

Drug Resistance Is Not Directly Affected by Mating Type Locus Zygosity in *Candida albicans*

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Recently, evidence was presented that in a collection of fluconazole-resistant strains of *Candida albicans* there was a much higher proportion of homozygotes for the mating type locus (*MTL*) than in a collection of fluconazole-sensitive isolates, suggesting the possibility that when cells become *MTL* homozygous they acquire intrinsic drug resistance. To investigate this possibility, an opposite strategy was employed. First, drug susceptibility was measured in a collection of isolates selected for *MTL* homozygosity. The majority of these isolates had not been exposed to antifungal drugs. Second, the level of drug susceptibility was compared between spontaneously generated *MTL*-homozygous progeny and their *MTL*-heterozygous parent strains which had not been exposed to antifungal drugs. The results demonstrate that naturally occurring *MTL*-homozygous strains are not intrinsically more drug resistant, supporting the hypotheses that either the higher incidence of *MTL* homozygosity previously demonstrated among fluconazole-resistant isolates involved associated homozygosity of a drug resistance gene linked to the *MTL* locus, or that *MTL*-homozygous strains may be better at developing drug resistance upon exposure to the drug than *MTL*-heterozygous strains. Furthermore, the results demonstrate that a switch by an *MTL*-homozygous strain from the white to opaque phenotype, the latter functioning as the facilitator of mating, does not notably alter drug susceptibility.

The great majority of *Candida albicans* strains are heterozygous at the mating type-like locus *MTL*, carrying *MTLa1* on one chromosome and *MTL α 1* and *MTL α 2* on the homologous chromosome (7, 14). Fusion has been demonstrated to occur both in vivo (8) and in vitro (16, 17) between engineered homozygous *MTLa* (*a/a*) and *MTL α* (*α/α*) cells, but not between parental *MTL*-heterozygous (*a/ α*) cells. Recently, Miller and Johnson (17) discovered an extraordinary and unexpected relationship between mating and white-opaque switching. They demonstrated that while cells of the *MTL*-heterozygous parent strain CAI4 (*a/ α*) did not undergo white-opaque switching, derivative homozygous *MTLa* and homozygous *MTL α* cells underwent white-opaque switching at high frequency, and that when homozygous cells were in the opaque phase, the efficiency of mating increased 10⁶-fold. Lockhart et al. (14) subsequently demonstrated that all white-opaque switchers obtained from a collection of clinical isolates were *MTL* homozygous and, conversely, that the majority of *MTL* homozygotes obtained from a collection of clinical isolates underwent white-opaque switching. Lockhart et al. (15) subsequently demonstrated that fusion occurred only between naturally occurring homozygous *MTLa* and homozygous *MTL α* strains in the opaque phase. Finally, Lockhart et al. (15) described for the first time the cell biology of *C. albicans* mating, including shmooing, chemotropism of conjugation tubes, fusion of tubes, nuclear association, vacuole expansion and nuclear separation in the conjugation bridge, asynchronous nuclear division in the zygote, daughter cell growth, nuclear migration in the daughter cell, septation, and daughter cell budding.

Recently, Rustad et al. (27) presented evidence suggesting a link between homozygosity at the *MTL* locus and fluconazole resistance in *C. albicans*. An analysis of 46 fluconazole-sensitive and 50 fluconazole-resistant strains revealed that while only 2% of the former were homozygous for the *MTL* locus (i.e., either *a/a* or *α/α*), 22% of the latter were homozygous. Rustad et al. (27) hypothesized that the association of fluconazole resistance and *MTL* homozygosity could be due to two possible mechanisms. First, drug resistance could be the direct result of the loss of either *MTLa1* or *MTL α 1/MTL α 2* (i.e., the direct result of *MTL* homozygosity), which in turn suggests that *MTL*-homozygous strains may be intrinsically resistant. Alternatively, they hypothesized that drug resistance could be the result of homozygosity of an allele linked to *MTL* on chromosome 5, through mitotic crossing over. Rustad et al. (27) demonstrated that homozygous *MTLa* and homozygous *MTL α* cells, engineered from the heterozygous *MTLa/ α* strain CAI4 by Hull et al. (8), were as susceptible to fluconazole as the parent *MTL*-heterozygous strain, which supported the hypothesis that the high level of *MTL* homozygosity among fluconazole-resistant strains was not due to the immediate acquisition of drug resistance through *MTL* alleles becoming homozygous without drug exposure.

To investigate further the relationship between fluconazole resistance and *MTL* homozygosity, we have used a strategy opposite that of Rustad et al. (27). Rather than measure the proportion of *MTL* homozygotes among identified drug-resistant strains, we measured drug susceptibility among identified *MTL*-homozygous strains, the majority of which were collected from patients prior to drug therapy. In addition, we tested the susceptibility of the *MTL*-homozygous progeny of three *MTL*-heterozygous strains that spontaneously formed *MTL*-homozygous progeny at high frequency. If resistance is directly associated with the loss of either *MTLa1* or *MTL α 1/MTL α 2*, then

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TABLE 1. Antifungal susceptibility of 17 *C. albicans* strains homozygous at the mating type-like locus (*MTL*)

<i>MTL</i> genotype	Strain	MIC ($\mu\text{g/ml}$) ^a							
		Flu.	Ravu.	Posa.	Vori.	Itra.	Caspo.	Ampho. B	5-FC
a	L26	2.00	0.060	0.120	0.030	0.500	0.12	1.0	2.00
	12C	0.25	≤0.007	0.015	≤0.007	0.030	0.06	0.5	0.25
	G106	0.50	≤0.007	0.030	≤0.007	0.120	0.06	1.0	0.25
	OKP90	≤0.12	≤0.007	0.015	≤0.007	0.030	0.12	1.0	16.00
	P60	0.25	≤0.007	0.015	≤0.007	0.060	0.06	1.0	0.25
	P37005	0.25	≤0.007	0.030	≤0.007	0.060	0.06	1.0	1.00
	P75063	0.25	≤0.007	0.015	≤0.007	0.030	0.12	1.0	0.12
α	WO-1	≤0.12	≤0.007	0.030	0.015	0.060	0.12	1.0	0.12
	19F	0.25	≤0.007	0.015	≤0.007	0.030	0.12	1.0	≥64.00
	GC75	≤0.12	≤0.007	≤0.007	≤0.007	0.030	0.25	1.0	0.25
	P87	0.25	≤0.007	0.030	≤0.007	0.060	0.12	1.0	0.12
	P37035	0.50	0.015	0.015	≤0.007	0.060	0.06	1.0	0.25
	P37037	0.25	≤0.007	0.015	≤0.007	0.030	0.25	1.0	0.25
	P37039	≤0.12	≤0.007	0.015	≤0.007	0.030	0.06	1.0	0.25
	P57072	0.25	≤0.007	≤0.007	≤0.007	0.015	0.06	1.0	0.12
	P78048	0.25	≤0.007	0.015	≤0.007	0.030	0.06	1.0	0.25
	P80001	0.50	≤0.007	0.030	≤0.007	0.120	0.06	1.0	0.12
All isolates									
	MIC ₅₀ ^b	0.25	≤0.007	0.015	≤0.007	0.030	0.06	1.0	0.25
	MIC ₉₀ ^b	0.50	≤0.007	0.030	≤0.007	0.120	0.12	1.0	2.00
a/α (97%)^c									
	MIC ₅₀ ^b	0.25	≤0.007	0.015	≤0.007	0.030	0.12	1.0	0.25
	MIC ₉₀ ^b	0.50	0.015	0.060	0.015	0.060	0.25	1.0	1.00

^a Flu., fluconazole; Ravu., ravuconazole; Posa., posaconazole; Vori., voriconazole; Itra., itraconazole; Caspo., caspofungin; Ampho.B, amphotericin B.

^b The MIC₅₀ and MIC₉₀ values represent the concentration of each antifungal agent that inhibited 50% and 90%, respectively, of the tested isolates.

^c Data were obtained from Pfaller et al. (22) for a collection of 486 isolates of *C. albicans* tested for caspofungin and from Diekema et al. (6) for a collection of 148 isolates of *C. albicans* tested for the remaining antifungals. The collections used as controls were assumed to be 97% a/α based on the results of Lockhart et al. (14).

MTL homozygotes randomly selected from a large collection of clinical isolates should be intrinsically drug resistant without exposure to drugs, and *MTL* homozygotes spontaneously generated by naturally occurring, unstable *MTL* heterozygotes should be more drug resistant than their heterozygous parent strains without exposure to drugs. The results presented here demonstrate that in the absence of previous antifungal drug exposure, naturally occurring *MTL*-homozygous strains of *C. albicans* are not intrinsically more drug resistant.

MATERIALS AND METHODS

***C. albicans* isolates.** A general collection of over 300 clinical isolates representing the five major clades distinguished by the complex DNA fingerprinting probe Ca3 (5, 14, 23, 25) were tested for *MTL* zygosity by PCR methods (14, 15). A total of 16 *MTL*-homozygous strains were identified, including 7 *MTL*_α-homozygous and 9 *MTL*_α-homozygous strains. The majority of isolates were previously described (14). Two additional isolates, P37037 and P37039, were both collected from healthy individuals in Wisconsin and New Jersey, respectively. To this collection was added strain WO-1, which is *MTL*_α homozygous. This population represents a mixed collection of isolates from the United States (nine isolates), Canada (one isolate), and South Africa (five isolates) from diverse clinical origins (14). With the exception of 19F and WO-1, these isolates were not exposed to any antifungal agent prior to collection. 19F was exposed to terconazole, and WO-1 was exposed to 5-fluorocytosine and amphotericin B prior to collection. Isolates were stored in 20% glycerol at 80°C prior to experimental use.

MIC testing. Antifungal susceptibility testing was performed by the reference broth microdilution method as described in the National Committee for Clinical Laboratory Standards document M27-A (18). Caspofungin (Merck Research Laboratories, Rahway, N.J.), amphotericin B (Sigma, St. Louis, Mo.), flucytosine (5-FC; Sigma), fluconazole (Pfizer Inc., New York, N.Y.), voriconazole (Pfizer), itraconazole (Janssen, Beerse, Belgium), posaconazole (Schering-Plough, Ken-

ilworth, N.J.), and ravuconazole (Bristol-Myers Squibb, Wallingford, Conn.) were obtained from their respective manufacturers. Serial dilutions were made in RPMI 1640 broth medium buffered to pH 7.0 with 0.165 M morpholinepropane-sulfonic acid. Microdilution trays were prepared in a single lot as previously described (21), with the addition of an array of caspofungin at final dilutions of 0.007 to 8 $\mu\text{g/ml}$. The trays were stored at -70°C prior to use.

Prior to testing, each isolate was passaged on supplemented Lee's agar medium (4, 11) to assess colony phenotypes and once on potato dextrose agar (Remel, Lenexa, Kans.) to ensure optimal growth characteristics. One hundred microliters of a suspension of cells varying between 0.5×10^3 and 2.5×10^3 cells per ml was added to each well of a microdilution tray. The trays were incubated in air at 35°C, and MIC endpoints were read after 48 h. Drug- and yeast-free controls were included in each tray. Following incubation, the growth in each well was compared with that in the control wells. The MICs of each of the triazoles, caspofungin, and 5-FC were defined as the lowest concentration resulting in approximately 50% growth inhibition. The MIC of amphotericin B was defined as the lowest concentration resulting in 100% growth inhibition. Quality control of the susceptibility assays was performed on the reference isolates *Candida parasilosis* ATCC 22091 and *Candida krusei* ATCC 6258 (3, 18).

DNA fingerprinting. The complex DNA fingerprinting probe Ca3 (1, 13, 24, 28) was used to confirm the identity of the progeny isolates from the parental strains P37037par and P37039par and to exclude the possibility of contaminations. DNA was extracted from cells (31), digested with *EcoRI*, and electrophoresed in a 0.8% agarose gel at 50 V according to methods previously described (12, 25, 32, 34). The DNA was then transferred to a Hybond N⁺ membrane (Amersham, Piscataway, N.J.) by capillary blotting. Prehybridization and hybridization with ³²P-labeled Ca3 probe were performed as previously described (32). Southern blots were then autoradiographed.

PCR analysis of the *MTL* locus. The PCR protocol used to analyze the *MTL* locus was previously described (14). The presence of *MTL*_α was ascertained by amplifying *MTL*_{α1} and the associated gene *OBPa* (7). The presence of *MTL*_α was ascertained by amplifying *MTL*_{α1} and *MTL*_{α2} and the associated gene *OBPa*. *MTL*_{α1}, *MTL*_{α2}, and *MTL*_{α1} are the *C. albicans* homologues of the *Saccharomyces cerevisiae* mating type genes *MAT*_{α1}, *MAT*_{α2}, and *MAT*_{α1}, respectively (7).

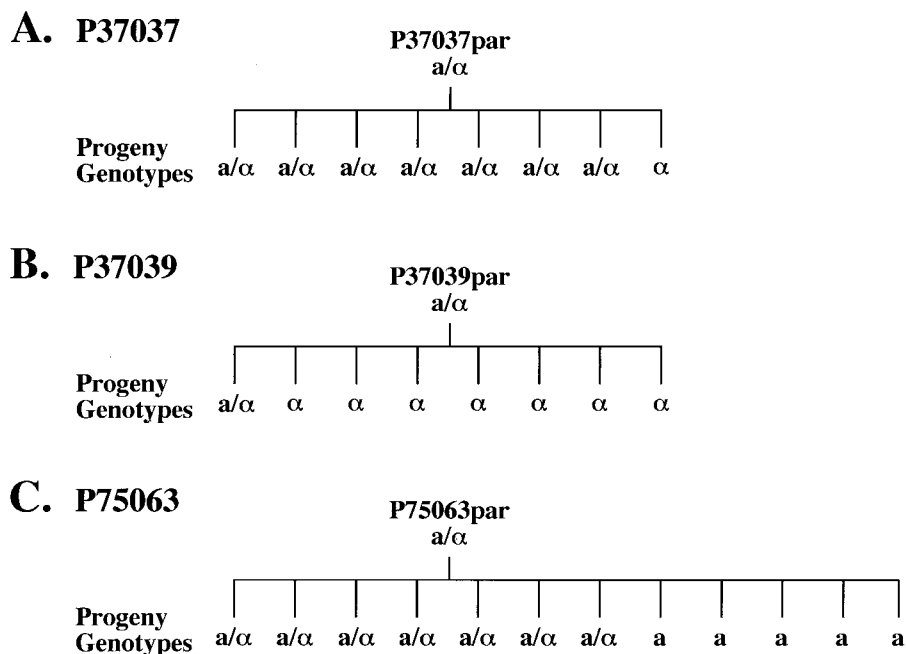


FIG. 1. Pedigrees demonstrating spontaneous in vitro loss of heterozygosity at the *MTL* locus. Selected progeny of three *MTL*-heterozygous parental strains, P37037par, P37039par, and P75063par, that formed opaque-phase sectors were analyzed by PCR amplification of *MTL* genes. Their *MTL* genotypes are coded a/α for *MTL* heterozygotes, a for *MTL*a homozygotes, and α for *MTL*α homozygotes.

RESULTS

Drug susceptibility of a and α cells. To test whether naturally occurring *MTL*-homozygous strains are more resistant to antifungal drugs than naturally occurring *MTL*-heterozygous strains, we first compared the susceptibility of 7 a strains and 10 α strains identified in a large collection of clinical isolates with the susceptibility of an independent set of unselected isolates from two large collections of naturally occurring isolates previously analyzed (6, 22). Since Lockhart et al. recently demonstrated that approximately 97% of naturally occurring *C. albicans* isolates are heterozygous at the *MTL* locus (14), we assumed here that the large collections previously analyzed for drug susceptibility were predominantly *MTL* heterozygous. In Table 1, the MICs of fluconazole, ravuconazole, posaconazole, voriconazole, itraconazole, caspofungin, amphotericin B, and 5-FC are presented for each of the 17 homozygous strains. In addition, the MIC₅₀ (the concentration that inhibited 50% of isolates) and MIC₉₀ (the concentration that inhibited 90% of isolates) of the entire collection of 17 *MTL*-homozygous strains and of the previously studied collections of predominantly *MTL*-heterozygous strains are provided in Table 1.

Of the 17 *MTL*-homozygous strains tested, only one strain, L26, exhibited increased resistance to fluconazole (Table 1). This strain also exhibited increased resistance to the other tested azoles and a slight increase in 5-FC resistance, but no resistance to caspofungin or amphotericin B. Two strains, OKP90 and 19F, exhibited increased resistance to 5-FC but normal susceptibility to all other drugs. With the exception of resistance to 5-FC observed for 19F, the two *MTL*-homozygous strains that had been exposed to drug therapy prior to collection, strains WO-1 and 19F, exhibited no increases in

resistance to the tested drugs. These results suggest that becoming homozygous in and of itself is not sufficient to confer drug resistance in *C. albicans*.

Drug susceptibility of spontaneous *MTL*-homozygous progeny of *MTL*-heterozygous strains. We next tested whether *MTL* heterozygotes gave rise to drug-resistant *MTL*-homozygous progeny. In an analysis of 30 *MTL*-heterozygous isolates, 3 were identified that formed opaque-phase sectors at high frequency. Since white-opaque switching is suppressed in *MTL*-heterozygous strains (14, 17), it appeared likely that these particular strains gave rise spontaneously to *MTL*-homozygous progeny at high frequency. To test this possibility, randomly selected progeny of the three such strains were tested for *MTL* zygosity by PCR analysis of mating type genes. For strains P37037 and P37039, *MTL*a/α and *MTL*α progeny were identified and, for strain P75063, *MTL*a/α and *MTL*a progeny were identified (Fig. 1). In Fig. 2A, PCR amplification patterns of *MTL*a1 and *MTL*α2 are presented for the *MTL*-heterozygous parental strains P37037par and P37039par and both *MTL*-heterozygous and *MTL*-homozygous progeny of each. A similar analysis of strain P75063 was previously reported (14). To demonstrate that *MTL*-heterozygous and *MTL*-homozygous isolates were progeny of the respective parent strains and not contaminants, isolates were DNA fingerprinted by Southern blot hybridization with the complex probe Ca3 (32, 34). The Southern blot hybridization patterns of the parent strain and the progeny of each strain were identical (Fig. 2B).

The drug susceptibilities of the three selected *MTL*-heterozygous parent strains and a single *MTL*-homozygous progeny of each were compared. For strains P37039 and P75063,

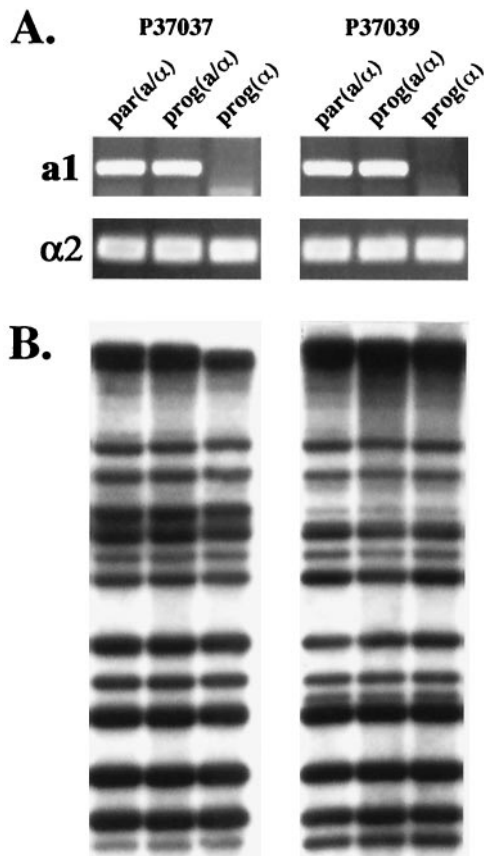


FIG. 2. Demonstration by PCR of spontaneous in vitro loss of heterozygosity at the *MTL* locus by strains P37037 and P37039. (A) PCR analysis of the *MTL* locus demonstrating *MTL* heterozygosity of the parent strains [par(a/α)] and *MTL* homozygosity of the progeny isolate (α/α). The *MTL*a1 and *MTL*α2 genes of each test isolate were amplified by PCR and run in a 1% agarose gel. (B) Southern blot analysis with the complex fingerprinting probe Ca3 confirmed that, in both strains, the heterozygous and homozygous isolates were progeny of the original parental strain. *Eco*RI-digested DNA was run in a 0.8% agarose gel, Southern blotted, and hybridized with radiolabeled Ca3 fingerprinting probe.

there was no significant difference (i.e., more than twofold increase) between the MICs for the parental *MTL* heterozygotes and their *MTL*-homozygous progeny. For strain P37037, there was no significant difference between the *MTL*-heterozygous parental isolates and *MTL*-homozygous progeny for seven of the eight antifungal drugs. In the case of 5-FC, however, the *MTL* heterozygote was 16-fold less susceptible than the *MTL* homozygote. Their respective 5-FC MICs were 4.00 and 0.25 μg per ml. Together, these data demonstrate that spontaneous *MTL*-homozygous progeny that have not been exposed to antifungal drugs are not more resistant to fluconazole or the other tested drugs than their *MTL*-heterozygous parents.

Drug susceptibility and the white-opaque transition. It has been demonstrated that while *MTL*-homozygous strains of *C. albicans* undergo white-opaque switching, *MTL*-heterozygous strains do not. Since high-frequency phenotypic switching has been shown to alter drug susceptibility levels (38), the possibility was entertained that the higher proportion of flucon-

azole-resistant strains in the collection of *MTL*-homozygous strains analyzed by Rustad et al. (27) may have been due to expression of the opaque-phase phenotype (9, 26, 33, 36, 37). To test this possibility, we compared the susceptibility of white- and opaque-phase cells of six *MTL*-homozygous strains to the eight antifungal drugs. For five of the six strains, there was no significant difference between white- and opaque-phase cells in their susceptibility to the eight antifungal drugs. One strain, 19F, exhibited increased resistance to 5-FC in both white- and opaque-phase cells but showed relatively low MICs of the seven other antifungal drugs. In this one strain, white-phase cells were significantly more resistant than opaque-phase cells to 5-FC. Their 5-FC MICs were ≥ 64.00 and 16.00 μg per ml, respectively. Together, these results indicate that, on average, white- and opaque-phase cells exhibit similar drug susceptibility patterns. Hence, the increase in the proportion of homozygous strains among fluconazole-resistant strains in the collection analyzed by Rustad et al. (27) was not due to increased expression of the opaque-phase phenotype.

DISCUSSION

Although it does not appear that specific drug-resistant strains of *C. albicans* have established themselves as significant components of the general colonizing populations of *C. albicans* (19, 20, 30, 35), drug-resistant strains have emerged in specific cases of candidiasis. In recent years, a number of genes involved in drug resistance have been identified and cloned (29, 39). Among these, genes of the ABC transporter family have been implicated in clinical resistance to the azoles. Two of these genes, CDR3 and CDR4, are regulated by white-opaque switching (2; D. Sanglard, F. Ischer, M. Monod, S. Dogra, R. Prasad, and J. Bille, Abstr. 5th ASM Conf. *Candida* Candidiasis, abstr. C27, 1999). In addition, a number of other putative drug resistance genes have been recently shown to be differentially regulated by the white-opaque transition (10). The report by Rustad et al. (27) that 22% of fluconazole-resistant strains were *MTL* homozygous while only 2% of fluconazole-susceptible strains were *MTL* homozygous was interesting in light of the recent discovery that only *MTL*-homozygous strains of *C. albicans* undergo white-opaque switching (14, 17).

Rustad et al. (27) proposed that the increase in the proportion of *MTL*-homozygous strains among fluconazole-resistant strains was either a direct result of becoming homozygous at the *MTL* locus or homozygous at a gene linked to *MTL*. To distinguish between these alternatives, we used a strategy opposite that of Rustad et al. (27) in which we tested whether strains selected for *MTL* homozygosity were more resistant to antifungal drugs. Because homozygosity in natural populations is relatively rare in *C. albicans* (14), the *MTL*-homozygous isolates analyzed here were obtained from different geographical locales and different clinical settings. This collection was distinct from the collection analyzed by Rustad et al. (27) in that isolates of their collection were selected solely for fluconazole resistance. If resistance in *C. albicans* isolates was a direct consequence of *MTL* homozygosity, it would be expected that naturally occurring *MTL*-homozygous isolates that have not been exposed to fluconazole or other antifungal drugs would nevertheless show intrinsic resistance. None of the 17 isolates had been exposed to fluconazole prior to collection, while two

were exposed to antifungal drugs other than fluconazole. We found no elevated levels of resistance to fluconazole and very limited resistance to the seven additional drugs among the 17 *MTL*-homozygous isolates. The two *MTL*-homozygous isolates that had been exposed to drug therapy prior to collection were not resistant to the drugs they had been exposed to. These results suggest that the relation between *MTL* homozygosity and fluconazole resistance is not direct, and they are consistent with the alternative hypothesis proposed by Rustad et al., namely, that a gene linked to *MTL*, which becomes homozygous when *MTL* becomes homozygous, is responsible for the acquisition of drug resistance (27). An analysis of the region of chromosome 5 harboring the *MTL* locus in the Stanford genome database, however, failed to reveal a possible gene candidate (i.e., potential drug resistance gene). *ERG11*, which is the only known gene involved in fluconazole resistance on chromosome 5, does not appear to be a viable candidate since it is not closely linked to the *MTL* locus and has been found to be heterozygous in some *MTL*-homozygous strains (27).

To further test whether *MTL* homozygosity leads to drug resistance in the absence of drug exposure, we analyzed *MTL*-homozygous progeny of three *MTL*-heterozygous strains that produce *MTL*-homozygous progeny at high frequency. In no case did we observe fluconazole resistance or general drug resistance in the *MTL*-homozygous progeny. Finally, we tested the possibility that the increase in *MTL*-homozygous isolates among drug-resistant isolates was the result of their *MTL*-homozygous-associated characteristic of switching from white to opaque (14, 17). A comparison of white- and opaque-phase cells (9, 26, 33, 36, 37) from six *MTL*-homozygous strains revealed no opaque-phase-specific resistance, thus eliminating this possibility as an explanation of the results reported by Rustad et al. (27).

Among the 17 *MTL*-homozygous isolates analyzed, one was resistant to 5-FC and a second one exhibited intermediary resistance to this drug. In addition, significant differences in susceptibility to 5-FC were observed between white- and opaque-phase cells of one strain and between the *MTL*-heterozygous parent and the *MTL*-homozygous progeny of another strain. These changes, however, did not support the acquisition of drug resistance as a result of becoming homozygous.

Together, our results demonstrate that *MTL* homozygosity in and of itself is not sufficient to confer fluconazole or general drug resistance. In addition, *MTL*-homozygous progeny formed spontaneously from *MTL*-heterozygous strains exhibit the same levels of susceptibility as their *MTL*-heterozygous parent strains. These results are consistent with an alternative hypothesis proposed by Rustad et al. (27) that the higher incidence of *MTL*-homozygous strains among fluconazole-resistant strains is the result of associated homozygosity of an unidentified gene linked to the *MTL* locus. These results are also consistent with the possibility that *MTL*-homozygous strains develop drug resistance more rapidly than *MTL*-heterozygous strains when exposed to antifungal drugs, a hypothesis now under investigation.

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