LETTER TO THE EDITOR

Phenotypic switching of Candida tropicalis is associated with cell damage in epithelial cells and virulence in Galleria mellonella model

Alane T.-P. Moraleza, Hugo F. Perinia, Luciana Furlaneto-Maiab, Ricardo S. Almeida, Luciano A. Panagiao, and Marcia C. Furlanetoo

aDepartment of Microbiology, Paraná State University of Londrina, Londrina, Paraná, Brazil; bTechnological Federal University of Paraná, Londrina, Paraná, Brazil

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Candida species are the most common causes of human fungal infection worldwide. The pathogenic status of Candida spp. is associated with modulation of virulence determinants in response to environmental changes, and impairment of host defenses.

Among Candida putative virulence factors, phenotypic switching is associated with fungal adaptability to environmental changes during invasion of host organism. Switching is a biological event associated with generation of phenotypic heterogeneity that occurs in a small fraction of the population, is random, reversible and represents an epigenetic state. In yeast, phenotypic switching drives variable changes, leading to the emergence of colonies with altered morphologies.

Our group has previously described a high-frequency switching of colony morphology in several clinical isolates of Candida tropicalis. The isolates could switch spontaneously, heritably and reversibly between at least six different phenotypes, not including the white-opaque transition. One of the identified switching system that include three colony phenotypes: smooth (parental phenotype) and two related switch variants (crepe and rough) was associated with in vitro changes in virulence traits, including biofilm formation, morphogenesis and hemolysis, as well as switching from itraconazole susceptibility to resistance.

Soll et al. were the first to assess switch phenotypes in C. tropicalis during the course of a prolonged Candida infection in a compromised host. Further, a white-opaque phenotypic switch was described for C. tropicalis that shows some similarities to that in Candida albicans. Although these studies have begun to explore the mechanism of phenotypic switching in C. tropicalis, the role of switching in this species remains an open question. Porman et al. demonstrated that a white-opaque phenotypic switching in C. tropicalis regulates a cryptic program of sexual mating, potentially giving rise to strains with increased virulence. The white-opaque transition also is associated with sexual biofilm formation in C. tropicalis, where biofilms are formed exclusively by opaque cells.

C. tropicalis together with the majority of the medically relevant Candida species belongs to the CTG clade and is genetically close to C. albicans. The frequency of infections caused by C. tropicalis has increased over the last years, particularly in tropical regions in some settings, bloodstream infections due to C. tropicalis have been associated with higher mortality than other Candida species.

In the present study, we address for the first time in the literature, if phenotypic switching affects virulence in C. tropicalis. To this end, we employed all three colony phenotypes (smooth, crepe and rough) described previously and revertants (strains that switched back from variant to parental phenotype) for the evaluation of the capacity of damaging epithelial cells and virulence in Galleria mellonella. The switched strains (crepe and rough variants) were obtained as individual subclones from a clinical isolate exhibiting a smooth colony dome. The crepe and rough variants exhibited an irregular and structured dome surface and were obtained at frequencies of $5 \times 10^{-3}$ and $3 \times 10^{-2}$, respectively.

In the present study, we initially determined the reversibility of crepe and rough variants phenotypes to the smooth phenotype (parental phenotype) by visually scoring of four days old growth colonies on YPD agar medium (Gibco) at 28°C. To calculate the reversibility frequencies, a total of 6,000 colonies from each variant

CONTACT Marcia C. Furlaneto furlaneto@uel.br Department of Microbiology, Paraná State University of Londrina, Paraná, C.P. 6001, 86051990, Brazil. Color versions of one or more figures in this article can be found online at www.tandfonline.com/kvir.
were scored. Colonies that arose from variants and exhibited morphology similar to the parental phenotype (smooth pattern) were considered revertant colonies. Successive culture of revertant colonies on YPD medium for up to 10 times was used to assess the stability of the phenotype.

To verify the ultrastructure of strains colonies, whole yeast colonies were removed from YPD agar plates after an incubation period of 4 d using a scalpel blade. Colonies were fixed for approximately 18 h at 4°C in 3.5% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M phosphate buffer, pH 7.2. They were then immersed in liquid nitrogen for 30 sec and freeze-dried for 2 h at 0.12 mbar MPa (Alpha 1-2 LD plus). Then, colonies were coated with gold (BALTEC SDC 050 Sputter Coater) and viewed in a FEI Quanta 200 Scanning Electron Microscope at 30kV.

The degree of cell damage caused by C. tropicalis parental, switch and revertant strains during interaction with monolayers of FaDu (ATCC® HTB-43™; human epithelial cell line, from a squamous cell carcinoma of the hypopharynx) cells was evaluated quantitatively through the measurement of lactate dehydrogenase (LDH) activity. FaDu cells were grown on RPMI 1640 medium (Difco) with serum 10% until 95% confluence in 24 well culture plates. Subsequently this cell was infected with 1×10⁶ cells in RPMI 1640 (Difco) in the absence of serum. For control samples, FaDu were incubated with RPMI 1640 (Difco) medium only. Additionally cells of the C. tropicalis strains were incubated without epithelial cells. After 24 h extracellular LDH release into medium was measured spectrophotometrically at 490 nm using a LDH Cytotoxicity Assay Kit (Cayman Chemical Company, www.caymanchem.com). The LDH activity of Candida cells in cell culture medium devoid of tissue was subtracted from the LDH activity of the tissue infected with yeasts. The assays were performed according to the manufacturer instructions and the measurements were performed in triplicates for each condition and repeated twice.

Galleria mellonella survival assays were carried out as described previously by Mylonakis et al. Briefly, groups of 10 larvae (0.3–0.6 g) were each infected with 10μl yeast cells suspensions at a final inoculum concentration of 1×10⁶, 2×10⁶, 6×10⁶ and 1×10⁷ yeast cells per larvae. The yeast cells were directly instilled into the haemocoel of the larvae by injection with a 10 μl Hamilton syringe in the last right proleg. The larvae were incubated at 37°C after inoculation, and survival was monitored every day by 240 h with intervals of 12 h.

For the analyzes of the effect of switching on virulence, C. tropicalis parental, switch and revertant strains were used to infect G. mellonella larvae at concentration of 2×10⁶ yeast cells per larvae. The assay was carried as described above. The larvae were considered to have died when they did not respond to physical stimulation (slight pressure with forceps). As controls, a group of larvae injected with PBS were studied in parallel in every infection investigation. Each experiment was repeated at least three times.

Fungal burden was determined by colony-forming unit (CFU) counts at 12 post-infection. Groups of 10 larvae were infected with 2×10⁶ cells per larvae of each strain and incubated at 37°C. Three larvae infected with each strain were selected and macerated. A volume of 1 ml of PBS ampicillin [200 mg/ml] was added and the mix was gently homogenized in a vortex with glass beads for 3 minutes. Different dilutions were made for each sample and 50 μl of these dilutions were placed on YPD-chloramphenicol agar plates [50mg/ml]. The plates were incubated at 37°C for 96 h and the number of colony forming units (CFUs) was determined.

Differences in cell damage potential and fungal burden were estimated using Student’s t-test. P value ≤ 0.05 was considered significant. Killing curves were plotted and estimation of differences in survival (log rank test and Wilcoxon tests) analyzed by the Kaplan-Meier method using Graph Pad Prism 3 software (La Jolla CA, USA). P value < 0.05 was considered significant.

We obtained spontaneously revertant strains from both switched variants. The crepe variant exhibited higher reversibility rate (5.2×10⁻¹) than that observed for rough variant (2.2×10⁻²). Reversibility rates were determined by dividing the number of colonies exhibiting parental phenotype by the total number of counted colonies (total of 6,000). Revertant colonies from crepe variant were named revertant of crepe (RC) and from rough variant were named revertant of rough (RR). Both revertants colonies exhibit high morphological similarity to the smooth phenotype (Fig. 1A, J, M), although RC colonies display slight variation in morphology, including less bright surface and slightly irregular edges (Fig. 1J).

Unlike the parental phenotype that exhibit smooth dome colony both switched variant strains exhibit more structured colonies morphologies (Fig. 1D, G). The ultrastructure of these colony phenotypes is presented in Figure. 1E, H. The crepe variant was characterized by the presence of a deep central depression with a highly wrinkled surface and an irregular periphery (Fig. 1E). The rough phenotype exhibit more complex architecture and is characterized by the presence of bundles of hyphae converging together and peripheral depressions areas (Fig. 1H). On the other hand, the parental phenotype and revertants strains (RC and RR) display a typical smooth colony phenotype with a flatly convex profile (hemispherical shape) (Fig. 1B, K, N).
Figure 1. Representative colonies of five phenotypes from isolate 49.07 of C. tropicalis (A) parental smooth colony; (D) crepe colony; (G) rough colony; (J) revertant of crepe (RC) and (M) revertant of rough (RR) were photographed using a stereoscopic microscope (Nikon SMZ-745). Scanning electron micrographs showing the microarchitecture of colonies following 96 h incubation on YPD agar at 28°C: (B) electron micrograph of the parental smooth colony; (E) electron micrograph of crepe variant colony; (H) electron micrograph of rough variant colony; (K) electron micrograph of the revertant of crepe colony and (N) electron micrograph of revertant of rough colony. Electron micrographs of cells types: (C) cells from parental smooth colony; (F) cells from crepe variant colony; (I) cells from rough variant colony; (L) cells from revertant of crepe colony and (O) cells from revertant of rough colony. White bar: 2 mm (A,D,G,J,M); 1 mm (B,E,H,K,N); 20μm (C,F,I,L,O).
The ultrastructural analysis also allowed the observation of the arrangement of individual cells within the colonies. After 4 d of colony development, colonies presenting smooth-pattern (parental and revertants strains) phenotype consist entirely of budding yeast cells (Fig. 1C, L, O). In contrast, cell morphologies in colonies of the switched variants consist of budding yeast cells and filamentous forms (hyphae and pseudohyphae) (Fig. 1F, I).

Analysis of cell-damaging potential of the switch variants (crepe and rough) and their revertants (RC and RR) in comparison to parental strain (isolate 49.07) is shown in Figure 2. All strains of the switching system showed detectable cellular damage. The crepe variant promoted greater cellular damage than its parental counterpart \( (P = 0.00002) \). Similarly, the revertant of crepe (RC) exhibited higher citotoxicity than that observed for the parental strain \( (P = 0.0000005) \), despite of its smooth-like colony morphology. On the other hand, the rough variant and its revertant strain (RR) showed the same extent of cytotoxicity to FaDu cells than parental strain.

We also aimed to compare in vivo virulence between switch strains and parental strain using the invertebrate model G. mellonella. To this end, we first assessed the pathogenicity of the parental strain in G. mellonella larvae. As shown in Figure 3, there was a dose-dependent effect on the killing of the insect. Larvae injected with PBS (control) did not die during the time of the experiment (6 days), presenting 100% survival. With the lower inoculum concentration \( (1 \times 10^6 \text{ cells per larvae}) \), the median survival time was 66 h post-infection. When the inoculum concentration was increased to \( 2 \times 10^6 \) and \( 6 \times 10^6 \text{ cells per larvae} \), 50% survival time decreased to 24 h after infection. With the higher inoculum levels of \( 1 \times 10^7 \text{ cells per larvae} \), the median survival time was 12 h post-infection, demonstrating that this concentration was significantly more lethal than the lower concentrations tested. At this inoculum concentration 100% of infected larvae were dead at 24 h post-infection. Therefore, inoculums of \( 2 \times 10^6 \text{ cells per larvae} \) were used in subsequent trials. As shown in Figure 4, the crepe variant was more virulent than the parental strain (49.07 isolate) \( (P = 0.0001) \). Besides, this phenotype was also more virulent than the rough variant \( (P < 0.0001) \). On the other hand, the rough variant was less virulent compared to the parental strain \( (P = 0.0001) \). Thus, the order of virulence observed from highest to lowest was crepe>smooth>rough cells. We observed no differences in larvae mortality between revertant of crepe (RC) and the parental strain \( (P = 0.6158) \) exhibiting a medium survival time of 36h. Differently, the revertant of rough (RR) was less virulent compared to the parental strain \( (P = 0.0154) \) (Fig 4). The virulence of the RR did not

![Figure 2. Lactato dehydrogenase (LDH) measured in the epithelial cell (FaDu) culture supernatant after 24 h incubation with C. tropicalis strains. Control, monolayers preincubated in normal cell culture medium (RPMI 1640 medium with 10% FBS); Parental; switch variants (Crepe and Rough) and respective revertants (RC and RR) were incubated in normal cell culture medium (RPMI 1640 medium). The experiment was performed in triplicates for each condition and repeated twice. Significant differences between parental and switch strains (variants and revertants) were determined by the Student’s t –test \( (P \leq 0.05) \).](image-url)
differ to that observed for its correspondent rough variant ($P = 0.2575$), in spite of its smooth-like colony morphology. In all infected larvae with different strains, a typical dark color due to accumulation of melanin was observed after infection. In contrast, there was no melanization in larvae injected with PBS (control) (data not shown).

Fungal burden was assessed in addition to virulence. At 12 h post-infection, the number of CFUs recovered from infected larvae varied among strains. As shown in Figure 5, larvae infected with crepe variant and its revertant (RC strain) exhibited higher fungal burden than that observed for parental strain ($P = 0.05$ for crepe variant and $P = 0.01$ for RC strain). Infected larvae with crepe and RC strains had increased CFUs by approximately 6.1 and 3.5-fold, respectively. On the other hand, larvae infected with rough variant and its revertant (RR strain) showed no statistical difference in fungal burden in relation to parental strain. At 24 h post-infection, 100% of larvae-infected with crepe variant were dead, making unfeasible comparative analysis of fungal burden at this infection time.

Phenotypic switching is a putative Candida virulence factor most extensively examined in C. albicans, where pleiotropic effects on virulence have been described.\textsuperscript{17-19} Differently, information regarding the influence of phenotypic switching on C. tropicalis virulence is largely unexplored to date. Reversibility is a trait of phenotypic switching event where variants can revert to the parental phenotype.\textsuperscript{3,20} Although we have previously evaluated the microstructure of switched colonies of C. tropicalis,\textsuperscript{4,21} in the present study, analysis of revertants colonies at ultrastructural level was reported for the first time. Colonies of revertants strains exhibited a smooth pattern consisting of budding yeast cells, differently to the structured morphology presented by their counterpart switched strains where filamentous growth was marked (Fig. 1). These data raise the hypothesis of a possible association between filamentation and formation of the architecture of switch phenotypes in C. tropicalis.

To date, there are few studies regarding the pathogenic potential of non-albicans Candida species using human cell lines as alternative model of ex vivo analyses.\textsuperscript{22,23} According to the literature, C. tropicalis has ability to adhere and proliferate into different epithelial cell
lines, invading and damaging these cells. In the present study, the employment of human epithelial cell line (FaDu) revealed to be a useful tool for the analysis of virulence of related phenotypic switching-derived strains. One of interesting finding was that the revertant of crepe exhibited higher cytotoxicity than that observed for the unswitched parental, despite its smooth-like colony phenotype. This result suggests that although there are similarities between colonies morphologies of parental and revertant of crepe, probably there are fundamental differences between the transcriptional programs in the two strains. The epigenetic state (switched state) of these strains may be associated with distinct physiological status and virulence traits that are independent of their colony phenotype.

Compared to C. albicans, relatively few investigations have been performed to assess in vivo virulence of C. tropicalis. It has already been showed that C. tropicalis can kill the lepidopteran G. mellonella larvae, which offers a useful tool to study its virulence. In the present study, with inoculum concentration of $2 \times 10^6$ cells per larvae a specific virulence-strain was observed where the crepe variant infection exhibited faster rate of killing and higher fungal burden compared to the remaining strains of the switching system. Taken together, our data revealed that the crepe switch variant exhibit higher cytotoxicity to FaDu cells and virulence in G. mellonella in comparison to its parental counterpart (unswitched strain). These data may reflect differential expression of virulence traits by this switched strain. In previous study, we reported that the crepe variant colonies had increased percentage of filamentous growth forms; besides, the crepe variant formed higher amount of biofilm and increased MIC to the antifungal itraconazole compared to that observed for its unswitched parental (original smooth). These altered traits could potentially affect virulence. Of note, in the present study, the employment of revertants strains raised interesting data. For instance, although the colonial morphology of the revertants resembles the original phenotype of the parental strain (smooth dome), virulence of revertants was either found to be at the same extent of that observed for their switch-variants counterparts or was restored to the level observed for the parental strain, depending on the virulence model tested. In addition, although no differences in the capability to kill G. mellonella larvae were detected between parental strain and the revertant of crepe, the latter exhibited higher fungal burden. These data suggest the occurrence of differences in their proliferation capabilities within the host, possibly as a result of their distinct transcriptional programs.

In conclusion, we report that phenotypic switching in C. tropicalis is associated with changes in virulence. Although revertants strains exhibit a parental smooth-

**Figure 5.** Fungal burden in G. mellonella infected with C. tropicalis strains. Galleria mellonella larvae were infected with $2 \times 10^6$ cells per larvae of smooth (original parental), crepe variant, rough variant; RC (revertant of crepe) and RR (revertant of rough), and CFUs recovered from G. mellonella after 12 h of infection. Fungal burden was quantified from pools of three homogenized larvae. Significant differences between parental and switch strains (variants and revertants) were determined by the Student’s t-test ($P \leq 0.05$).
like colony phenotype, the level of virulence and fungal burden may be the same of that observed for their corresponding switch variants. This is the first report of the employment of the *G. mellonella* infection model for the analyses of virulence of related strains belonging to a phenotypic switching system in *Candida* species. Further studies on the molecular basis of this phenotypic switching system and its role into host adaptation will be important in search of new strategies to manage infections caused by *C. tropicalis*.

**Disclosure of potential conflict of interest**

No potential conflicts of interest were disclosed.

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