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# Significance of hyphae formation in virulence of *Candida tropicalis* and transcriptomic analysis of hyphal cells

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ABSTRACT

Recently, the proportion of *Candida tropicalis* in clinical isolates has significantly increased. Some *C. tropicalis* strains colonize the skin or mucosal surfaces as commensals; others trigger invasive infection. To date, the pathogenicity of *C. tropicalis* has not been thoroughly researched. This study reports several virulence factors, including biofilm and hyphae formation, proteinase, phospholipase, lipase and hemolytic activity, in 52 clinical isolates of *C. tropicalis* collected from five hospitals in four provinces of China. Some *C. tropicalis* tended to produce more hyphae than others in the same circumstance. Six *C. tropicalis* strains with different morphologies were injected into mice via the tail vein, and the survival proportions and fungal burdens of the strains were evaluated. Hyphal production by *C. tropicalis* was associated with stronger virulence. RNA sequencing revealed that *C. tropicalis* with more hyphae up-regulated several genes involved in morphological differentiation and oxidative response, including IF2, Atx1, and Sod2. It appears that hyphal formation plays a vital role in the pathogenicity of *C. tropicalis*, and interacts with the oxidative stress response to strengthen the organism's virulence.



## 1. Introduction

*Candida* species have progressively become an important opportunistic pathogen, causing invasive infection and several diseases in immunocompromised patients, especially those with cancer or hematological malignancies (Gamaletsou et al., 2014; Pfaller and Diekema, 2007). Although *Candida albicans* remains the most common cause of *Candida* infections, the morbidity and mortality of infections by other *Candida* species has increased significantly over the past two or three decades (Pfaller et al., 2014; Wisplinghoff et al., 2014). Among the non-*albicans Candida*, the strongly pathogenic species *Candida tropicalis*, which is well-known for its high resistance to azole antifungals, ranks first or second in isolation rate (Kothavade et al., 2010).

Pathogenesis of *Candida* species is a three-step process of adhesion, invasion and cell damage (Hebecker et al., 2014), mediated by virulence factors such as hyphae and biofilm formation, and extracellular enzyme activity (Mayer et al., 2013). Biofilm, which often establishes on the surfaces of implanted devices such as urinary catheters, venous catheters and dentures, forms a barrier to the diffusion of antifungals (Cuéllar-Cruz et al., 2012). Aspartic proteinases are secreted onto the surfaces of the fungal cell walls, facilitating tissue invasion in disseminated infections (Kumar et al., 2015). Phospholipase helps to maintain the function of the fungal cell membrane, and contributes to the invasion of host tissue by hydrolyzing the ester linkages in glycerophospholipids (Schaller et al., 2005). Lipase promotes the survival of fungal cells in macrophages and mitigates the inflammatory response of the host (Bader, 2014). Furthermore, the fungal cells secrete hemolysin, which lyses red blood cells, and sequester the iron to facilitate hyphae invasion and establish disseminated infection in the host (Chin et al., 2013). Along with these virulence factors, the morphologic transitions and hyphae formation of *Candida* species are attracting increasing attention. *C. tropicalis* exhibits three main morphologies; yeast, pseudohyphae and hyphae. However, although morphologic transitions have been widely investigated in *C. albicans*, similar studies in *C. tropicalis* are limited.

Hyphae formation of *Candida* species is triggered by multiple environmental cues, including (but not limited to) serum, *N*-acetylglucosamine, neutral pH, high temperature, and nutrient starvation (Mayer et al., 2013; Yang et al., 2014). Hyphal initiation is negatively regulated by the Nrg1 gene, dependent on the cAMP-PKA pathway (Thompson et al., 2011), whereas the elongation and maintenance of hyphae is mediated by hyphal-specific transcription factors such as Brg1, Ume6 and Hgc1 (Lackey et al., 2013). The stronger cell–cell and cell–surface adhesion capability of *C. albi-*

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*cans* was consistent with its overexpression of Als3, Ssa1 and Hwp1 (Sherry et al., 2014). During its yeast-to-hyphae transition, *C. albicans* secretes several extracellular enzymes (Soll, 2014; Yang et al., 2014). In *C. tropicalis*, hyphae and biofilm formation are regulated by the Wor1 gene, which also regulates the white–opaque switch (Porman et al., 2013). Chen et al. (2014) demonstrated that calcineurin can control the hyphal growth and virulence of *C. tropicalis*. Oxidative stress can also trigger morphogenesis in *Candida* species (Dantas et al., 2015; Schröter et al., 2000). Eukaryotic pathogens rapidly encounter superoxide radicals ($O_2^-$) from several sources. $O_2^-$, the byproduct of electron transport (mainly generated in mitochondria or chloroplasts) (Chauhan et al., 2006), is known as a reactive oxygen species (ROS). Antioxidant enzymes such as superoxide dismutase catalyze $O_2^-$ into $H_2O_2$ and $O_2$, and strengthen the fungal resistance in an oxidative environment (Li et al., 2015; Dantas et al., 2015).

In this study, we evaluated the properties of 52 clinical isolates of *C. tropicalis*, and investigated the virulence of the strains with different morphogenesis in a mouse model. The genes associated with *C. tropicalis* pathogenicity were then identified by RNA-Seq.

## 2. Material and methods

### 2.1. Strains and medium

Fifty-two clinical isolates of *C. tropicalis* were collected from five hospitals in Shanghai, Jiangsu, Guangdong and Anhui Provinces in China. All of the isolates were maintained by biweekly passages on yeast peptone dextrose (YPD) agar containing 10 g/l yeast extract, 21 g/l peptone and 21 g/l dextrose.

### 2.2. Biofilm formation and quantification assay

Biofilms were produced in sterile polystyrene 96-well tissue culture plates (Corning, NY, USA) as previously recorded (Alnuaimi et al., 2013). Briefly, $5 \times 10^6$ yeast cells were two-fold diluted with YPD medium, and 200-μl cell suspensions was added to each well of the culture plates. The plates were incubated at 37 °C for 2 h in an orbital shaker. After the adhesion phase, the cell suspensions were removed and each well was washed twice with PBS. Finally, 200 μl YPD medium was added to each well, and the plates were again incubated at 37 °C for 48 h with gentle shaking to form biofilms. A blank control with YPD medium alone was prepared in parallel, and every isolate of *C. tropicalis* was detected in triplicate. After incubation for 48 h, the YPD medium was removed and each well was co-cultured with 200 μl methanol for 15 min. Biofilms were dyed by 0.2% crystal violet for 5 min, then each well was rinsed three times with PBS. After destaining with 33% acetic acid for 3 min, the plates were spectrophotometrically determined on KHB ST-360 Microplate Reader (KeHua Biology, Shanghai, China) at 570 nm.

### 2.3. Hyphae formation and assay

*Candida tropicalis* was cultured overnight in 5 ml YPD with 200 rpm shaking at 30 °C. The cultured cells were washed twice with PBS and the $OD_{600}$ was adjusted to 1.0. Hyphae of *C. tropicalis* were induced in 24-well plastic cell culture plates (Corning, NY, USA) containing 1 ml RPMI 1640 medium (Gibco, NY, USA) supplemented with 10% Fetal Bovine Serum (Gibco, NY, USA). A total of $5 \times 10^6$ *C. tropicalis* cells (20 μl) were inoculated in this inducing medium at 37 °C for 12 h. After Gram staining, their hyphae formations were observed under light microscopy at 100× magnification.

### 2.4. Proteinase activity assay

Proteinase activity was detected as reported in Bramono et al. (2006). Briefly, 0.2 ml of the culture filtrate was incubated with 3.0 ml of 0.1 M sodium acetate buffer (pH 4.0) containing 5 mg/ml of BSA. *Candida* cells were cultured at 37 °C for 48 h and the reaction was terminated by adding 500 μl 10% trichloroacetic acid. After centrifugation, the proteinase activity was assayed by the Lowry method at 750 nm. Each isolate was performed in duplicate.

### 2.5. Phospholipase, lipase and hemolytic activity assays

Phospholipase, lipase and hemolytic activities were estimated as previously described (Samaranayake et al., 1984; Silva et al., 2011; Deorukhkar et al., 2014). Briefly, 5 μl ($5 \times 10^6$) *C. tropicalis* was inoculated onto respective plates, and incubated at 37 °C for 48 h. The expression of phospholipase and lipase enzymes and hemolysin production were identified by a precipitation zone. The phospholipase activity (Pz), lipase activity (Lz) and hemolytic activity (Hz) were calculated as the ratio of the colony diameter to the total diameter of the colony plus precipitation zone (mm). The lower the ratio, the greater the enzyme production. Each isolate was performed in duplicate.

### 2.6. In vivo *virulence studies*

The *in vivo* virulence studies were performed on six- to eight-week-old female BALB/c mice from Shanghai SLAC Laboratory Animal Cooperation. *Candida tropicalis* cells harvested from the abovementioned hyphae-inducing medium were washed twice with 5 ml PBS, then resuspended in 2 ml of PBS. Three days before infection, all mice were intraperitoneally injected with 200 mg/kg cyclosporine. For each strain, three mice were injected via the tail vein with 200 μl ($5 \times 10^6$) yeast and monitored daily for survival over 15 days. Moribund mice were euthanized with $CO_2$. Another three mice per strain were similarly infected and dissected after 6 days. The fungal burdens of the liver, kidney and spleen were analyzed by quantitative culture as previously described (Chen et al., 2014). Briefly, the organs were weighed and homogenized for 10 s at 13,600 rpm/min (Branson Inc., Shanghai, China). The tissue homogenates were serially diluted and plated (100 μl) onto YPD. After incubation at 30 °C for 48 h, the CFUs were counted and their viability was assessed.

### 2.7. RNA-Seq

The RNA library construction and sequencing were performed at Shanghai Biotechnology Cooperation. The cDNA libraries were constructed following the TruSeq™ RNA Sample Preparation Guide (Illumina, San Diego, USA). Briefly, the total RNA was isolated from the abovementioned hyphae-inducing medium using the Yeast RNAiso Reagent kit (Takara, Tokyo, Japan) and the mRNA was isolated with the Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany). The mRNAs were fragmented by incubation in Elute, Prime, Fragment Mix at 9 °C for 8 min, yielding 120- to 200-bp inserts. First-strand cDNA was synthesized with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, USA) using random primers. Double-stranded cDNA was synthesized in Second Strand Master Mix and isolated by Ampure XP beads. The adapter was ligated to the A-Tailing fragment, and the DNA fragments with their adapter molecules at both ends were enriched through 12 cycles of PCR. The purified library was quantified by Qubit® 2.0 Fluorometer, and the insert size was validated by an Agilent 2100 Bioanalyzer. A cluster was generated by the cBot Clonal Amplification System, and then sequenced on the Illummina HiSeq 2500.