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The gray phenotype and tristable phenotypic transitions in the human fungal pathogen *Candida tropicalis*

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ABSTRACT

Phenotypic plasticity, the ability to switch between different morphological types, plays critical roles in environmental adaptation, leading to infections, and allowing for sexual reproduction in pathogenic *Candida* species. *Candida tropicalis*, which is both an emerging human fungal pathogen and an environmental fungus, can switch between two heritable cell types termed white and opaque. In this study, we report the discovery of a novel phenotype in *C. tropicalis*, named the gray phenotype. Similar to *Candida albicans* and *Candida dubliniensis*, white, gray, and opaque cell types of *C. tropicalis* also form a tristable switching system, where gray cells are relatively small and elongated. In *C. tropicalis*, gray cells exhibit intermediate levels of mating competency and virulence in a mouse systemic infection model compared to the white and opaque cell types, express a set of cell type-enriched genes, and exhibit both common and species-specific biological features. The key regulators of white-opaque transitions, Wor1 and Efg1, are not required for the gray phenotype. A comparative study of the gray phenotypes in *C. tropicalis*, *C. albicans*, and *C. dubliniensis* provides clues to explain the virulence properties and niche preferences of *C. tropicalis*.

1. Introduction

Candida tropicalis is one of the most prevalent pathogenic yeast species of humans. Infections caused by this fungus have increased dramatically in the recent decade worldwide, likely due to the common use of azole-derivatives and *C. tropicalis*' innate resistance to these antifungals (Kothavade et al., 2010).

An important feature of *C. tropicalis* and its related *Candida* clade species, such as *C. albicans*, is their ability to switch between different morphological phenotypes (Huang, 2012; Porman et al., 2011; Xie et al., 2012). Morphological plasticity confers these pathogens with the abilities to adapt to diverse and changing environments. For example, both *C. albicans* and *C. tropicalis* can undergo a switch between two heritable cell types termed white and opaque (Huang, 2012; Porman et al., 2011; Slutsky et al., 1987; Xie et al., 2012). The two cell types differ in a number of biological aspects, including colony and cellular appearances, virulence in different infection systems, mating competency, and global gene expression profiles (Porman et al., 2011; Soll, 2009; Tao et al., 2014). Recently, we discovered a novel heritable cell type, named the gray phenotype, in *C. albicans* and its closely related species *Candida dubliniensis* (Tao et al., 2014; Yue et al., 2016). The gray cell type, together with the white and opaque cell types, forms a white-gray-opaque tristable switching system. The cellular morphologies of white, gray, and opaque cell types are distinguishable in the two *Candida* clade species. In general, white cells are relatively round and form white and shiny colonies on nutrient agar plates; opaque cells are large and elongated in cell shape and form dark and rough colonies; and gray cells are also elongated in cell shape, but are much smaller than opaque cells (Tao et al., 2014; Yue et al., 2016). In *C. albicans*, gray cells are more virulent than white and opaque cells in superficial infection models. However, this is not the case in *C. dubliniensis*. This difference could be due to the distinct expression features of secreted aspartyl proteases (Saps) in the two species. In both *C. albicans* and *C. dubliniensis*, gray cells are less virulent than white and opaque cells in mouse systemic infection models and exhibit an intermediate level of mating competency between that of white and opaque cells (Tao et al., 2014; Yue et al., 2016). Interestingly, deletion of *HXK1*, a gene encoding the N-acetylglucosamine kinase, in the

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non-switchable derivatives of *C. albicans* SC5314 induces the gray and opaque phenotypes (Cao et al., 2016).

In this study, we report the discovery of the gray phenotype in *C. tropicalis*. Gray cells of *C. tropicalis* are similar to their counterparts in *C. albicans* and *C. dubliniensis* in several aspects, such as cellular morphology and mating competency. However, the gray cell type of *C. tropicalis* has distinct species-specific biological characteristics, including colony morphology, virulence in the mouse model of systemic infections, and global gene expression profile differences. A comparative analysis of the gray phenotypes in these three *Candida* clade species provides clues to explain their differences in terms of prevalence and virulence.

2. Materials and methods

2.1. Strains, culture conditions and plasmids

The strains used in this study are listed in Table S1. *C. tropicalis* strain JX1012 was isolated from sputum of a hospitalized patient. The species identity was verified by sequencing the internal transcribed spacer (ITS) region 1 DNA sequences. YPD (20 g/L glucose, 20 g/L, peptone, 10 g/L yeast extract, w/v) and Lee's GlcNAc medium were used for the routine growth of *C. tropicalis*. Lee's glucose and Lee's GlcNAc media (Tao et al., 2014) were used for phenotypic switching assays. For positive selection of transformants, 100 μg/ml nourseothricin or 100 μg/ml hygromycin were added to the YPD medium. The solid medium contained 2% agar and 5 μg/mL of the dye phloxine B for staining of opaque colonies.

Two fragments of the 5′ and 3′ ends of ct*URA3* genes were amplified by PCR and subcloned into pSFS2A (Reuss et al., 2004), generating plasmid pSFS2A-ctURA3. To generate the *ura3*/*ura3* mutant of JX1012, we deleted the first allele of the *URA3* gene using the *Apa* I and *Sac* I-linearized plasmid pSFS2A-ctURA3. The second allele of the *URA3* gene was deleted using the fusion PCR strategy (Noble and Johnson, 2005). The *cahph* marker was used for positive selection on plates containing medium supplemented with hygromycin (the *Candida hph* hygromycin-resistance gene, *cahph*, was a gift from Dr. Xaioqin Lai, Institute of Microbiology, CAS, Beijing, China) (Zhang et al., 2016). Similarly, the knockout plasmids, pSFS2A-ctWOR1 and pSFS2A-ctEFG1, were constructed and were used to deleted the first allele of *WOR1* and *EFG1* in *C. tropicalis* JX1012, respectively. The second allele of *WOR1* or *EFG1* was then deleted using the fusion PCR strategy. *cahph* was used as a selective marker. Primers used for this study are listed in Table S2.

2.2. White-gray-opaque switching assays

The switching assays of white-gray-opaque phenotypes were performed as described previously with slight modifications (Tao et al., 2014). Briefly, white, gray, or opaque cells were first grown on solid Lee's GlcNAc medium at 30 °C for two days. The temperature choice of 30 °C was used for most experiments in this study because both the gray and opaque phenotypes are stable at this temperature. Homogeneous white, gray, or opaque colonies were replated onto solid Lee's GlcNAc plates and incubated in air for two days at 30 °C or 37 °C, or three days at 25 °C. The numbers of colonies representing different cell types were counted, and the switching frequencies between different phenotypes were calculated (switching frequency = number of colonies with an alternative cell type/total colony number x 100%). Three biological replicates were performed for the switching assays.

2.3. Virulence assays

All of the animal experiments were performed according to the guidelines approved by the Animal Care and Use Committee of the Institute of Microbiology, Chinese Academy of Sciences. Systemic infection and fungal burden assays were performed according to previous reports (Tao et al., 2014). Six female BALB/c mice aged four to five weeks were used for the fungal burden determinations of each *C. tropicalis* cell type in two different assays. In the first assay, cells of each cell type (2×10^6 in 200 μL of PBS) were injected into each mouse via the tail vein, and the mice were sacrificed after 24 h of injection. In the second assay, cells of each cell type (1×10^7 in 200 μL of PBS) were injected into each mouse, and the mice were sacrificed after seven days of injection. Fungal burden assays in five organs (liver, kidney, spleen, lung, and brain) were performed.

2.4. RNA-Seq and quantitative real-time PCR (RT-PCR) assays

White, gray, and opaque cells were grown at 30 °C on solid Lee's GlcNAc medium for two days. Cells were collected from homogenous white, gray, or opaque colonies and total RNA was extracted as described previously (Tao et al., 2014). The RNA-Seq analysis was performed by the company BGI-Shenzhen according to the company's protocol (http://www.genomics.cn/). Approximately 10 million (M) reads were obtained by sequencing each library. The gene expression level was calculated using the FPKM method (http://www.genomics.cn/). Quantitative RT-PCR assays were performed to verify a selection of cell-type enriched genes. Relative gene expression levels of *SAP* genes in different cell types were examined by quantitative RT-PCR assays. Lee's GlcNAc and YCB-BSA media (Tao et al., 2014) were used for cell culture. For each cell type, 1×10^6 cells in 20 μL ddH_2O were inoculated into 2 mL of liquid medium. Cells were collected after incubation for 24 h at 30 °C and total RNA was extracted for quantitative RT PCR assays. The signal from each experimental sample was normalized to the expression level of the *ACT1* gene. Three biological replicates were performed.

2.5. Mating assays

Quantitative mating assays were performed as described previously (Tao et al., 2014). The JX1012u (*MTL***a**/**a** *ura3*/*ura3*) mutant was used as the experimental strain and opaque cells of CAY2061 (*MTLα*/*α his1*/*his1*) served as the tester mating partner (Porman et al., 2011). White, gray, and opaque cells of JX1012u and opaque cells of CAY2061 were collected from solid Lee's GlcNAc plates. Cells were counted, and 1×10^7 opaque cells of CAY2061 and 1×10^7 white, gray, or opaque cells of JX1012u were mixed in 10 μL ddH_2O, spotted onto Lee's GlcNAc plates, and cultured for 48 h in air at 30 °C. The mating mixtures were then resuspended, diluted, and plated onto three types of selectable plates (SC-uridine, SC-histidine, and SC-uridine-histidine) for growth. The mating efficiencies were calculated according to previous reports (Miller and Johnson, 2002). Mating efficiency = (conjugants)/(limiting parent + conjugants) = the greater of (-Uri -His)/(-Uri) or (-Uri -His)/(-His).

3. Results and discussion

3.1. Identification of the gray phenotype in C. tropicalis

In a previous study, we observed an intermediate cell type between white and opaque in some clinical isolates of *C. tropicalis* (Xie et al., 2012). We suspected that this intermediate cell type