Fluconazole induces rapid high-frequency \(MTL\) homozygosis with microbiological polymorphism in \(Candida albicans\)

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**KEYWORDS**

\(Candida albicans\); fluconazole; loss of heterozygosity; mating type-like gene

**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/\(a\)). 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 \(\times\) \(10^{-5}\)) 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
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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**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. 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. 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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**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%). However, fluconazole resistance is not limited to clinical strains. A significantly higher proportion of *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. 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.

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. A significantly higher proportion of *MTL* homozygosity was found in the fluconazole-resistant group than in the fluconazole-susceptible group among clinical strains. 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 strain with fluconazole for a short term, calculated the frequency of LOH, and analyzed microbiological polymorphism.
The ratios correspond to first-generation FI-FGDSs (see Methods section). 100 V for 30 minutes and stained with SYBR Safe DNA gel stain running buffer and in agarose gel (Bio-Rad, California, USA) at 12 hours at 30°C for 2 hours. The samples were stored in 0.5 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 1 mM 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 MTL gene, 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
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.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parent strain</th>
<th>MTL gene</th>
<th>Filamentous</th>
<th>DNA content&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fluconazole MICs</th>
<th>Amphotericin B MICs</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC5314</td>
<td>SC5314</td>
<td>a1a2</td>
<td>No</td>
<td>1.0</td>
<td>0.125</td>
<td>0.125</td>
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<tr>
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<td>SC5314</td>
<td>a1a2</td>
<td>Yes</td>
<td>0.51</td>
<td>0.125</td>
<td>0.19</td>
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<tr>
<td>FI-SGDS-A12-2</td>
<td>FI-FGDS-A12</td>
<td>a1</td>
<td>Yes</td>
<td>0.53</td>
<td>0.19</td>
<td>0.125</td>
</tr>
<tr>
<td>FI-FGDS-A14</td>
<td>SC5314</td>
<td>a1</td>
<td>Yes</td>
<td>0.48</td>
<td>0.125</td>
<td>0.19</td>
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<tr>
<td>FI-FGDS-A17</td>
<td>SC5314</td>
<td>a1a2</td>
<td>Yes</td>
<td>0.98</td>
<td>0.19</td>
<td>0.125</td>
</tr>
<tr>
<td>FI-SGDS-A17-1</td>
<td>FI-FGDS-A17</td>
<td>a1a2</td>
<td>No</td>
<td>0.65</td>
<td>0.25</td>
<td>0.125</td>
</tr>
<tr>
<td>FI-TGDS-A17-1-L2</td>
<td>FI-SGDS-A17-1</td>
<td>a1a2</td>
<td>Yes</td>
<td>0.69</td>
<td>0.19</td>
<td>0.064</td>
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<tr>
<td>FI-TGDS-A17-1-R2</td>
<td>FI-SGDS-A17-1</td>
<td>a1</td>
<td>Yes</td>
<td>0.49</td>
<td>0.5</td>
<td>0.125</td>
</tr>
<tr>
<td>FI-FGDS-A25</td>
<td>SC5314</td>
<td>a1a2</td>
<td>No</td>
<td>0.58</td>
<td>0.19</td>
<td>0.19</td>
</tr>
</tbody>
</table>

<sup>a</sup> Based on the G1 peak value of the flow cytometry analysis of the DNA content, the value was normalized to that of SC5314 (2N).

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...
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. 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

![Figure 2](https://example.com/fluconazole-induced-high-frequency-LOH-in-C-albicans)

**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.
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 non-financial interests associated with this article.

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