Deciphering Colonies of Phenotypic Switching-Derived Morphotypes of the Pathogenic Yeast Candida tropicalis

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Abstract
Background Phenotypic switching generates fungal colonies with altered morphology and allows pathogens to adapt to changing environments.
Objective This study investigated the structure and genetic factors of switched morphotypes colonies in Candida tropicalis.
Methods Morphotypes of C. tropicalis comprised the clinical strain 49.07 that exhibited smooth colony phenotype and switched (crepe and rough) morphotypes that showed colonies with marked structural variations, including wrinkled surface, depressions areas, and irregular edges (structured morphology). The morphotypes were analyzed for the presence and distribution of the extracellular matrix (ECM) at the ultrastructural level-SEM. The composition of the ECM and the percentage of hyphae in colonies were evaluated. The expression of EFG1 (Enhanced filamentous growth protein 1), WOR1 (White-opaque regulator 1), and BCR1 (Biofilm and cell wall regulator 1) in the morphotypes was measured by RT-qPCR.
Results Colonies of the switched variants exhibited distinct arrangements of ECM compared to the smooth phenotype (clinical strain). In addition, rough variant colonies showed higher amounts of total carbohydrates and proteins in ECM (p < 0.05). Switched (crepe and rough) colonies exhibited a higher percentage of hyphae throughout their development (p < 0.05). The mRNA expression levels of EFG1, WOR1, and BCR1 in the rough morphotype were significantly higher than they were in the smooth morphotype. In addition, there was a positive correlation between the expression of these genes and filamentation (hyphae formation) of the rough morphotype (r² > 0.9472, p < 0.05).
Conclusion Structural variations in switched morphotypes colonies of C. tropicalis seem to be associated with increased hyphae growth and the amount and distribution of ECM. Switched colonies have distinct expressions of the EFG1, WOR1, and BCR1 master regulators genes.

Keywords Colony structure · Phenotypic switching · Hyphae · Extracellular matrix · Transcription factors
Introduction

*Candida tropicalis* is an opportunistic yeast pathogen with an ability to cause both superficial and systemic infections in humans [1]. This species has been the second most prevalent *Candida* species in bloodstream infections, particularly in tropical regions, promoting high mortality [2–5]. The success of *C. tropicalis* as a human pathogen can be attributed to its vast repertoire of virulence determinants, such as adhesion, morphogenesis, biofilm-forming ability, and secretion of lytic enzymes [1]. *C. tropicalis* is a polymorphic fungus, existing in a unicellular yeast cells form, pseudohyphae, and hyphae [1], where hyphal growth plays a vital role in the pathogenicity of this species [6].

A notable feature of *C. tropicalis* is its ability to undergo phenotypic switching. This phenomenon is associated with a reversible change in colony morphology at rates higher than somatic mutation rates [7]. Phenotypic switching confers plasticity within isogenic populations and enables pathogens to adapt to a constantly changing microenvironment [7–9]. Switching systems that comprise white-opaque and white–gray-opaque transitions, as well as multiple forms of reversible switch phenotypes, were described for *C. tropicalis* [10–15].

Soll et al. [10] first described that *C. tropicalis* possesses a varied repertoire of switch phenotypes. In previous studies we described that phenotypic switching promotes changes in the colony architecture of *C. tropicalis* clinical isolates, leading to the development of colonies with structured morphological patterns exhibiting depressions and elevations areas, wrinkled surface, and irregular edges [11, 14, 16]. Ultrastructural analysis of colonies of structured morphological patterns revealed the presence of high amounts of extracellular matrix (ECM), suggesting a possible role for ECM in *C. tropicalis* switching events [11]. For the yeast *Saccharomyces cerevisiae*, the ability to form an abundant ECM is one of the features typical for colonies with structured architecture [17]. This morphological pattern was characterized as “biofilm-like” due to the presence of relevant amounts of ECM [11, 17]. In addition, colonies of switched phenotypes showing structured morphological patterns were associated with higher percentages of filamentous growth compared to colonies of unstructured (smooth) phenotype [14, 16].

Phenotypic switching can translate into changes in the virulence of the pathogen. Switched variant phenotypes of *C. tropicalis* have altered virulence, showing variations in hemolytic activity, adhesion to biotic and abiotic surfaces, biofilm formation, and recognition by *Galleria mellonella* hemocytes [11, 14, 16, 18, 19].

Although the presence of hyphae and ECM in switch colonies of *C. tropicalis* may be associated with the architecture of these colonies [11, 16], the composition of the matrix and the genetic factors that mediate multiple forms of switch phenotypes, including structured colonies remain unknown. In contrast, molecular mechanisms that regulate the white-opaque and tristable switching system in *C. tropicalis* are reported [12, 15]. Therefore, we evaluated hyphae production throughout colony development, as well as the presence, distribution, and composition (total carbohydrates and proteins) of the ECM in *C. tropicalis* colonies of smooth phenotype and switched phenotypes of structured morphological patterns (crepe and rough). The expression of transcriptional regulatory genes (*WOR1*, *EFG1* and *BCR1*) by colonies cells of these morphological patterns was also investigated.

Materials and Methods

*Candida tropicalis* Morphotypes and Culture Conditions

Morphotypes employed in this study comprise a *C. tropicalis* clinical strain (49.07), obtained from a patient admitted at a tertiary-care hospital at Londrina—Parana State [20], and two switch variants (crepe and rough) that arose from the 49.07 strain [14]. The strain 49.07 exhibited a smooth dome colony with a flatly convex profile (hemispherical shape), and was characterized by colony of “smooth morphology”. The variants (crepe and rough) exhibited marked differences in colony morphologies with wrinkled surface, depressions areas, and irregular edges, and were characterized by colonies of “structured morphology” [16] (Fig. 1, I and II). SEM analysis were made as previously described by our group, using the FEI Quanta 200 Scanning Electron Microscope at 30 kV [16]. These morphotypes were obtained as a stock culture from the Fungal Genetics Laboratory,
The State University of Londrina-Brazil. The morphotypes were stored as frozen stocks with 15% (w/v) glycerol at \(-80^\circ\text{C}\) and cultured on yeast extract-peptone-dextrose (YPD) (DIFCO) agar plates at 28 °C for 96 h.

**Matrix Composition of the Colonies**

The extracellular matrix (ECM) of the colonies was extracted as described by Azeredo et al. [21], with modifications. Prior to the extraction procedure, portions of the colonies were pretreated with glutaraldehyde (GTA). Portions (0.3 g wet weight) of each of the phenotypes were incubated at 4 °C for 3 h and 30 min in 30 ml of glutaraldehyde (1.8% GTA). After this period, the samples were centrifuged at 9000 g for 10 min, resuspended in phosphate-buffered saline (PBS) and centrifuged again. Then the final cell pellet was once again resuspended in PBS. The suspensions were then sonicated for 30′, 1′ and 2′. The tubes were kept on ice during sonication.

Subsequently, the supernatant was filtered (nitrocellulose filter, 0.2 μm) and stored at \(-20^\circ\text{C}\). The cell pellets were freeze-dried to determine the dry weight of cells after extraction of matrix. Portions of 0.3 g (wet weight) of each phenotype (parental and variants) were also freeze-dried to determine the control dry weight (dry weight of cells not subjected to matrix extraction). At the end of the extraction procedure, 30 ml of matrix suspension were obtained. The experiments were performed in triplicate, in three independent experiments.

The total carbohydrate content of the colony ECM was estimated according to Dubois et al. [22], using...
Table 1  *Candida tropicalis* primers used for quantitative PCR (qPCR)

| Sequence (5′–3′)          | Primer | Target | Source       |
|---------------------------|--------|--------|--------------|
| GCCATATGAACCCCAAGTTG      | Forward| BCR1   | This study   |
| AGGTTTGGCAACTGTCCCTG      | Reverse|        |              |
| CCGTCTAAATGTTACCTGCATCAA | Forward| WOR1   | [12]         |
| TTCGTCGTACTTTAGTAAATGTCTTCT | Reverse|        |              |
| TCTACGTGCTGCAACACAC       | Forward| EFG1   | This study   |
| TACCAGGAGGTTGAATTG        | Reverse|        |              |
| GGGACGATATGGAGAAGATCTG    | Forward| ACT1   | This study   |
| CACGCTCTCTGTGAGGATCTTC    | Reverse|        |              |

glucose as a standard. The protein content of the matrix was measured by employing the BCA kit (Bicinchoninic Acid, Sigma-Aldrich, St Louis, USA), using bovine serum albumin (BSA) as a standard. The content of both components was relativized with the dry weight of the colonies.

Determination of the Percentage of Hyphae in Colonies with Different Phenotypes

Throughout colony development, the percentage of hyphae was determined at 48, 72, and 96 h of culture. At each time, three colonies from each morphological group were suspended in PBS (1x) and the percentage of hyphae was determined by direct counting of 1000 cells per experiment in a Neubauer counting chamber under a light microscope (Oleman). Three independent experiments were conducted. Each hyphae was considered 1 multicellular unit.

Gene Expression

*EFG1* (Enhanced filamentous growth protein 1), *WOR1* (White-opaque regulator 1) and *BCR1* (Biofilm and cell wall regulator 1) expression was determined by quantitative PCR (qPCR). Cells of colonies with typical morphology were selected, transferred to microtubes, frozen in liquid nitrogen, and their RNA was extracted using the Trizol-chloroform method (Thermo Fisher Scientific, USA). RNA was quantified and the quality was assessed using a NanoDrop spectrophotometer (ThermoScientific, Loughborough, UK). Complementary DNA (cDNA) was synthesized from 200 ng of extracted RNA using an RT-PCR kit (Invitrogen, Carlsbad, CA, USA) in a GeneAmp® PCR (Eppendorf, Gradient Mastercycler) following the manufacturer’s instructions. The cDNA obtained was stored in a freezer at −20 °C. Primers used for qPCR are described in Table 1.

Each sample was analyzed in duplicate by real-time PCR performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems®). The reaction was performed using Platinum® SYBR® Green qPCR Supermix-UDG (Invitrogen, Carlsbad, CA, USA) with a final volume of 20 µl. Gene expression data were normalized by the expression of the constitutive β-Actin gene (*ACT1*). Once the data were normalized, the relative gene expression levels were analyzed by the equation $2^{-(\Delta \Delta CT)}$.

Statistical Analysis

The paired t-test was used to compare the means, considering statistical significance *p < 0.05, **p < 0.01 and ***p < 0.001, by GraphPad Prism 5 software. Pearson’s correlation, determined by the R statistical software, was used to correlate the variables (p < 0.05).

Results

Evaluation of Extracellular Matrix and Morphogenesis in Smooth and Switched Colonies

As shown in Fig. 1, colonies of smooth morphology (clinical strain) exhibited homogeneous distribution of extracellular matrix, similar to a thin film, covering the cells (Fig. 1—Smooth III). In contrast, colonies of crepe and rough (structured morphology) had a greater amount of ECM. In crepe variant colonies, the matrix was arranged in “lamellae”, filling many of the depressions along the colony surface (Fig. 1—Crepe III). In rough variant colonies, the matrix appeared in a polarized form, arranged in dense networks of
different thicknesses, covering and connecting the cells (Fig. 1—Rough III).

The ECM preparations obtained were evaluated for total carbohydrate and protein content by colorimetric methods. Comparative analyses between phenotypes showed that the total carbohydrate and protein content in rough variant colonies (structured phenotype) (33.74 ± 12.47 mg carbohydrate and 3.47 ± 1.42 mg protein/g colony dry weight) is higher than that observed in the smooth phenotype (clinical strain) (13.75 ± 11.05 mg carbohydrate and 1.11 ± 0.26 mg protein/g colony dry weight) (\(p < 0.05\)). The crepe variant exhibited no differences from the parental strain (\(p > 0.05\)) (Fig. 2a).

To evaluate morphogenesis throughout the growth of switched colonies, the number of hyphae was counted at 48, 72, and 96 h. The results represent the average of three independent experiments with 1000 counted cells each (B).

Expression of WOR1, EFG1, and BCR1 Varied Among Switched Morphotypes of C. tropicalis

The expression levels of transcriptional regulatory genes (WOR1, EFG1 and BCR1) were quantified from colony cells of smooth, crepe and rough morphotypes.

### Table 1

| Morphotypes | Carbohydrate\(^a\) | Protein\(^b\) |
|-------------|---------------------|--------------|
| Smooth      | 13.75±11.05         | 1.11±0.26    |
| Crepe       | 12.50±6.94          | 1.49±0.19    |
| Rough       | 33.74±12.47 *       | 3.47±1.42 *  |

\(a\) (mg carbohydrate/g colony dry weight) and total proteins; \(b\) (mg protein/g colony dry weight) (a). The percentage of hyphae in the phenotypes was evaluated throughout colony development at 48, 72, and 96 h. The results represent the average of three independent experiments with 1000 counted cells each (B).

Fig. 2 Extracellular matrix and filamentation of C. tropicalis morphotypes. Composition of the matrix of C. tropicalis colonies in terms of amounts of total carbohydrates and proteins—\(^a\) (mg carbohydrate/g colony dry weight) and total proteins; \(^b\) (mg protein/g colony dry weight) (a). The percentage of hyphae in the phenotypes was evaluated throughout colony development at 48, 72, and 96 h. The results represent the average of three independent experiments with 1000 counted cells each (B).

Representative cells of morphotypes colonies (C). Bar scale: 10 \(\mu m\). (\(*) P < 0.05\), (\(**) P < 0.01\), (\(***) P < 0.001\) compared to the smooth phenotype (clinical strain)
Switched colonies showed distinct gene expression profiles. The rough morphotype showed a significant increase in the expression of \( EFG1 \) (11.3 ± 1.1) and \( WOR1 \) (2.7 ± 0.3), important transcriptional regulators of filamentous growth when compared to the smooth phenotype. \( BCR1 \) expression (4.6 ± 0.5), a regulator of biofilm formation, was also higher in the rough morphotype than in the smooth strain (\( p < 0.05 \)) (Fig. 3). Differently, the crepe morphotype showed a decrease in the expression levels of \( EFG1 \) (0.02 ± 0) and \( BCR1 \) (0.01 ± 0) compared to the expression of the smooth phenotype (\( p < 0.001 \)), while no significant difference was observed on the level of \( WOR1 \) expression between crepe and smooth morphotypes (Fig. 3). In addition, the expression of all three transcription factors was positively correlated (\( r^2 > 0.9472, \ p < 0.05 \)) to the hyphal formation profile of the rough morhotype.

Discussion

In this study, we evaluate the structural and genetic factors associated with switching-derived morphotypes of \( C. \) tropicalis. During the ultrastructural analysis, we observed that, although present in colonies of all morphotypes (smooth, crepe and rough), extracellular matrix exhibits differences in abundance and distribution (Fig. 1, III). These variations suggest an association with the complexity of the phenotypes since both crepe and rough variants, characterized by colonies of structured morphology [16], have dense matrix networks covering and connecting the cells, in contrast to that observed for the smooth phenotype. This evidence reinforces the possible role and biological importance of the matrix in maintaining the architecture of structured colonies derived from phenotypic switching in \( C. \) tropicalis, classified as biofilm-like colonies [11]. For the yeast \( Saccharomyces \) cerevisiae, the formation and architecture of structured biofilm colony phenotype are related to the abundant presence of ECM [17].

Currently, studies related to ECM in colonies of \( Candida \) species are largely unexplored. In the present study, we showed for the first time that the amounts of total carbohydrates and proteins in ECM extracted from colonies were variable between switched phenotypes of \( C. \) tropicalis (Fig. 2a) indicating that macromolecular components may vary in matrix composition in a morphotype-dependent fashion. ECM extracted from biofilms of \( C. \) tropicalis has been evaluated [23–25]. According to these authors, the amounts of carbohydrate and protein in the \( C. \) tropicalis biofilm matrix are variable compared to other \( Candida \) species. In addition, it was demonstrated that the composition of the matrix contributes to drug resistance in \( C. \) tropicalis biofilms [23].

In addition, our data also revealed that structured phenotypes vary in cellular differentiation throughout colony development. The switch phenotypes (crepe and rough) exhibited the highest percentages of hyphae compared to the smooth phenotype. The high proportion of hyphae was maintained throughout 48, 72 and 96 h of culture (Fig. 3a, c). These results suggest that phenotypic switching may act on the yeast-to-hyphae transition at the early stages of colony development reflected in structured colonies (crepe and rough), corroborating our previous findings [16]. For \( C. \) albicans white-opaque switching system, opaque cells do not undergo filamentation under conditions that induce hyphae formation in white cells, suggesting that switching may have an effect on infection as hyphal growth is a virulence determinant that facilitates tissue invasion [26].

Using RT-PCR, we analyzed the expression of \( EFG1 \), \( WOR1 \) and \( BCR1 \) transcription factors by colonies cells of \( C. \) tropicalis morphotypes. Our data showed higher levels of gene expression in the rough
than in the smooth colonies, being more than 11-fold for \textit{EFG1} and near three-fold for \textit{WOR1} (Fig. 3). Both genes are important regulators of the white-opaque transition [27–29], and play key roles in regulating cell differentiation of tristable switching [30] in \textit{C. tropicalis}. The expression level of \textit{BCR1}, a regulator of biofilm formation, was 4.6-fold higher in the rough than in the smooth colonies. In \textit{C. tropicalis}, \textit{BCR1} also acts as a regulator of the white-opaque transition [29], and as an activator of filamentation [28]. Here, we show for the first time that the expression levels of \textit{EFG1}, \textit{WOR1} and \textit{BCR1} were increased in a switch morphotype (rough variant) of \textit{C. tropicalis} that is characterized by colonies of structured morphology, as illustrated in Fig. 1. In addition, gene expression of all three transcription regulators correlated to the high capacity of the rough morphotype to form hyphae throughout colony development.

In contrast, although no significant difference was observed in the level of \textit{WOR1} expression between the crepe and smooth colonies, the former had a higher percentage of hyphae in relation to the smooth morphotype. According to Zhang et al. [28] and Porman et al. [31], \textit{WOR1} overexpression led to an increase in filamentous growth in \textit{C. tropicalis}, however, deletion of \textit{WOR1} had no prominent effect on filamentation, suggesting that \textit{WOR1} is not an essential gene for filamentation in \textit{C. tropicalis} [28]. In the crepe variant, the phenotypic switching transiently induced the expression of \textit{EFG1}, since its expression was high in colonies at 24 h of culture (data not shown) and absent in colonies at 48 h of culture (Fig. 3). Therefore, switched morphotypes of \textit{C. tropicalis} characterized by colonies of structured morphologies (crepe and rough) seem to have distinct gene expression profiles throughout their development.

Yeast-filamentous growth transition is a strategy by which \textit{Candida} species may increase their virulence. In previous studies, we showed that structured switch phenotypes of \textit{C. tropicalis} with higher amounts of filamentous forms exhibit greater success in colonizing biotic and abiotic surfaces [19], higher biofilm formation [14] as well as elevated virulence against \textit{G. mellonella} larvae [18] compared to smooth counterpart morphotypes.

In conclusion, our data suggest that structured morphological patterns of switched colonies in \textit{C. tropicalis} may be due, at least in part, to changes in the matrix arrangements and increased filamentous growth. In addition, switched colonies have distinct gene expression profiles. The transcriptional factors \textit{EFG1}, \textit{WOR1} and \textit{BCR1} are likely to influence filamentation in colonies of the rough variant of \textit{C. tropicalis}. This is the first study to evaluate genetic factors that may mediate the switching event in \textit{C. tropicalis} that originate colonies of structured morphology.

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Author Contributions CMS and MCF conceived and designed the experiments. CMS, ATPM and MMS performed the experiments. CMS, MCF, MSM and LF-M analyzed the data. CMS and MCF drafted the manuscript. CMS, MMS, LF-M and MCF reviewed and edited the manuscript. All authors read and approved the final manuscript.

Declarations

Conflict of interests The authors declare that they have no competing interest. The authors alone are responsible for the content and the writing of the paper.

Consent for Publication All authors agree to the submission of the manuscript in its actual format.

Ethical Approval This article does not contain any studies with human participants or animal experiments.

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