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The role of the *de novo* pyrimidine biosynthetic pathway in *Cryptococcus neoformans* high temperature growth and virulence

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ABSTRACT

Fungal infections are often difficult to treat due to the inherent similarities between fungal and animal cells and the resulting host toxicity from many antifungal compounds. *Cryptococcus neoformans* is an opportunistic fungal pathogen of humans that causes life-threatening disease, primarily in immunocompromised patients. Since antifungal therapy for this microorganism is limited, many investigators have explored novel drug targets aim at virulence factors, such as the ability to grow at mammalian physiological temperature (37 °C). To address this issue, we used the *Agrobacterium tumefaciens* gene delivery system to create a random insertion mutagenesis library that was screened for altered growth at elevated temperatures. Among several mutants unable to grow at 37 °C, we explored one bearing an interruption in the *URA4* gene. This gene encodes dihydroorotase (DHOase) that is involved in the *de novo* synthesis of pyrimidine ribonucleotides. Loss of the *C. neoformans* Ura4 protein, by targeted gene interruption, resulted in an expected uracil/uridine auxotrophy and an unexpected high temperature growth defect. In addition, the *ura4* mutant displayed phenotypic defects in other prominent virulence factors (melanin, capsule and phospholipase) and reduced stress response compared to wild type and reconstituted strains. Accordingly, this mutant had a decreased survival rate in macrophages and attenuated virulence in a murine model of cryptococcal infection. Quantitative PCR analysis suggests that this biosynthetic pathway is induced during the transition from 30 °C to 37 °C, and that transcriptional regulation of *de novo* and salvage pyrimidine pathway are under the control of the Ura4 protein.

1. Introduction

Cryptococcus neoformans is a pathogenic fungus with a world-wide distribution. This yeast is found in the environment on rotting wood, and it is often associated with avian excreta. It can cause life-threatening respiratory and neurological infections, especially in immunocompromised patient populations. In fact, recent surveys estimate greater than 500,000 deaths due to *C. neoformans* each year, mainly in patients with AIDS and other immune compromising conditions (Park et al., 2009). The immunocompromised population has increased world-wide due to many factors including the AIDS pandemic and growing numbers of transplant patients. Together, these factors have turned this yeast into a major pathogen (Mitchell and Perfect, 1995; Singh and Husain, 2000).

Options for antifungal therapies for cryptococcosis are limited. Among the common agents used for this infection are polyenes (amphotericin B-based drugs), antimetabolites (flucytosine), and azoles (Perfect et al., 2010). However, drug toxicity and antifungal resistance remain important issues in the treatment of all fungal infections (Rex et al., 2001; Paiva and Pereira, 2013). To address potential novel strategies for antifungal therapy, we and others have studied virulence-associated phenotypes that allow *C. neoformans* to survive within the infected host and to cause disease. These factors include polysaccharide capsule, melanin, phospholipase, and growth at 37 °C (Heitman et al., 2006; Brown et al., 2007; Li and Mody, 2010; Lam et al., 2013). The ability of this fungus to grow at human physiological temperature is controlled by several cellular factors, including the effectors of the calcineurin and Ras signal transduction pathways (Odom et al., 1997; Alspaugh et al., 2000; Kozubowski et al., 2009). To identify additional elements that are

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required for high-temperature growth, we used a random insertion mutagenesis strategy mediated by *Agrobacterium tumefaciens* to generate mutants of *C. neoformans* unable to grow at 37 °C (Idnurm et al., 2004; McClelland et al., 2005). Using this method, we identified temperature-sensitive strains containing mutations; one of these was defective in pyrimidine biosynthesis.

The *de novo* synthesis of uracil monophosphate (UMP) is essential for the synthesis of nucleic acids, and it is a common biosynthetic pathway for all organisms. Some cells can also make UMP using pyrimidine (Norager et al., 2002). In addition to basic cell growth, many organisms, including pathogenic parasites, require efficient pyrimidine biosynthesis for rapid cell proliferation and adaptation to cell stress (Fairbanks et al., 1995; Fox and Bzik, 2010; Ali et al., 2013; Hegewald et al., 2013; Ong et al., 2013). In *S. cerevisiae*, transcription of genes linked to the uracil biosynthesis pathway can increase three to eight times the yeast cells are subjected to pyrimidine limitation (Roy et al., 1990). The enzyme dihydroorotate dehydrogenase (DHOase-URA1) converts dihydroorotate into orotate, and DHOase inhibitors have been successfully tested as antiproliferative agents in neoplastic growth, as suppressors of immunological responses (Chen et al., 1992; Greene et al., 1995; Liu et al., 2000; Khutornenko et al., 2010), and also as inhibitors of the growth of *Toxoplasma gondii* (Hegewald et al., 2013). In the basidiomycete *Ustilago maydis*, the *pyr4* gene encodes dihydroorotate dehydrogenase, and its absence results in loss of pathogenicity and uracil auxotrophy (Banuett, 1995; Bölker, 2001; Zameitat et al., 2007). Previous work by Morrow et al. (2012) showed that *de novo* synthesis of purine derivatives such as GTP is also important for virulence in *C. neoformans*.

C. neoformans encodes genes for pyrimidine *de novo* (*URA1* through *URA6*) and salvage (*URH1*, *URK1* and *CDD1*) pathways (Fig. 1A and B, respectively), suggesting this yeast can use both strategies to obtain this essential nutrient. One pyrimidine biosynthetic gene that has been previously characterized in *C. neoformans* is *URA5*, and this has been primarily used as selectable marker for transformation (Edman and Kwon-Chung, 1990; Kwon-Chung et al., 1992; Varma et al., 1992). However the virulence phenotypes associated with the *ura5* mutation have not been extensively characterized. In this paper we studied the role of the *URA4* gene in *C. neoformans*, the impact of its interruption on virulence factors and survival *in vitro* and *in vivo*. Also, the relationship between the pyrimidine *de novo* and salvage pathways and high temperature growth was established by transcription analysis. Our results showed that these pathways are not only important for the high temperature growth trait but also for the expression of several virulence factors, as well as efficient responses to cell stresses.

2. Material and methods

2.1. Strains, media and reagents

All wild-type, deletion and reconstituted strains were generated in the *C. neoformans* var. *grubii* KN99α background (MATalpha) donated by Dr Joseph Heitman. The *ura5* mutans were obtained after selection of *C. neoformans* var. *grubii* strains baring spontaneous mutation of the *URA5* gene after incubation on YPD medium containing 5-FOA (5-fluoroorotic acid, 10 μg/mL). Oligonucleotide primers are presented in Tables 1 and 2. The media used were standard YPD medium (1% yeast extract, 2% peptone and 2% dextrose, 2% agar), synthetic medium (6.7 g/l Sigma Yeast Nitrogen Base with amino acids and ammonium sulfate, 2% dextrose, 2% agar) with or without addition of 20 μM uracil (Sigma). Geneticin (Invitrogen) and Hygromycin (Invitrogen) were added to the selection medium for transformation at 200 μg/mL final concentration, as indicated.

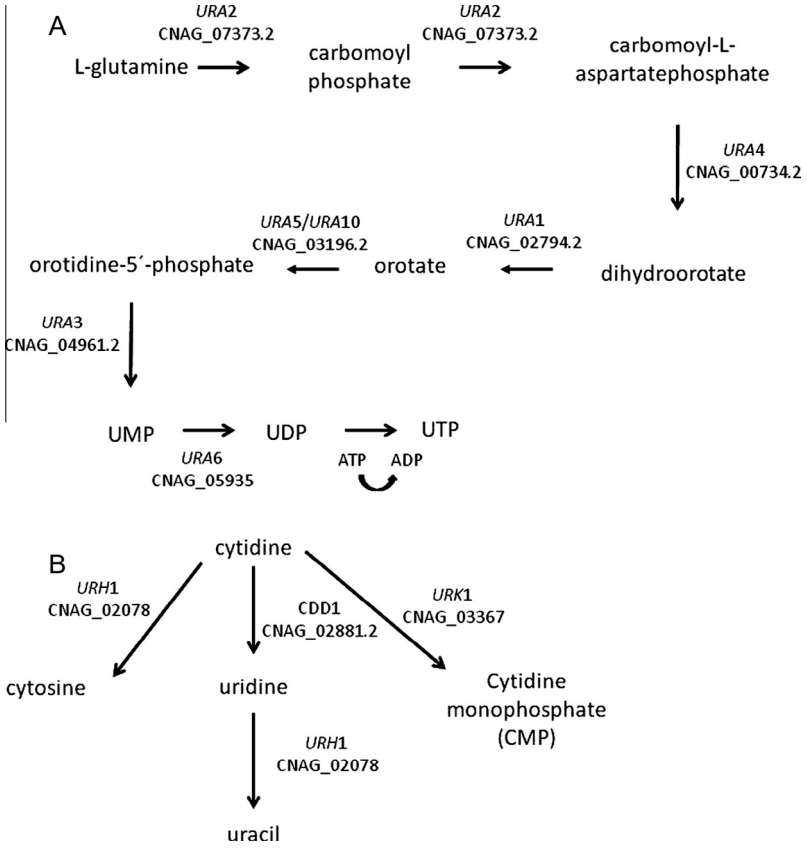

Fig. 1. Representation of the pyrimidine ribonucleotide *de novo* biosynthetic (A) and salvage (B) pathways (adapted from http://www.yeastgenome.org/).