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Short-term exposure to fluconazole induces chromosome loss in *Candida albicans*: An approach to produce haploid cells

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ABSTRACT

Candida albicans is considered to be an obligate diploid fungus. Here, we describe an approach to isolate aneuploids or haploids induced by the short-term (12–16 h) exposure of diploid reference strains SC5314 and CAI4 to the most commonly used antifungal drug, fluconazole, followed by repeated single-cell separation among small morphologically distinct colonies in the inhibition zone. The isolated strains had altered cell morphology and LOH events in the *MTL* and other marker alleles of the analyzed loci at 8 chromosomes of *C. albicans* with decreased DNA content. The present study employed next-generation sequencing (NGS) combined flow cytometry analysis of the DNA content to analyze the haploid, autodiploid, and aneuploid strains that arose from the fluconazole treatment instead of using the conventional single nucleotide polymorphism/comparative genome hybridization (SNP/CGH) method. A multiple-alignment tool was also developed based on sequenced data from NGS to establish haplotype mapping for each chromosome of the selected strains. These findings revealed that *C. albicans* experiences 'concerted chromosome loss' to form strains with homozygous alleles and that it even has a haploid status after short-term exposure to fluconazole. Additionally, we developed a new platform to analyze chromosome copy number using NGS.

1. Introduction

Candida albicans is a member of the flora in humans, but it becomes an opportunistic fungal pathogen for the immune-compromised, such as patients with acquired immunodeficiency syndrome (AIDS), cancer patients undergoing chemotherapy, and patients receiving intestinal surgical intervention (Jones et al., 2004; Poulter, 1987). *C. albicans* accounts for 50–60% of invasive fungal infections in humans (Ruan and Hsueh, 2009). *C. albicans* is considered to be an obligate diploid yeast and its meiosis and sexual organs have not been identified. The *MTL* (Mating Type-Like) locus was identified in 1999 (Hull and Johnson, 1999). The opaque cells were homozygous for the *MTL* gene and opaque phase cells and colonies were readily identified on agar containing 5 ppm phloxine B to be stained red (Anderson and Soll, 1987; Lockhart et al., 2002). The mating behavior between diploids of opposite mating type produces tetraploid cells that subsequently undergo 'concerted chromosome loss' to become diploid cells. The parasexual cycle provides a potential way to enhance the evolution of *C. albicans*, although it does not easily occur in nature (Bennett and Johnson, 2003; Forche et al., 2008). Moreover, a recent study demonstrated that *C. albicans* has a viable mating-competent haploid state derived from diploid cells and that the highly heterozygous *C. albicans* genome contains recessive alleles that are detrimental but are not necessarily lethal. Similar to the chromosome number reduction that occurs in tetraploids, diploid *C. albicans* undergoes 'concerted chromosome loss' to become haploid, which facilitates the elimination of lethal alleles from the population (Hickman et al., 2013). In this previous study, one of the haploids, the progenitor of Haploid IV, and an autodiploid strain were the isolates that were obtained from the small colonies growing in the presence of fluconazole in our laboratory.

Abbreviations: LOH, loss of heterozygosity; NGS, next-generation sequencing; SNP-RFLP, single nucleotide polymorphism-restriction fragment length polymorphism; INDEL, INsertion/DELetion; CAHCA, Comparative Allele Haplotyping for *C. albicans*; RD_{mean}, mean depth of reads; SNV, single nucleotide variation; MNV, multiple nucleotide variation.

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Fluconazole, one of the most commonly used antifungal agents, inhibits ergosterol biosynthesis in *C. albicans* but also in other yeasts and thus causes cell membrane instability. Clinical isolates with resistance to fluconazole were more commonly found to be *MTL* homozygotes in comparison with cells without fluconazole resistance (Rustad et al., 2002). *C. albicans* developed resistance to fluconazole under long-term serial propagation under fluconazole stress (Cowen et al., 2000). In further studies, fluconazole was also found to be one of the stressor that induced *C. albicans* to change its chromosomes, including loss of heterozygosity (LOH) of genes, and produced an isochromosome of the left arm of chromosome 5 (i5L) and aneuploid cells (Selmecki et al., 2006, 2008).

In this study, using a regular fluconazole E-test assay, the reference strain SC5314 was exposed to fluconazole, and morphologically distinct, small colonies were generated in the inhibition zone. These cells obtained from the small colonies were spread onto phloxine B-containing plates. The purpose of this study was to detect whether the phenotypic change in the *C. albicans* strains is correlated with a genotypic alteration. In the initial of screening step, *MTL* homozygote strains were selected by PCR for *a* and α1/α2 alleles, and then these selected strains were tested for single nucleotide polymorphism-restriction fragment length polymorphism (SNP-RFLP) to determine whether the LOH of other alleles marked on all eight chromosomes occurred after fluconazole treatment. The associated DNA content was analyzed by flow cytometry, and the diversity of morphological phenotypes was shown by growing the isolated strains on Spider agar.

The nucleus of *C. albicans* is extremely small; therefore, chromosome number counting by conventional Giemsa staining for karyotyping becomes impractical. Currently, accurate chromosome copy number determination relies on SNP/CGH array and combined flow cytometry analysis of the DNA content (Abbey et al., 2011). However, single nucleotide polymorphism (SNP) detection and INsertion/DELetion (INDEL) by reads coverage are widely analyzed in next-generation sequencing (NGS) combined with bioinformatics of the human genome and other species (Lindner et al., 2012; Qi et al., 2009). With the increasing popularity and decreasing cost of NGS, the use of NGS for the analysis of ploidy and haplotype mapping becomes feasible. Therefore, we employed this new approach to SNP analysis instead of using CGH arrays because the highly heterozygous diploid genome sequence of *C. albicans* has been established since 2004 (Jones et al., 2004; Legrand et al., 2008). In this study, the chromosome copy number was estimated by SNP detection, and the reads were determined using NGS. The haplotype mapping of each chromosome for the isolated strains was established with a multiple alignment tool, namely CAHCA (Comparative Allele Haplotyping for *C. albicans*), which was developed based on a database of the diploid sequence.

2. Materials and methods

2.1. C. albicans strains and isolates

The strains used in this study are listed in Table 1. The *C. albicans* reference strain SC5314 was used for its complete genome sequence (Jones et al., 2004). *C. albicans* T2-FL, T2-L3, T4-F2, T4-L, T5-F1, T6-N6, T7-R2, LN208B-LS, and 17-1-LS strains were derived from strain SC5314, and strains U30-N1, U37-R2, U46-N1, U46-L1, and U46-L2 were derived from CAI4 after treatment with fluconazole.

2.2. Fluconazole-induced assay and strain purification

The fluconazole-induced assay was performed by treating strains SC5314 and CAI4 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^{-1} phloxine B) for 12–16 h at 30 °C. In this period, the inhibition zone was formed (Fig. 1A), and peapod-shaped elongated cells in the inhibition zone were observed using a microscope (Fig. 1B). Phloxine B was added to distinguish viable cells; the dead cells were stained red, and the viable cells remained unstained. The unstained peapods usually containing four cell compartments were rescued from the fluconazole plate and seeded onto fresh PB-YPD plates by a micromanipulator (Narishige, Japan) under an inverted microscope (Olympus IMT2, Japan). The rescued peapods were incubated at 25 °C for 8–12 h for germination. At this stage, new cells emerged from each compartment of the peapod (Fig. 1C), and separation of the emerging single cells was again performed using a micromanipulator. From each peapod, approximately 10–20 single cells were isolated. The individual strains derived from a single cell developed into morphologically distinct colonies that appeared to be different from the parental strain SC5314, especially in the initial stage (Fig. 1D). For each isolate, further purification was required for 2–3 repeats until all initial colonies reached stable morphological homogeneity (Fig. 1E).

2.3. Initial colony morphology

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

2.4. Single-cell colony morphology

Each strain was diluted to 100–500 cfu mL^{-1} and was plated to yield 5–30 colonies on a single Spider agar plate at 30 °C. After 3 and 7 days, each colony was photographed with a Nikon SMZ800 stereoscope microscope. The images were captured by a digital CCD camera (Pixera Penguin 150 CL, Pixera Corp., Los Gatos, CA) (Homann et al., 2009).

2.5. Genomic DNA extraction

Strains were inoculated in YPD broth overnight at 30 °C, and the cells were harvested by centrifugation for 10 min at 5000 rpm. Yeast genomic DNA was extracted using a reagent genomic DNA kit (Geneaid) and a DNA purification kit (Geneaid).

2.6. PCR analysis of the Mating Type-Like (MTL) loci

We used 30 ng of pure genomic DNA. The *MTL*a1, *MTL*α1, and *MTL*α2 primer sequences used in this study were the same as the sequences used in the study by Rustad et al. (2002). Briefly, reaction mixtures were typically heated for 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 60 °C, and 1 min at 72 °C. After a final incubation period of 10 min at 72 °C, we stored the reaction mixtures at 4 °C. The PCR fragments were resolved by electrophoresis on a 1.5% agarose gel in 1× TAE buffer (BIO-RAD) at 100 V for 30 min and stained with SYBR Safe DNA gel stain (Invitrogen).

2.7. 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 h in YPD agar and prepared by fixing in 70% ethanol at 4 °C. The cells were washed and suspended in 1 mL PBS, sonicated on low power, and then resuspended in 0.5 mL of 2 mg/mL RNaseA solution and incubated