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ORIGINAL ARTICLE

Fluconazole induces rapid high-frequency *MTL* homozygosis with microbiological polymorphism in *Candida albicans*

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KEYWORDS
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Abstract *Background: Candida albicans*, a common fungal pathogen that can cause opportunistic infections, is regarded as an apparently asexual, diploid fungus. A parasexual cycle was previously found between homozygotes with opposite mating type-like loci (*MTL*a/α). Fluconazole-resistant strains had a higher proportion of *MTL* homozygotes, whereas *MTL* homozygous *C. albicans* was found in only about 3.2% of clinical strains. *MTL* heterozygotes had a low frequency (1.4×10^{-4}) of white—opaque switching to *MTL* homozygotes in nature.
Methods: Here, a reference *C. albicans* strain (SC5314) was used in a fluconazole-induced assay to obtain standard opaque *MTL* homozygous strains and first-generation daughter strains from the fluconazole inhibition zone. Further separation methods were employed to produce second- and third-generation daughter strains. Polymerase chain reaction analysis based on *MTL* genes was used to define *MTL* genotypes, and microscopic observations, a flow-cytometric assay, and an antifungal E-test were used to compare microbiological characteristics.
Results: MTL homozygotes were found at a high frequency (17 of 35; 48.6%) in fluconazole-induced first-generation daughter strains, as were morphological polymorphisms, decreased DNA content, and modified antifungal drug susceptibility. High-frequency *MTL* homozygosity

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was identified inside the fluconazole inhibition zone within 24 hours. The DNA content of fluconazole-induced daughter strains was reduced compared with their progenitor SC5314 and standard *MTL* homozygous strains.
Conclusion: Treatment with fluconazole, commonly used to treat invasive candidiasis, inhibited the growth of *C. albicans* and altered its microbiological characteristics. Our results suggest that fluconazole treatment induces the high frequency of loss of heterozygosity and microbiological polymorphism in *C. albicans*.
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Introduction

Candida albicans, a common fungal pathogen that can cause opportunistic infections, is increasingly being recognized as a human pathogen in immunocompromised hosts, such as premature infants, cancer chemotherapy patients, solid-organ transplant recipients, and patients coinfected with human immunodeficiency virus or undergoing immunosuppressive treatment. *C. albicans* accounts for 50–60% of invasive fungal infections in humans, and the mortality rate of candidemia is higher (up to 61%).[1,2] *C. albicans* is regarded as a diploid, apparently asexual fungus. The frequency of spontaneous white-to-opaque cell switching is about 1.4×10^{-4} for *C. albicans* and about 10^{-2} for the specific WO-1 strain, as revealed by phloxine B-containing Lee's medium. Mating type-like loci *MTL*(a/α) identified in *C. albicans* are orthologous genes to mating-type loci *MAT*(a/α) in *Saccharomyces cerevisiae*. Only *MTL* homozygotes undergo white-to-opaque cell switching, and all individuals of strain WO-1 and 3.2% of 220 clinical isolates were found to be *MTL* homozygotes.[3–6] Opaque cells are 10^6 times more mating competent than white cells. The tetraploid parasexual cycle of *C. albicans* consisting of mating followed by chromosome loss has been described previously.[7–9]

Fluconazole is among the most common antifungal drugs to treat invasive fungal diseases caused by *C. albicans*; thus, much attention has been paid to fluconazole-resistant *C. albicans* strains.[10,11] A significantly higher proportion of *MTL* homozygosity was found in the fluconazole-resistant group than in the fluconazole-susceptible group among clinical strains.[12,13] However, fluconazole resistance is not directly affected by *MTL* homozygosity. One study of the evolution of fluconazole resistance was conducted to obtain a series of 330-generation daughter strains by treating one clinical strain with and without fluconazole. Further studies of the 330-generation series of *C. albicans* strains show that they appeared to have gradually developed adaptations against fluconazole through genomic evolution with corresponding fluconazole-resistant genes, which has indirectly correlated with *MTL* homozygosity.[14,15] However, recent analyses of these evolved strains indicated that rapid acquisition of aneuploidy and fluconazole resistance occurred within the first few generations.[16] Fluconazole resistance in *C. albicans* is due to both accumulated point mutations and loss of heterozygosity (LOH); therefore, *MTL* homozygosity is coincident with LOH on the left arm of chromosome 5, where two genes corresponding to fluconazole resistance, *ERG*11 and *TAC*1, are located. Recent analyses of these evolved strains indicate that rapid acquisition of aneuploidy and fluconazole resistance occurred within the first few generations.[17,18]

In a recent study, we found that fluconazole induced random chromosome loss and LOH in *C. albicans*, but the frequency of LOH is unclear.[19] Therefore, we collected isolates and offspring that were treated by fluconazole for a short term, calculated the frequency of LOH, and analyzed microbiological polymorphism.

Methods

C. albicans strains

The strains used in this study are listed in Table 1. The *C. albicans* reference strain SC5314 was used for its complete genome sequence.[6] A fluconazole-induced assay was used to obtain 35 fluconazole-induced first-generation daughter strains (FI-FGDSs) of SC5314. FI-FGDSs were analyzed MTL locus, and the heterozygotes were further separated and isolated by plating culture and micromanipulation to selected 87 fluconazole-induced second-generation daughter strains (FI-SGDSs) and 141 fluconazole-induced third-generation daughter strains (FI-TGDSs).

Fluconazole-induced assay and strain purification

The strain SC5314 was treated with the fluconazole-induced assay as described previously.[19] Phloxine B distinguishes opaque sectors and colonies by differentially staining them red. The strain SC5314 was treated with a fluconazole-containing disc (100 mg fluconazole per disc) or a fluconazole E-test strip (AB BIODISK, Solna, Sweden) on PB–YPD agar (YPD plus 5 μg/mL phloxine B) for 12–16 hours at 30°C. In this period, an inhibition zone was formed, and cells in the inhibition zone were observed using a microscope. The 35 FI-FGDSs were isolated from the inhibition zone with needle and each colony was spread on YPD agar for 6–8 hours. The initial cell morphology was observed to ensure that the strain was pure; if the morphology was different, we would isolate each different cell by a micromanipulator to be an FI-SGDS. In the 87 FI-SGDSs also, each colony was spread on YPD agar for 6–8 hours and the initial cell morphology was observed; each different cell was isolated to be an FI-TGDS.

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Table 1 Strains used in this study.

Strain	Parent strain	*MTL* genotype	No. of strains	Ratio of DNA content decrease	Ratio of *MTL* homozygosity[a]
SC5314 (*n* = 1)		a1α1α2	1		
FI-FGDS (*n* = 35) (first generation)	SC5314	a1α1α2	31	4/35 (11.4%)	4/35 (11.4%)
		a1	2		
		α1α2	2		
FI-SGDS (*n* = 87) (second generation)	FI-FGDS	a1α1α2	74	12/35 (34.3%)	11/35 (31.4%)
		a1	6		
		α1α2	7		
FI-TGDS (*n* = 141) (third-generation)	FI-SGDS	a1α1α2	100	20/35 (57.1%)	17/35 (48.6%)
		a1	24		
		α1α2	17		

[a] The ratios correspond to first-generation FI-FGDSs (see Methods section).
FI-FGDS = fluconazole-induced first-generation daughter strain; FI-SGDS = fluconazole-induced second-generation daughter strain; FI-TGDS = fluconazole-induced third-generation daughter strain.

Initial cell morphology

Each strain was spread, at a density of 100–200 cells/mm^2, on a YPD agar plate and incubated for 6–12 hours at 30°C to observe the initial colony morphology. The initial colony morphology of each strain was observed using a microscope (Leica DM2500, Leica Microsystems, Germany) and was photographed using a QICAM Fast1394 device (Qimaging, Canada).

Polymerase chain reaction analysis of MTL gene and frequency of MTL homozygosis

We used 30 ng of pure genomic DNA. The *MTL*a1, *MTL*α1, and *MTL*α2 primer sequences and polymerase chain reaction condition used in this study were according to those used in previous studies.[12,19] Reaction mixtures were typically heated for 5 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 1 minute at 60°C, and 1 minute at 72°C. After a final incubation period of 10 minutes at 72°C, we stored the reaction mixtures at 4°C. The polymerase chain reaction fragments wer observed by electrophoresis on a 1.5% agarose gel in 1× Tris-acetate-EDTA (TAE) buffer which is used as both a running buffer and in agarose gel (Bio-Rad, California, USA) at 100 V for 30 minutes and stained with SYBR Safe DNA gel stain (Invitrogen, Thermo Fisher Scientific Inc, Waltham, USA). The frequency of *MTL* homozygosis corresponded to FI-FGDSs.

Flow cytometry analysis of DNA content

The DNA content of the SC5314 strain and its daughter strains was analyzed using Sytox Green dye-stained flow cytometry. The tested strains were propagated at 30°C for 24 hours in YPD agar and prepared by fixing in 70% ethanol at 4°C. The cells were washed and suspended in 1 mL phosphate buffered saline, sonicated on low power, and then resuspended in 0.5 mL of 2 mg/mL RNaseA solution and incubated at 37°C for 2 hours. The samples were treated with 5 mg/mL pepsin for 30 minutes at 37°C, and were then suspended in 0.5 mL phosphate buffered saline and sonicated at low power. The samples were stained in 1mM Sytox Green dye for 1 hour at 4°C and then analyzed by flow cytometry. A total of 10,000 cells were analyzed for each tested strain. Cell and colony morphologies of all the DNA-decreasing strains were observed and the minimum inhibitory concentrations (MICs) were determined by the E-test method, following Lu et al.[20]

Results

Frequency of MTL homozygotes

In total, 263 strains, 35 FI-FGDSs (harvested from phloxine B-stained colonies inside the inhibition zone of the fluconazole-induced assay), 87 FI-SGDSs, and 141 FI-TGDSs, were obtained and analyzed to determine their *MTL* genotypes (Table 1). In the 35 FI-FGDSs, four (11.4%) had lost one of the *MTL* genes, and all four of these were *MTL* homozygous. In 87 FI-SGDSs, there were 13 *MTL* homozygous strains, corresponding to FI-FGDSs; the frequency was 11/35 (31.4%). In 141 FI-TGDSs, there were 41 *MTL* homozygous strains, corresponding to 35 FI-FGDSs; the frequency was 17/35 (48.6%) (Table 2). The colonies inside and outside the fluconazole inhibition zone were collected, and replated on PB–YPD agar. The percentages of phloxine B-stained colonies inside and outside were 24.6% (118/479) and 0.449% (2/445), respectively. The frequency of phloxine B-stained colonies increased about 55-fold with fluconazole stress (Figure 1).

Microbiological polymorphism in fluconazole-induced daughter strains: cell/colony morphology, DNA content, and antifungal drug susceptibility

Compared with the reference strain SC5314, single colony and cell morphology were different. All the daughter strains examined had a lower DNA content than the progenitor SC5314 strain. The six fluconazole-induced *MTL* homozygous daughter strains, FI-SGDS-A12-2, FI-FGDS-A14, FI-SGDS-A17-1, FI-TGDS-A17-1-L2, FI-TGDS-A17-1-R2, and FI-FGDS-A25, investigated had significantly lower DNA contents, decreased by more than 40% (Figure 2, Table 2). Changing microbiological characteristics were found in fluconazole-induced daughter strains with and without *MTL*

Table 2 Microbiological characteristics of *C. albicans* strains with fluconazole treatment.

Strain	Parent strain	*MTL* gene	Filamentous	DNA content[a]	Fluconazole MICs	Amphotericin B MICs
SC5314		a1α1α2	No	1.0	0.125	0.125
FI-FGDS-A12	SC5314	a1α1α2	Yes	0.51	0.125	0.19
FI-SGDS-A12-2	FI-FGDS-A12	a1	Yes	0.53	0.19	0.125
FI-FGDS-A14	SC5314	a1	Yes	0.48	0.125	0.19
FI-FGDS-A17	SC5314	a1α1α2	Yes	0.98	0.19	0.125
FI-SGDS-A17-1	FI-FGDS-A17	a1α1α2	No	0.65	0.25	0.125
FI-TGDS-A17-1-L2	FI-SGDS-A17-1	α1α2	Yes	0.69	0.19	0.064
FI-TGDS-A17-1-R2	FI-SGDS-A17-1	a1	Yes	0.49	0.5	0.125
FI-FGDS-A25	SC5314	α1α2	No	0.58	0.19	0.19

[a] Based on the G1 peak value of the flow cytometry analysis of the DNA content, the value was normalized to that of SC5314 (2N). FI-FGDS = fluconazole-induced first-generation daughter strain; FI-SGDS = fluconazole-induced second-generation daughter strain; FI-TGDS = fluconazole-induced third-generation daughter strain; MIC = minimum inhibitory concentration.

homozygosity. In the antifungal drug susceptibility, the MICs of fluconazole in strains FI-SGDS-A17-1 and FI-TGDS-A17-1-R2 were increased to 0.25 and 0.5, respectively, and the MIC of amphotericin B in the strain FI-SGDS-A17-L2 was decreased to 0.064.

Discussion

In this study, a fluconazole-induced assay identified four *MTL* homozygotes in the 35 first-generation FI-FGDSs, and, upon further propagation of these strains, we ended up

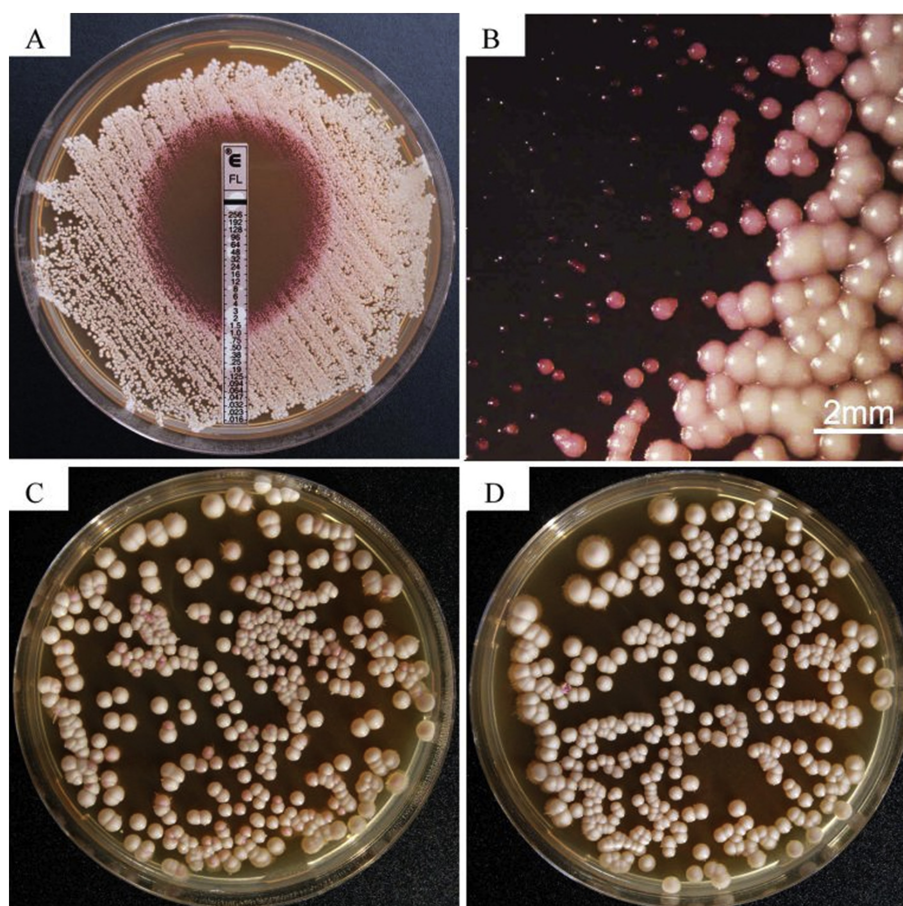

Figure 1. Phloxine B-stained colonies by fluconazole-induced assay. Phloxine B distinguishes opaque sectors and colonies by differentially staining them red. (A) Strain SC5314 was treated with a fluconazole E-test strip on PB–YPD agar. (B) A photomicrograph of the transitional area was taken with a microscope using a 10× objective to show the colonies inside and outside the inhibition zones. Colonies were collected from (C) inside and (D) outside the inhibition zone, and replated on PB–YPD agar for 7 days at 30°C. Colonies inside the inhibition zones in (A) and (B) were all phloxine B-stained colonies (red). Regrown colonies, taken from (C) inside and (D) outside the inhibition zone, were partially phloxine B-stained (red), and the ratios were 118/479 (24.6%) and 2/445 (0.00449%), respectively.

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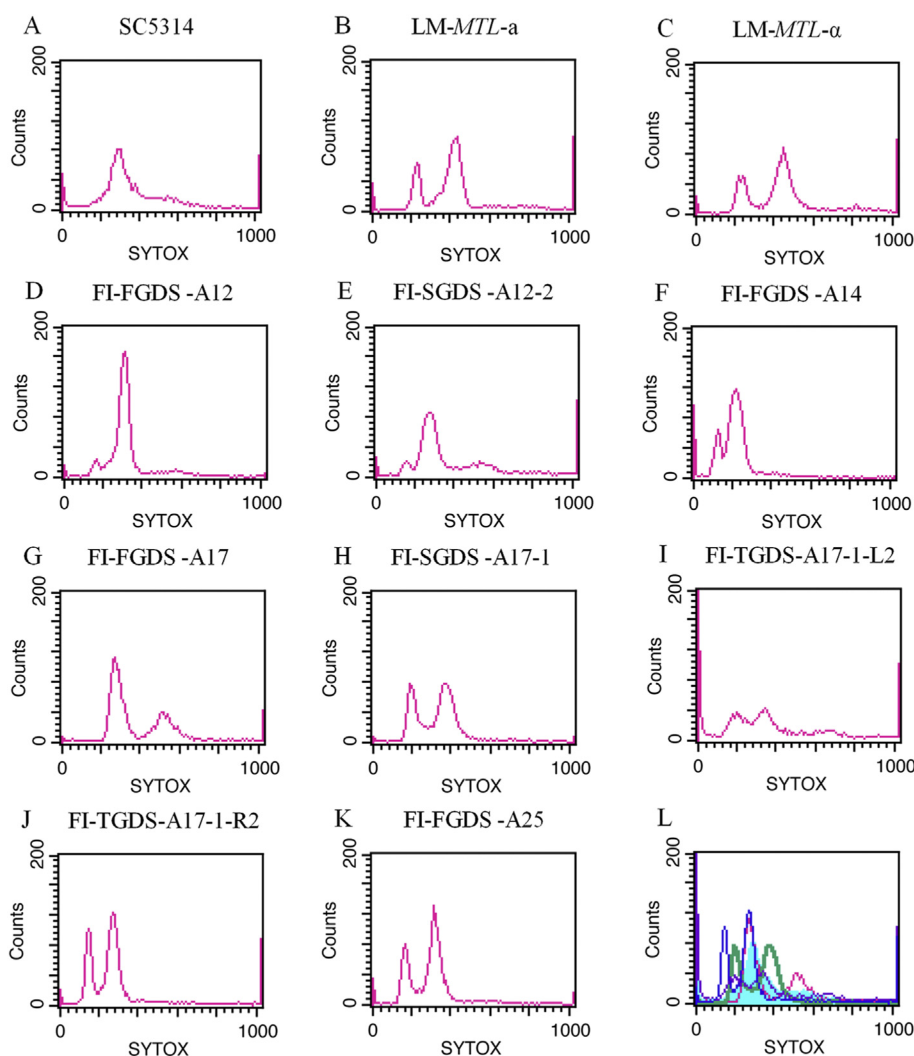

Figure 2. Flow cytometry analysis of DNA content. A was reference diploid strain SC5314, B and C were *MTL* homozygous strains without fluconazole treatment. D–K were strains with fluconazole treatment. L was the merged of all strains. The fluconazole-induced daughter strains, FI-FGDS-A14, FI-SGDS-A17-1, FI-FGDS-A17-1-L2, FI-TGDS-A17-1-R2, and FI-FGDS-A25, had more significant decreases in DNA content compared with SC5314, however nonfluconazole-induced daughter strains, LM-MTL-a and LM-MTL-α, did not. FI-FGDS = fluconazole-induced first-generation daughter strain; FI-SGDS = fluconazole-induced second-generation daughter strain; FI-TGDS = fluconazole-induced third-generation daughter strain.

with 17 third-generation homozygotes, with a high occurrence (48.6%) of *MTL* homozygosity in *C. albicans*. The FI-FGDSs were collected from fluconazole stress for 12–16 hours, but the FI-SGDSs and FI-TGDSs were collected from PB–YPD plates without fluconazole. These results revealed that short-term treatment with fluconazole affected at least three generations of *C. albicans*. We speculate that *C. albicans* may encounter a genetically unstable period in changing chromosome number, together with possibly decreasing their DNA content, under fluconazole stress. Thus, the early-stage colonies may not have been able to survive fluconazole stress unless they were rescued from the stress in a timely manner. In our study, fluconazole-induced SC5314 daughter strains were found to have a much higher *MTL* homozygosity ratio (48.6%) than that found in nature using Lee's medium assay (0.14%) or observed with clinical strains (3.2%).

Fluconazole, one of the most common antifungal drugs used to treat fungal infections caused by *C. albicans*, is known to be an inhibitor of lanosterol 14-alpha-demethylase, which interferes with ergosterol biosynthesis.[11] The proportion of *MTL* homozygotes in our study was much higher than that found in clinical isolates, although the clinical use of fluconazole to treat *C. albicans* infections is very common. The mechanisms by which *MTL* homozygosity is rapidly induced by fluconazole in *C. albicans* are random chromosome loss and unequal division in mitosis. Two hypotheses may explain the different proportions of *MTL* homozygotes in our study compared with clinical isolates. The first assumes that fluconazole-induced *MTL* homozygotes have good mating ability. Therefore, most of them mate quickly with homozygotes for the complementary allele in the host environment, and their offspring are identified as *MTL* heterozygotes that actually genetically differ from the initial heterozygotes. The second hypothesis is that most of the fluconazole-induced homozygotes vanish in the host environment because of fluconazole

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stress, host innate immunity, and poor competition ability. In previous studies, *MTL*-a and *MTL*-α homozygotes were demonstrated to mate in a mouse model, and heterozygotes adapted better to the host environment, thereby becoming the predominant population. These observations partially support our two hypotheses of the homozygotes either mating quickly or dying out.

Additional microbiological characterization of the progenitor strain SC5314 and some of the fluconazole-induced daughter strains was conducted. Dramatic microbiological changes were found in the daughter strains investigated. These alterations included cell/colony polymorphism in some of the daughter strains, decreased or increase DNA content in the strains, and modified antifungal drug susceptibility. Rapid microbiological changes with polymorphisms and a high frequency of *MTL* homozygosity in the fluconazole assay suggest a capacity of this yeast for rapid evolution, especially if fluconazole-induced *MTL* homozygotes are mating competent. In summary, fluconazole treatment inhibited the growth of *C. albicans* and altered its microbiological characteristics. High-frequency *MTL* homozygosity was identified inside the fluconazole inhibition zone within 24 hours. The DNA content of the daughter strains was reduced compared with that of the progenitor SC5314 strain and heterozygous strains, suggesting chromosome loss induced by fluconazole treatment. The mechanism will induce the rapid evolution and produce fluconazole-resistant strains in *C. albicans*. The present study provides new insights into the interactions between the pathogenic *C. albicans* fungus and the antifungal drug fluconazole. In clinical treatment, inappropriate dosage of fluconazole will increase the evolution and produce fluconazole-resistant strains. These novel data may be helpful in the future to better manage patients infected with *C. albicans*. In addition, the unexpected action of fluconazole on *C. albicans* can be leveraged in future models in microbiological studies.

Conflicts of interest

None of authors have any competing financial or nonfinancial interests associated with this article.

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