed *PDR5* and *SNQ2* at a level between that of the progenitor and parents evolved for 400 generations in FLC. Three tetrads representing parental ditypes, nonparental ditypes, and tetratypes for the two segregating genes from experiment 1 were also assayed for expression of the same set of four genes (Figure 3). In all cases, the putative genotype interpreted as containing the first mutation expressed *PDR5* and *SNQ2* at a high level approximately equal to the evolved parents and all genotypes interpreted as lacking the first mutation expressed *PDR5* and *SNQ2* at basal levels.

Spores of these tetrads were also examined for ability to reproduce in 128 μg/ml FLC on agar medium. Genotypes from these tetrads containing only the first resistance mutation showed the same fitness deficit in 128 μg/ml FLC as did D2, D3, and D4 at generation 100 (see Figure 2), at which time only the first mutations had become established.

Mapping of mutations: The semidominant nature of the first mutations from experiment 1 and their effects on the expression of *PDR5* and *SNQ2* were consistent with the action of known mutations in *PDR1* or *PDR3* (KOLACZKOWSKA and GOFFEAU 1999; DERISI *et al.* 2000). For these crosses, the first mutation from the D2 line in experiment 1 was placed in a *MAT*α background lacking G418 resistance. This strain was then crossed with the *PDR1* and *PDR3* knockout strains, both of which carried the KanMX4 cassette with G418 resistance at the deletion site and had MICs of 16 μg/ml FLC or lower. In the cross with the *PDR1* knockout strain, all 30 tetrads were parental ditype with two FLC-resistant spores and two FLC-sensitive, G418-resistant spores. The unknown resistance mutation was therefore tightly linked to *PDR1*. In the cross with the *PDR3* knockout strain, all tetrads also segregated 1:1 for resistant *vs.* sensitive, but the G418 resistance (encoded at the *PDR3* knockout site) did not cosegregate with low resistance. This indicates that the first mutations from experiment 1 were not in the same chromosomal region as *PDR3*.

The first resistance mutations in D2, D3, and D4 were further pinpointed to *PDR1* by sequencing both DNA stands of the entire *PDR1* ORF plus the flanking intergenic regions in the progenitor P1 and in D2, D3, and D4 at generations 100 and 400. A single mutation was observed in each of the three evolved lines at generations 100 and 400. Each mutation was near the carboxy

TABLE 3

Expression of three genes (mean ±SD) relative to *YEF3*

Strains (n = no. measured)	MIC (μg/ml) FLC	*PDR5*	*SNQ2*	*ERG11*
Haploid progenitors (n = 3)	16	1.2 ± 0.3	1.1 ± 0.2	1.2 ± 0.2
Haploid D2, D3, and D4, gen. 100 (n = 3)	64	4.8 ± 0.8	2.7 ± 0.5	1.2 ± 0.1
Haploid D2, D3, and D4, gen. 400 (n = 3)	256	3.5 ± 0.3	2.3 ± 0.5	2.5 ± 0.9
Diploid D2, D3, and D4, gen. 400 (n = 3)	256	3.2 ± 0.2	2.9 ± 0.6	1.3 ± 0.3
Hybrid diploids D2, D3, and D4 × P1 (n = 6)	64 or 128	1.8 ± 0.3	1.8 ± 0.2	1.3 ± 0.3
Haploid O1, O2, and O3 (n = 3)	256	0.8 ± 0.5	1.3 ± 0.4	3.5 ± 0.3
Hybrid diploid O1, O2, O3 × D2, D3, D4 (n = 9)	64 or 128	3.3 ± 0.8	2.0 ± 0.5	1.2 ± 0.2
Hybrid diploid O1, O2, O3 × P1 (n = 3)	16	1.5 ± 0.5	1.5 ± 0.2	1.0 ± 0.2

Gen., generation.

TABLE 4

Fitness of progenitor haploid and FLL-resistant haploids from experiments 1 and 2

Haploid	No. of doublings ±SD (n = 3 replicates)				
	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5
O1/**O2**/**O3**/P5	**13.9 ± 0.4**	**14.1 ± 0.3**	**13.7 ± 0.4**	—	8.0 ± 0.2
D2/D3/D4/**O1**/P5	**11.3 ± 0.3**	14.7 ± 0.1	14.6 ± 0.2	14.3 ± 0.1	9.0 ± 0.1
O2/D3/D4/P5	—	**11.8 ± 0.2**	14.6 ± 0.3	14.4 ± 0.4	8.7 ± 0.2
O3/D2/D4/P5	—	14.7 ± 0.2	**11.5 ± 0.4**	14.1 ± 0.4	8.3 ± 0.3

Mean number of doublings for all strains in 0.5× YPD in the absence of FLC was 13.3 ± 0.3 (data not shown). Boldface type indicates one-step mutant strain. Underlining indicates D strain. No underlining indicates P5. —, no tag present. Slashes (/) separate the designations of the strains competed.

terminus of the predicted polypeptide, near the activation domain (Kolaczkowska *et al.* 2002) of this transcriptional regulator: D2, T817K; D3, C862W; and D4, L722P.

The second mutations in experiment 1: Although the second mutations in experiment 1 remain unidentified, certain genes can be excluded as candidates. Since the second mutations were semidominant, they are not likely to be the result of simple loss of function and are therefore not likely to correspond to any of the FLC-resistant, gene-deletion strains described below under experiment 2. Also, because the second mutations did not affect the expression of *PDR5* and *SNQ2*, the regulator *PDR3* is not a likely candidate. Further transcriptional profiling may provide clues about the nature of the second mutations.

Experiment 2: *Mutation and fitness:* Three haploid *MAT***a**-tagged strains, O1, O2, and O3 ("O" means selection in a single high concentration of FLC; the number indicates the tag), were spread directly on medium containing 128 μg/ml FLC. On each plate, 10^4 cells from an overnight culture in 0.5× YPD were distributed as evenly as possible. Because wild-type cells undergo seven to nine doublings in the presence of FLC, cell numbers on each plate reached 1–5 × 10^6 within 2 days (see below). Large, continuously growing colonies were picked after 4–5 days of incubation. Three mutants of O1, O2, and O3, respectively, all had MICs of 256 μg/ml of FLC.

The fitness of the strains from experiment 2 was compared to that of the progenitor and the resistant strains from experiment 1 (Table 4) in competitive growth assays containing 128 μg/ml FLC. The fitness of O1, O2, and O3 was substantially higher than that of P5, but less than that of D2, D3, and D4. The fitness of O1, O2, and O3 in the absence of FLC was the same as that of all of the other strains, including the progenitor; no fitness cost of resistance was detected.

Genetic analysis: The following results showed that O1, O2, and O3 each contained a single recessive mutation for resistance that mapped to the same chromosomal region. The MICs of the heterozygous diploid hybrids of O1, O2, and O3 with P1 were all 16 μg/ml, indicating that the resistance determinant was recessive with respect to MIC. The fitness of these same hybrid diploids, however, was somewhat higher (9.7 ± 0.5 doublings) than that of the competitor progenitor diploid P5 (7.4 ± 0.3 doublings) in 128 μg/ml FLC, indicating that the resistance determinant was not completely recessive with respect to this criterion. These same heterozygous hybrid diploids were allowed to sporulate and all 30 tetrads dissected showed a 1:1 ratio for MICs of 16 and 256 μg/ml; this indicated the segregation of a single mutation. When O1 and O3 were crossed with a *MAT*α, FLC-resistant (MIC 256 μg/ml) segregant from the cross of O2 with P1, the hybrid diploids had MICs of 256 μg/ml and none of 29 meiotic tetrads examined showed any segregation; all spores showed MICs of 256 μg/ml. This showed that the mutations in O1, O2, and O3 were each located in the same chromosomal region. O1, O2, and O3 consistently overexpressed *ERG11* relative to P1, but *PDR5*, *SNQ2*, and *FLR1* were all expressed at levels similar to that of P1 (Table 3).

Mapping of the mutation: To identify the recessive mutation from experiment 2, we first took a comprehensive and unbiased approach to identify FLC-resistant yeast mutants. Approximately 4700 viable haploid deletion mutants were screened for FLC resistance. Because these strains are viable, this set should be highly enriched for loss-of-function mutations with minimal fitness defects. In total, 13 deletion mutants displayed a FLC-resistant phenotype (Figure 4). This set of FLC-resistant strains is significantly enriched for genes classified within the Munich Information Center for Protein Sequences database (http://mips.gsf.de) as functioning in lipid, fatty-acid, and isoprenoid biosynthesis ($P = 7.7 \times 10^{-5}$; Robinson *et al.* 2002; http://funspec.med.utoronto.ca). The strength of the FLC-resistant phenotype was distinguished by colony size on FLC medium (Figure 4). Consistent with previously published observations (Sanglard *et al.* 1998; Lupetti *et al.* 2002), loss of function at *ERG3* resulted in a relatively strong FLC-resistant phenotype. Deletion

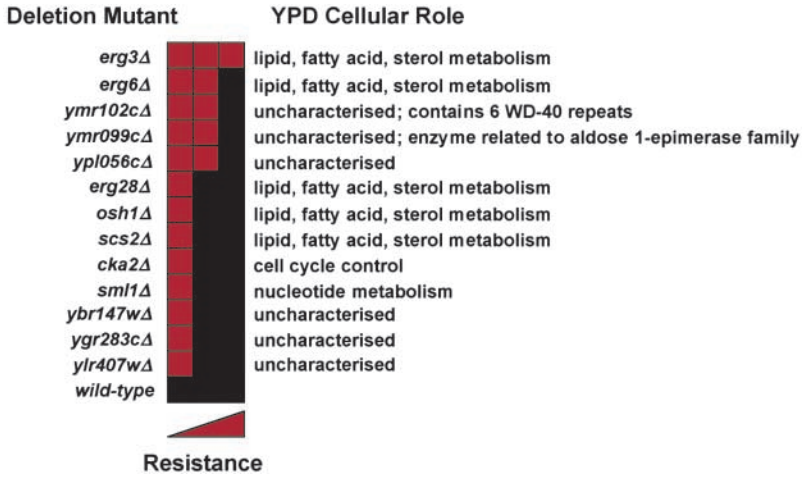

FIGURE 4.—Haploid deletion strains resistant to 64 μg/ml FLC. Cellular roles are as defined by the Yeast Proteome Database (http://www.incyte.com/proteome).

mutations of three other genes implicated in ergosterol biosynthesis, *ERG6*, *ERG28*, and *OSH1* (PALTAUF *et al.* 1992; BEH *et al.* 2001; GACHOTTE *et al.* 2001); a gene associated with inositol metabolism, *SCS2* (KAGIWADA *et al.* 1998); a gene associated with cell cycle control, *CKA2*; a gene associated with nucleotide metabolism, *SML1*; and six uncharacterized genes, *YMR099c*, *YMR102c*, *YPL056c*, *YBR147w*, *YGR283c*, and *YLR407w*, resulted in a FLC-resistant phenotype. All of these mutations provide clues about possible mechanisms for the evolution of a FLC-resistant phenotype. The genes *YMR099c* and *YMR102c* flank the gene that encodes Srt1p, a protein involved in the synthesis of dolichol, a family of long-chain polyprenols (SATO *et al.* 2001). Because gene deletions can cause overexpression of neighboring genes (HUGHES *et al.* 2000), it is possible that overexpression of *SRT1* causes the FLC-resistant phenotype observed for the *YMR099c* and *YMR102c* deletion strains.

On the basis of these screen data, the resistance mutations in O1, O2, and O3 from experiment 2 were placed in a *MAT*α background and then crossed with the *ERG3* knockout strain. From these crosses, all of the 35 tetrads tested showed no segregation for FLC resistance; all spores had MICs of 256 μg/ml FLC. The mutations in experiment 2 are therefore located in the same chromosomal region as *ERG3*. Like the O1, O2, and O3 mutants, the *ERG3* deletion strain has a MIC of 256 μg/ml FLC and overexpresses *ERG11* at greater than threefold. This effect of the *ERG3* knockout on the expression of *ERG11* was reported previously (HUGHES *et al.* 2000). The combined genetic linkage and phenocopy of the O1, O2, and O3 mutants to the *ERG3* deletion suggest that the mutations in O1, O2, and O3 likely reside in *ERG3*.

Hybrids from experiments 1 and 2: Hybrid diploids were constructed between O1, O2, and O3 and P1, D2, D3, and D4 (Table 5). The MIC of the hybrids of strains O1, O2, and O3 with D2, D3, and D4 was either 64 or 128 μg/ml, similar to hybrids of D2, D3, and D4 with P1 (Table 1). The fitness of these same hybrids was higher (mean 11.7 ± 0.9) than that of diploid P5 (7.9 ± 0.6 doublings) in 128 μg/ml FLC. Hybrids between O1, O2, and O3 and all other strains expressed *ERG11* at basal levels, a result consistent with the recessive nature of these mutations with respect to the MIC of FLC. In the hybrids between O1, O2, and O3 and D2 and D3, expression levels of *PDR5* and *SNQ2* were about equal to those of the hybrids of D2, D3, and D4 with P1, a result consistent with the semidominant nature of the first mutation in the evolved populations established above for experiment 1. In contrast, in the hybrids between O1, O2, and O3 and D2 and D3, the *PDR5* message was present at levels about equal to those of D2, D3, and D4, a result more consistent with full dominance. The reason for the difference in expression levels of *SNQ2* and *PDR5* in the hybrids between experiments 1 and 2 is not known.

Differential response of haploids and diploids to strong selection: Because the mutations for resistance in experiment 1 were semidominant, while those from experiment 2 were recessive, we examined the effect of ploidy on the frequency of resistance at 128 μg/ml FLC. In another version of experiment 2, equivalent numbers of isogenic haploid (*MAT***a** and *MAT*α) and diploid (*MAT***a**/α, *MAT***a**/**a**, and *MAT*α/α) strains were spread on 0.5× YPD containing 128 μg/ml FLC. More colonies appeared among haploid than among diploid cells subjected to this kind of selection (Figure 5, Table 6). Those colonies that did appear among diploid cells were observed after only 2 days. Among the haploid cells a few resistant colonies were evident by day 2, but the majority of colonies appeared later. We conclude that the haploids had a greater frequency of mutant phenotypes than the diploids. The nature of the mutations in these experiments has not yet been investigated.

DISCUSSION

Our results show that the mode of selection is a strong determinant of the mechanism of drug resistance that is favored in a fungal population. The two different

TABLE 5

Fitness of diploids constructed from the progenitor haploid and FLC-resistant haploids from experiments 1 and 2

Diploids[a]	MIC FLC	No. of doublings ±SD (n = 3 replicates)		
		Tag X[b]	Tag Y[c]	Tag 5 (all P5)
O2 × O1/P5	256	13.8 ± 0.4	13.8 ± 0.4	9.5 ± 0.5
O2 × O3/P5	256	13.2 ± 0.2	13.3 ± 0.2	8.7 ± 0.6
O1 × P1/P5	16	9.0 ± 0.9	−	7.2 ± 0.7
O2 × P1/P5	16	10.3 ± 1.2	10.1 ± 1.1	7.2 ± 0.7
O3 × P1/P5	16	9.4 ± 0.7	9.7 ± 0.5	7.8 ± 0.9
O1 × D2/P5	64	11.2 ± 0.5	11.2 ± 0.5	7.9 ± 0.4
O1 × D3/P5	128	12.5 ± 0.8	12.2 ± 0.6	8.6 ± 0.4
O1 × D4/P5	64	10.9 ± 0.1	11.3 ± 0.1	8.0 ± 0.1
O2 × D2/P5	64	11.4 ± 0.2	−	7.8 ± 0.4
O2 × D3/P5	128	12.7 ± 0.3	12.3 ± 0.1	8.6 ± 0.1
O2 × D4/P5	128	11.3 ± 0.3	11.1 ± 0.4	7.9 ± 0.4
O3 × D2/P5	128	11.2 ± 0.2	11.0 ± 0.2	7.7 ± 0.3
O3 × D3/P5	128	13.2 ± 0.1	13.2 ± 0.1	9.0 ± 0.2
O3 × D4/P5	128	10.8 ± 1.2	10.6 ± 1.2	7.3 ± 1.1

Mean number of doublings for all strains in 0.5× YPD in the absence of FLC was 13.6 ± 0.2 (data not shown).

[a] Diploids are listed with *MAT***a** strain first and the *MAT*α strain second. Progenitor diploid P5 after the slash (/) was the reference strain in each fitness assay.

[b] Fitness of each diploid carrying two tags (*e.g.*, D2 × D3) was measured with both tags (*e.g.*, tag 2 and tag 3).

[c] Minus (−) means that only one tag was present in the hybrid diploid.

selection regimens used in this study resulted in the appearance of completely different mechanisms of resistance. In both experiments 1 and 2, contamination was ruled out by the presence of the marker tags and parallel evolution was the rule. Under the stepwise selection regimen of experiment 1, two successive mutations that were semidominant and approximately additive in their effect on resistance appeared independently in three different populations. In contrast, under the single exposure to high concentrations of FLC in experiment 2, one recessive mutation appeared independently in three different populations. Although the population sizes and transfer regimens differed between experiment 1 and 2, additional evidence suggests that the different outcomes are not sensitive to these factors and that the nature of the selection applied is the main determinant. When progenitor cells were grown in liquid cultures at high FLC concentrations (64 or 128 μg/ml), the loss of function in *ERG3* was always the mutation favored (data not shown). Although additional loss-of-function mutations equivalent to the 12 other gene deletions in Figure 4 probably occurred in the experimental populations under strong selection, these would not be expected to rise to high frequency because their fitness is much less than that of the *ERG3* mutations in high FLC concentrations.

The recruitment of two divergent kinds of resistance to FLC in *S. cerevisiae* populations is reminiscent of the two divergent kinds of resistance found in experimental populations of *C. albicans* (Cowen *et al.* 2000, 2002a),

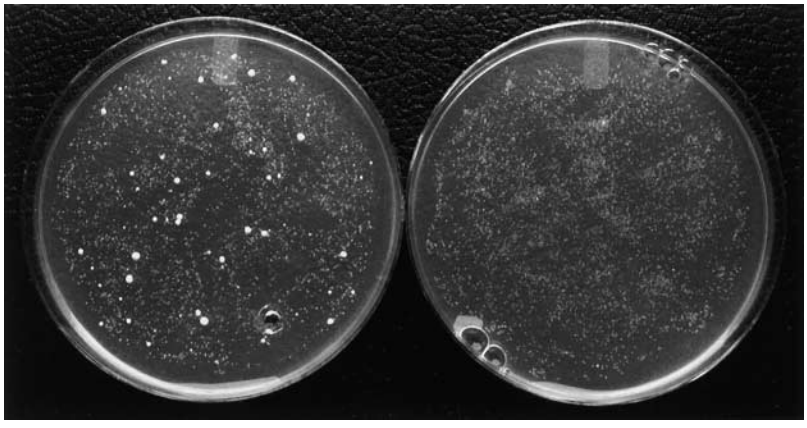

Figure 5.—Progenitor haploid (left) and its corresponding isogenic *MAT***a**/**a** diploid (right) after 5 days of incubation on 0.5× YPD with 128 μg/ml FLC. Inoculum for each plate consisted of 10^4 cells. The plate with haploid cells shows numerous resistant colonies while the plate with diploid cells does not.

TABLE 6

Number of colonies arising from 10^4 haploid and diploid cells spread on medium with 128 μg/ml FLC

Strains	Plates with 128 μg/ml FLC			Average	Standard deviation
	1	2	3		
*MAT***a** haploid	31	49	20	33.3	14.6
*MAT*α haploid	57	60	63	60.0	3.0
*MAT***a**/α diploid	8	8	1	5.7	4.0
*MAT***a**/α diploid	1	0	0	0.3	0.6
*MAT***a**/**a** diploid	5	3	5	4.3	1.2

but the actual resistance mechanisms documented here showed both similarities and differences to those found earlier in *C. albicans*. The resistance in *S. cerevisiae* resulting in overexpression of the ABC transporters *PDR5* and *SNQ2* in experiment 1 is very similar to the resistance in *C. albicans* resulting in the overexpression of the homologous ABC transporters, *CDR1* and *CDR2*. In contrast, the resistance in *S. cerevisiae* resulting in apparent loss of function in *ERG3* and overexpression of *ERG11* was not accompanied by any equivalent in the experimental populations of *C. albicans*. Also, the resistance in *C. albicans* accompanied by overexpression of the major facilitator gene *MDR1* had no equivalent in this study, as none of the resistant mutants overexpressed the homolog *FLR1*. Interestingly, in *S. cerevisiae*, it was the resistance pattern in experiment 1 that showed a temporal succession of changes, while in *C. albicans*, it was the *MDR1* pattern that showed a temporal succession. Additional mechanisms of resistance may well be possible in *S. cerevisiae*, but different selection regimens and/or smaller populations with greater replication from those used here may be necessary to find them.

The two divergent pathways of resistance recruited in populations under different kinds of selection showed no immediate advantage when combined in hybrids. The F_1 hybrids containing all three resistance mutations as heterozygotes had lower MIC and fitness in high concentrations of FLC than did either parent alone. Further evidence from meiotic offspring of these hybrids (data not shown) suggests that haploids containing all three mutations merely show the maximum MIC of the parents. That none of the mutations in experiments 1 and 2 are fully dominant with respect to MIC or fitness and that different modes of selection favor different kinds of resistance make it unlikely for both mechanisms to predominate together in diploid populations evolving in the presence of FLC and in the absence of genetic exchange between cell lineages.

In both experiments 1 and 2, fitness was measured in addition to MIC to detect any reduction of fitness in the absence of the drug that might accompany the evolution of resistance. Despite the rapid appearance of resistance in both experiments 1 and 2, where a fitness cost might have been expected, none was detected in any of the strains. If such a cost exists, it was too small to be detected under the conditions used here. This result is similar to that found in experimental populations of *C. albicans*, in which the majority of resistant strains showed no significant fitness cost, and for the few that did, the cost was slight and was eliminated with further evolution (Cowen *et al.* 2001).

Although no cost of resistance was found where it was expected, there was a strong and unexpected fitness deficit in D2, D3, and D4 at generation 100 of experiment 1 at the two highest concentrations of FLC. This fitness cost was statistically significant and repeatable even when the progenitor and generation 100 strains were grown separately (Figure 2). In effect, the first mutations (*PDR1*), which confer resistance to the lower concentrations of FLC, actually reduce the ability of cells to reproduce at higher concentrations of the drug, relative to the progenitor (Figure 2). The presence of these *PDR1* mutations can therefore render additional resistance mutations, including the *ERG3* mutations favored in experiment 2, unavailable in high concentrations of fluconazole due to insufficient population size. For example, when populations from generation 100 of experiment 1 were plated on medium with 128 μg/ml FLC (exactly as in experiment 2), population expansion was severely limited and accumulation of further resistance mutations did not occur (data not shown).

This discrepancy between MIC and fitness similar to that observed at generation 100 of experiment 1 has been noted before (Cowen *et al.* 2001) and may be due to differences in what capabilities the two assays measure. MIC measures the concentration at which the final cell density is reduced by half relative to the same medium without the drug, while the fitness assays measure the number of cell doublings over a defined period of time. The discordance in these measures (*i.e.*, when MIC is low/intermediate) suggests two ways of coping with the presence of FLC, one of which results in an increase in MIC and the other of which allows a residual number of cell divisions even at the highest concentrations of FLC. This residual growth at high concentrations of the drug is well known as the "trailing" phenotype shown by certain strains of *C. albicans* in MIC tests (Cowen *et al.* 2001, 2002b). Although the trailing phenotype of *C. albicans* in FLC is eliminated by cyclosporine (Marchetti *et al.* 2000) and may be mitigated by altering pH conditions (Marr *et al.* 1999), the reduction of the trailing phenotype here is due instead to the pleiotropic nature of the *PDR1* mutations in experiment 1. The trailing phenotype was not apparent at the later time points of experiment 1 because, with the presence of both mutations conferring high fitness in all concentrations of FLC, this phenotype is overwhelmed. If this kind of discrepancy, that is, low/medium MIC with moderately high fitness in high concentrations of FLC, is

common among pathogenic yeasts, then it could be an important factor in why determination of MIC for a clinical isolate often fails to predict therapeutic outcome (Reyes and Ghannoum 2000; Rex *et al.* 2001). In contrast to the discordance between MIC and fitness seen with the trailing phenotype, there was no discordance between fitness and MIC at the high end of the range of measurement in experiments 1 and 2.

If the two different kinds of resistance to FLC are representative of the evolutionary capabilities of *S. cerevisiae* in general, then ploidy may play a role in the rate of evolution of FLC resistance in *S. cerevisiae*. The effects of mutation availability and dominance on the evolution of haploids and diploids are discussed by Orr and Otto (1994) and our experiments provide one example of these effects. In high concentrations of FLC, haploids are expected to evolve resistance faster than diploids because the *ERG3* loss-of-function mutation strongly favored under these conditions is recessive. Although diploids should experience twice as many of these mutations as haploids, they would not benefit unless the recessive mutant allele becomes homozygous, for example, through mitotic recombination. Essentially, diploids would be subject to a delay before these recessive mutations for resistance would be expressed as homozygotes, while haploids would experience no such delay. The results in Table 6 and Figure 5 supported this prediction: greater numbers of resistant colonies appeared among haploids than among diploids. More detailed tests of this and other hypotheses about the effect of ploidy on the rate of evolution of drug resistance are now under way.

How might this study be relevant to the evolution of antifungal drug resistance by fungal pathogens in animal hosts? If divergent outcomes resulted from the different selection regimens in these experiments, then the host environment may be even more conducive to divergence in the evolution of antifungal drug resistance. An animal host represents a highly structured physical environment in which the concentration of a drug is anything but uniform throughout the body and over time. The potential for different kinds of selection to operate would therefore likely be far greater within an animal host than within the artificial and highly uniform experimental populations studied here. Another factor that may favor divergence in the evolution of drug resistance in an animal host is that the effective sizes of fungal populations in the highly structured environment are undoubtedly smaller than those studied here, lending even more of an element of chance to the local outcome of evolution. Finally, fungal pathogens vary in ploidy. For example, *C. albicans* is diploid, while *Cryptococcus neoformans* and *Aspergillus fumigatus* are haploid (Taylor *et al.* 1999). Ploidy may contribute to the success or failure of a fungal population to evolve resistance in any given environment. Testing these hypotheses will undoubtedly be more complicated in animal hosts than in artificial cultures. The kinds of resistance that predominate over time in an animal host will depend not only on the "fitness" of the resistant types in their local compartments, but also on their ability to survive during periods of stasis and to disseminate between compartments. Despite these complexities, however, there is no compelling reason why the underlying effects of selection, dominance, and ploidy on the evolution of antifungal drug resistance found here in artificial populations should not apply to the evolution of fungal pathogens within animal hosts.

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