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

Fabiano Assis de Gontijo[a], **Renata C. Pascon**[a], **Larissa Fernandes**[b], **Joel Machado Jr**[a], **J. Andrew Alspaugh**[c], and **Marcelo A Vallim**[a,*]

[a]Universidade Federal de São Paulo, Departamento de Ciências Biológicas, Diadema, SP, Brazil

[b]Universidade de Brasília, Faculdade de Ceilândia, Brasília, DF, Brazil

[c]Duke University School of Medicine, Department of Medicine, Durham, NC, USA

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.

Keywords

pyrimidine biosynthesis; thermal tolerance; basidiomycete yeast

[*]Corresponding author: Marcelo A. Vallim, Laboratório de Interações Microbianas, Departamento de Ciências Biológicas, Universidade Federal de São Paulo, Rua Arthur Ridel, 275, 09972-270 - Bairro Eldorado, Diadema, SP, Telephone: 55 11 3319-3549, marcelo.vallim@gmail.com.

1. Introduction

Cryptococcus neoformans is a pathogenic fungus with a worldwide 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 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 dihydrogenase (DHOase-URA1) converts dihydroooratate 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 (Figure 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, 10μg/mL). Oligonucleotide primers are presented in Table 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/liter Sigma Yeast Nitrogen Base with amino acids and ammonium sulfate, 2% dextrose, 2% agar) with or without addition of 20μM uracil (Sigma). Geneticin (Invitrogen) and Hygromycin (Invitrogen) were added to the selection medium for transformation at 200 μg/mL final concentration, as indicated.

2.2. Genetic manipulation

C. neoformans genomic DNA extraction was performed as published (Pitkin *et al.*, 1996). General molecular biology manipulations were performed according to Sambrook *et al.* (1989). Southern blots were performed using Roche PCR DIG Probe Synthesis kit, DIG Wash and blot kit, CDP-star Ready according to the manufacturer's instructions.

2.3. *Agrobacterium tumefaciens* random insertion mutagenesis

The *C. neoformans* mutant collection was created according to the method described by Idnurm *et al.* (2004). Briefly, *Agrobacterium* mediated-transformation was achieved by co-culture of the *C. neoformans* strain KN99α with *A. tumefaciens* LBA4404 strain harboring the plasmid pPZP-Hyg2 or pPZP-Neo1 (Walton et al, 2006, donated by Dr. J Heitman) and induced with 100 μM of acetosyringone. Co-culture was conducted for 48 hours and aliquots were plated on YPD medium supplemented with 200 μg/mL Geneticin or 200 μg/mL Hygromycin, and cefotaxime (100 μg/mL) to kill *Agrobacterium*. After 48 to 72 hours,

antibiotic resistant colonies were transferred to YPD medium supplemented with the appropriate antibiotic and incubated at 30°C and 37°C. Strains that grew at 30°C but that failed to grow at 37°C were chosen for further analysis. The selectable marker insertion site was identified by inverse PCR methods as previously described (Idnurm *et al.*, 2004). Briefly, we extracted genomic DNA from these strains, and digested with either *Hind*III or *Eco*RI, (Fermentas). The digested DNA was purified through a Qiagen column (Qiaquick PCR Purification Kit), and ligated to itself with T4 DNA ligase. PCR was performed using primers directed against selectable marker sequences (primers MAV30 and MAV32, MAV31 and MAV33 (Table 1). Gel purified PCR products obtained by inverse PCR were sequenced and compared to the Broad Institute *C. neoformans* strain H99 genome database to determine the position of the T-DNA insertion into the genome.

2.4. Gene deletion and reconstitution, and *C. neoformans* transformation

Independent *ura4* mutants were generated by biolistic transformation and homologous recombination. The *URA4* coding region was partially replaced with the Geneticin resistance cassette containing the Neo^R gene under control of the actin *ACT1* promoter and *TRP1* terminator (Walton, *et al.*, 2005). *In vitro* construction of the deletion cassette was performed using PCR overlap extension (Davidson *et al.*, 2002) using the following primers (left arm: primers MAV067 and MAV097, right arm: MAV098 and MAV 072, and Geneticin resistance gene: MAV099 and MAV070) described in Table 1. The cassette was introduced into *C. neoformans* KN99α by the biolistic method as described (Toffaletti *et al.*, 1993), and transformants were screened for the *ura4* mutation by PCR and Southern blots. The *ura4+URA4* reconstituted strain was generated by introducing the wild-type *URA4* allele into the *ura4* mutant strain by the biolistic method. Briefly, the reconstituted strain was selected on synthetic media (YNB) without uracil, and this strain also became sensitive to Geneticin. As a control, the same culture of mutant cells was subject to bombardment with gold particles without DNA and the selection on YNB without uracil did not yield any colony. The primers used to generate the deletion and reconstitution strains are described in Table 1.

2.5. High temperature growth ability, melanin and capsule production assay

Thermotolerance was evaluated on both rich (YPD) and synthetic media (YNB) at 30°C and 37°C. To assess melanin production, overnight cultures were incubated in YPD medium, spotted on Niger seed agar plates (75g/L Niger bird seed, 0.1% Dextrose, 2% agar, with 20μM uracil), and incubated at 30°C for up to 96 hours. Capsule was induced by inoculating fresh cells of each strain in CO_2-independent medium (Gibco, Cat 18C45-088) with and without 20μM uracil at 30°C at 150 rpm rotation (O'Meara, *et al.*, 2013). After 24 hours incubation, cells were stained with BactiDrop India Ink (Remel) and directly observed by light microscopy. Alternatively, induced cells were washed in 1x PBS, resuspended in PBS and 1% Bovine Serum Albumin, incubated with 1 μL of mAb 18B7 (Casadevall *et al.*, 1998) under gentle agitation for 1 hour at room temperature. Cells were washed in PBS + 1% BSA and incubated with 1 μL of fluorescent Alexa Fluor® 594 Goat Anti-Mouse IgG (Life Technologies) for 1 hour at room temperature with gentle rotation. After 2 washes in PBS, cells were observed by fluorescent microscopy with red filter (Texas Red). Differential interference microscopy (DIC), bright field, and fluorescent images were obtained with a

Zeiss Axio Imager.A1 fluorescent microscope and AxioCam MR digital camera. Also, we have cultured the strains on CO_2-independent medium supplemented with uracil and uridine (20 μg/mL) up to 72 hours at 30°C and 37°C.

2.6. Multi stress sensitivity assay

Osmotic, ionic and cell wall stress were assessed by spotting cells onto YPD plates supplemented with 0.75 M KCl, 0.75 M NaCl, 0.5% Congo Red, or pH 8.0. All cultures were incubated at 30°C.

2.7. Minimal inhibitory concentration assay

Sensitivity to antifungal drugs was determined for mutant, reconstituted and wild type strains by standard minimal inhibitory concentration (MIC) assays according to Clinical and Laboratory Standards Institute (CLSI M27-A2) protocols with modifications. Briefly, fresh cultures were diluted and 100 cells were inoculated in YNB plus 20μM of uracil. The concentration of fluconazole ranged from 16 to 0.0625 μg/mL, and that of amphotericin B from 1 to 0.03 μg/mL. A control with cells and without drugs was included, and a blank containing only medium was also applied in the assay. MIC80 was defined as the drug concentration in which growth was at least 80% inhibited compared to the drug-free control following incubation for 72 h at 30°C. Growth was determined by measuring the turbidity of the cell suspension in a Multiskan Ascent (Lab System) at 620 and 540 nm. After absorbance reading, the cells were stained with 0.001% vital dye Rezasurine (Sigma) for 1 hour at 30°C and 150 rpms. All experiments were performed in triplicate for fluconazole and amphotericin B, and two biological replicates were applied.

2.8. Transcriptional analysis by real time PCR

Total RNA for transcriptional pattern analysis was obtained from cells incubated for 2 hours in YPD and YNB at 30°C and 37°C. Cells were harvested, washed three times in PBS, flash frozen on dry ice, and lyophilized. RNA extraction was performed according to Yang *et al.* (2002). cDNA synthesis was performed with RevertAid H minus First Strand cDNA synthesis kit (Thermo Scientific) with Oligo dT and random hexamer primers from 5 μg of total RNA. Real time PCR amplifications were made from diluted templates (1:10) with 800 nM target primers, 300 nM *GPD1* (Glyceraldehyde-3-phosphate dehydrogenase, Varma and Kwon-Chung. 1999) as internal control primers, and 1X Power SYBR Green master mix (Life Technologies). Quantification of the transcript levels was performed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001), normalizing against *GPD1* as previously described by Vandesompele *et al* (2002).

2.9. Macrophage infection assay

Macrophage-like J744.A1 cells were used to assay survival of yeasts within mammalian cells according to the methods of Cox *et al.* (2003). In brief, mAb-opsonized (mAb 18B7 antibody (McFadden and Casadevall, 2004) *C. neoformans* cells were co-incubated in 96-well tissue culture plates with J774A.1 cells in 200 μL macrophage medium at a multiplicity of infection (MOI) of 10:1 (1×10^6 *C. neoformans* cells/200 μL, and 1×10^5 J774A.1 cells/200 μL). The J774A.1 cells were activated by adding LPS and INF-γ one day prior to

introducing the fungal cells. After an initial co-culture for 1 hour, the non-internalized fungal cells were removed by washing 3 times with PBS, and fresh medium was added. After 24 hours of incubation, the mammalian cells were disrupted with 0.5% SDS for 5 minutes, and the number of viable *C. neoformans* cells was determined by quantitative culture. Statistical validation was calculated for each macrophage co-cultivation experiment, and CFU counts were compared among strains by one-way ANOVA followed by Tukey's post-test for comparison of pairs. Differences were considered valid at a 95% confidence interval ($p<0.05$).

The phagocytic index was determined as follows: Separate plates containing macrophages where incubated with the three fungal strains under the same conditions, and after two hours of phagocytosis, the wells were washed twice with PBS to remove free yeast cells and stained with a commercial equivalent of Giemsa for fast processing of microscopic samples (Instant-Prov Quick Haematological Stain, New Prov Laboratory Products, Brazil) and observed in an inverted microscope for measurement of the phagocytic index. This was calculated as the percentage of cells containing at least one yeast to the total number of cells. For each strain, 900–1200 cells were counted in nine separate fields.

2.10. *In vivo* virulence assay

Virulence of the *C. neoformans* strains was assessed in the murine inhalation model of cryptococcal infection (Cox *et al.*, 2000). Briefly, groups of 4- to 6-week-old C57/BL6 mice were anaesthetized with 7.5μL/g i.p. of a mixture of ketamine (10.25 mg/mL) and xylazine (0.86 mg/mL). The mice were then infected via nasal inhalation with the wild-type strain KN99α, mutant (*ura4*) or reconstituted (*ura4*Δ+*URA4*) strains. The inoculum (10^5 cells in a 25μL per strain) was prepared from cultures which were incubated overnight in YPD at 30°C. Ten mice were infected for each strain. Mice were examined twice daily and sacrificed according to clinical measures predicting mortality (weight loss >15%, inability to access food and water). Survival data were analyzed by the Kruskal–Wallis test. The *ura4*-infected mice were sacrificed at 40 days post-infection; lungs from two of the surviving mice were harvested, homogenized, serially diluted, and plated on YPD to access the number of viable cells in the lungs. These experiments were performed at Duke University in accordance with institutional guidelines for animal experimentation.

G. mellonella killing assay. The infection of *Galleria mellonella* by *C. neoformans* was performed according to Mylonakis *et al* (2005). This assessment of virulence was performed at a permissive temperature (30°C) for the *ura4* mutant strain to assess temperature-independent aspects of survival in an infected host. Briefly, larvae with average body weight of 174 mg were separated in groups with 14 individuals. Each group was inoculated with 1×10^5 cells of wild type (KN99α), *ura4* (FGC003) or the reconstituted strain *ura4*Δ+*URA4* (FGC003R1) with a 10-μl Hamilton syringe. PBS (Phosphate buffered saline) was used as control. Ampicilin (20 mg/kg of body weight) was administrated with all inocula to avoid bacterial contamination. After infection, the *G. mellonella* larvae were incubated in a glass Petri dish (15mm diameter) at 30°C, and survival (as assessed by spontaneous or provoked motion) was accessed daily. Observation was completed when the larvae died or formed cocoons. Statistical analysis between treatment groups was preformed employing Prism

(GraphPad Software, La Jolla, USA). A p value of <0.05 was considered significant by Tukey's multiple comparison test.

3. Results

3.1. *C. neoformans* dihydroorotase activity is required for high temperature growth

To identify *C. neoformans* elements involved in high temperature growth, we used insertional mutagenesis to create a panel of *C. neoformans* mutant strains, screening for temperature sensitivity. Using the wild type strain KN99α as a recipient for mutagenesis, we identified 64 mutants unable to grow at 37°C. To define the sites of selectable marker insertion, we isolated genomic DNA from each strain and used inverse PCR to characterize the junction sequences between the inserted transfer DNA (t-DNA) and the interrupted *C. neoformans* DNA sequence. Typically, this method of transconjugation results in single, stable insertion mutations in the fungal genome (Idnurm *et al*, 2004). One of these strains, mutant G368, had an insertion present on chromosome I, which truncated a gene annotated as CNAG_00734 at amino acid residue 159 (Figure S2). BLAST search analysis at NCBI and SGD (*Saccharomyces* Genome Database) revealed that CNAG_00734 has similarity to the *S. cerevisiae* dihydroorotase (DHO) (EC 3.5.2.3) encoding gene *URA4* (YLR420W). DHO catalyzes a step in pyrimidine ribonucleotide biosynthetic pathway (Figure 1A), converting N-carbamoyl-L-aspartate into L-dihydroorotate by cyclical dehydration (Guyonvarch *et al*., 1988). No human homolog of this gene was identified.

The predicted protein sequence encoded by the *C. neoformans URA4* gene was aligned with orthologous sequences from *Ustilago maydis*, *S. cerevisiae* (S288c), *Candida albicans*, and *Schizosaccharomyces pombe*. The highest similarity is shared between the two basidiomycetes *C. neoformans* and *U. maydis* (61.2%), and the remaining similarities range between 47–59%. Moreover, the putative *C. neoformans* Ura4 protein has the two conserved signatures attributed to DHO enzymes (http://prosite.expasy.org/). The first domain spans residues 12-20, including two invariant histidines at positions 14 and 16. The second conserved domain spans residues 242-255 and has five highly conserved amino acids (D244, A246, P247, H248 and K253). Mehbood *et al.* (2010) described the crystal structure of *Bacillus anthracis* DHO. In this bacterium there is an active site, which includes a binuclear Zn center, where the H14 and H16 residues and the D224 and D244 residues all bind an α-Zinc atom, and two distant histidines and asparagines bind to a β-Zinc atom.

3.2 *C. neoformans URA4* gene deletion and reconstitution

To ensure that the temperature-sensitive phenotype of this strain was due to the *ura4* mutation, we created an independent *ura4* mutant strain in which we replaced 181 base pairs from the wild type locus, encoding residues 126 to 151, with the neomycin resistance gene. The *ura4* mutation and subsequent reconstitution were confirmed by Southern blot analysis (Supplemental Figure 1). These independently generated strains were used in all subsequent analyses.

3.3. Uracil auxotrophic mutants are sensitive to high temperature growth

In order to confirm the high temperature growth sensitivity is due to absence of a functional dihydroorotase in *C. neoformans*, serial dilution of cells from the mutant FGC003 (*ura4*), wild type (KN99α) and reconstituted strain FGC003R1 (*ura4*Δ*::URA4*) were inoculated on YPD plates and incubated at 30°C and 37°C for 48h. Figure 2A shows that wild type and reconstituted strains grow at the same rate in both temperatures, whereas the *ura4* mutant strain FGC003 is no longer able to grow at 37°C. We explored whether the *URA4* gene itself or the pyrimidine biosynthetic pathway in general is required for high-temperature growth by testing two *ura5* mutants for this phenotype. These strains have mutations in a distinct component of pyrimidine biosynthesis, and they were made in related but different strain backgrounds (KN99α and H99, both serotype A). Similar to the *ura4* mutants, both *ura5* strains were unable to grow at 37°C.

Consistent with its predicted function, mutation of the *C. neoformans URA4* gene caused the mutant strain to be auxotrophic for uracil, similar to the *ura5* mutant strain (Figure 2B). Similar to the wild type, the *ura4* mutant strain FGC003 was inhibited by the addition of 5-Fluoroorotic acid (5-FOA) to the growth medium. In contrast, the *ura5* mutant was able to grow well on 5-FOA-containing media, as expected considering that the two genes are predicated to act in different steps of the pathway, with the *C. neoformans* Ura5 converting 5-FOA to fluorodeoxyuridine, which is toxic to cells (Edman and Kwon-Chung, 1990; Kwon-Chung *et al.* 1992).

Taken together these observations suggest that: (i) the *URA4* gene (CNAG_00734) is part of the pyrimidine biosynthetic pathway encoding DHO in *C. neoformans*; and (ii) blocking pyrimidine ribonucleotide biosynthesis affects the ability of *C. neoformans* serotype A to grow at mammalian physiological temperature.

3.4. The *ura4* mutation affects multiple virulence factors in *C. neoformans*

Besides high temperature growth, “classical” virulence factors for *C. neoformans* include the production of melanin, capsule and phospholipase B activity. Melanized cells are more resistant to reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) than non-melanized cells (Wang and Casadevall, 1994). Strains that do not produce this pigment are reported to have attenuated virulence in animal models (Kwon-Chung *et al.* 1982; 1986). The *ura4* mutant strain produced melanin, however in a delayed fashion. While colonies of the wild type (KN99α), reconstituted strain (*ura4*Δ*+URA4*) and *ura5* mutant produce melanin within 48 hours of incubation (Figure 3A), the *ura4* strain colonies are pigmented only after 72 hours (data not shown). This was observed regardless of whether the medium was supplemented with uracil.

Another well-studied virulence factor is the production of a polysaccharide capsule which allows *C. neoformans* to evade phagocytosis by macrophages (Kozel and Gotschlich, 1982; Chang and Kwon-Chung, 1994, 1998; Chang *et al.* 1996). We evaluated this phenotypic trait by direct observation of India ink staining and light microscopy, as well as with immunofluorescence. Both methods reveal a dramatic decrease in capsule production by the *ura4* mutant in comparison to the wild type (KN99α) and reconstituted (FGC003R1) strains

(Figure 3B), regardless of uracil supplementation. Interestingly, two independent *ura5* mutants presented a similar capsule-poor phenotype as observed for the *ura4* mutant strain, irrespective of uracil addition to the medium (data not shown), however, addition of uridine (20μM) reverted this condition for the *ura4* mutant (Figure S3) [ANOVA, *t*-Student's and Scott Knout, $p < 0.05$]. At 30°C there was no significant variation in the capsular volume between KN99α, *ura4*Δ, or *ura4*Δ+*URA4*. Together, these data suggest that an intact pyrimidine biosynthesis pathway is required for the efficient induction of surface capsule expression

Phospholipase B is involved in tissue invasion by breaking down the host cell membrane leading to cell lysis (Ghannoum, 2000). Previous studies revealed that the *C. neoformans* phospholipase B mutant (*plb1*) has a slower intracellular growth inside macrophages (Cox *et al.* 2001). Phospholipase B activity can be assessed by measuring the zone of precipitation (Pz) on egg yolk-containing media (Price *et al.*, 1982). Nine independent colonies were spotted for each strain to calculate an average Pz index. Statistical analysis showed that the Pz index, while not significantly different between the wild type (1.76±0.26) and the *ura4+URA4* reconstituted strain (1.56±0.32), was significantly smaller for the *ura4* mutant (1.17±0.73, $p<0.05$), consistent with Ura4-dependent phospholipase B production. Figure S4 illustrates the capsule production of these strains. Addition of uracil (20μM) or uridine (20μM) in the medium did not rescue the phospholipase activity for the mutant.

3.5. *URA4* gene is involved in multi-stress resistance

The *C. neoformans* cell wall is an important organelle which provides protection from the environment and determines the multiple cell morphologies that this yeast assumes during its development (reviewed in Casadevall and Perfect, 1998). To assess the role of Ura4 in cell integrity and stress response, we incubated the wild type, *ura4* mutant and *ura4+URA4* strains on media containing various compounds known to affect cell wall phenotypes. The *ura4* mutant displayed a marked sensitivity to Congo Red and high salt concentrations (Figure 4A). Cell wall assembly is impaired by Congo Red (CR), which interferes with cell morphology and mother cell-bud separation in *S. cerevisiae* (for review see Kopecká and Gabriel, 1992; Ram and Klis, 2006). Additionally, resistance to salt stress has been correlated to *C. neoformans* survival *in vivo*, and genes involved and/or regulated by the HOG pathway must be expressed during osmotic stress (Idnurm *et al.*, 2009; Ko *et al.*, 2009; Jung *et al.*, 2012).

Another stress that *C. neoformans* is subject to during the infection of the host is altered pH. The fungal cells have a preference for acidic growth conditions, and during infection the mammalian tissues offer a slightly alkaline environment. Therefore, an array of genes must be transcribed to respond to this new environment (Casadevall, 2005). We observed that the *ura4* mutant has a dramatic growth defect at elevated pH compared to wild type and reconstituted strains (Figure 4B).

3.6. Uracil deficiency increases drug susceptibility

Antifungal agents with activity against cryptococcosis act mainly upon the cell membrane (van der Horst *et al.*, 1997). The azoles (e.g., fluconazole) interferes with the ergosterol

biosynthesis, whereas the polyenes (e.g., amphotericin B) binds the ergosterol in the fungal cell membrane leading to leakage of uni- and divalent cations resulting in cell death (for reviews see Bolard, 1986, Fromtling, 1988). To assess whether any of these drugs would act in association with the pyrimidine biosynthesis pathway defect, we tested them against the wild type, *ura4* mutant and *ura4+URA4* reconstituted strains. The three strains were equally sensitive to fluconazole in rich medium (YPD) as well as in synthetic medium (YNB supplemented with uracil). MIC testing with amphotericin B demonstrated that the *ura4* mutant strain had an increased sensitivity to this drug leading to growth arrest at 0.06 μg/mL, compared to the MIC for the wild type KN99α of 0.5 μg/mL. The reconstituted strain (*ura*Δ+*URA4*) demonstrated a higher MIC than the mutant, though not fully to the level of wild type. We also tested whether other defects in pyrimidine biosynthesis would result in similar changes in amphotericin susceptibility, and we noted that the *ura5* mutant strain behaved similar to the *ura4* mutant being inhibited by amphotericin B at 0.06 μg/mL. Banerjee *et al* (2014) tested the susceptibility of *C. neoformans* var *grubii* (H99) *ura5* mutant against fluconazole and amphotericin B. They also observed that mutants with a depleted pyrimidine pathway are more susceptible to amphotericin B compared to wild type and reconstituted, whereas fluconazole affected the wild type and mutant equally. This information suggests that inhibition of the pyrimidine biosynthetic pathway, in association with cell membrane-active agents, might have a positive impact on the treatment of cryptococcosis.

3.7. The *URA4* gene is essential for fungal survival within macrophages

All the results described above indicate that the *ura4* mutant strain would not likely succeed in avoiding macrophage phagocytosis and subsequent fungal killing. Therefore, we tested for intracellular survival in the macrophage lineage J744.A1. When co-cultured with these murine macrophages, the *ura4* mutant strain did not survive as well as the wild type (KN99α) or reconstituted (*ura4*Δ+*URA4*) strains. After 24 hours of co-incubation with J774.A1 cells, the *ura4* mutant strain demonstrated a marked decrease in survival (435.3±37.4 CFU/ml) compared to wild type (735.9±42.3 CFU/ml) or the reconstituted strain (814±82 CFU/ml) ($p < 0.05$ for each comparison). The *ura5* mutant similarly does not survive well inside the macrophage (214.5±74.2 CFU/ml). The altered survival was not attributable to varying rates of engulfment since the phagocytosis index was identical among these strains (data not shown). Taken together, these results link defects in the pyrimidine biosynthetic pathway with alterations in *C. neoformans* intracellular survival.

3.8. *URA4* gene is essential for *in vivo* survival

We compared the relative virulence of the wild type, ura4, and ura4 + URA4 reconstituted strains in a murine inhalational model of cryptococcosis (Cox *et al.*, 2000). As presented in Figure 5, infections with the wild type and reconstituted strains resulted in an average survival of 17.5 (±1.87) and 17.5 (±1.58) days post-infection, respectively. On the contrary, the *ura4* mutant strain-infected mice survived until the end of the experiment (40 days post-infection). Lungs from two of the surviving mice were harvested and homogenized, followed by serial dilution and quantitative cultures. Even though these animals appeared clinically healthy, there were still viable yeast cells in their lungs ($2.04\times10^5 \pm 5.6\times10^3$ CFU/mg).

Also, we checked whether the *ura4* mutant would display attenuated virulence at the permissive growth temperature (30°C) in a *Galleria mellonella* killing assay. The wild-type strain and the reconstituted (*ura4+URA4*) strains resulted in killing of all *Galleria* larvae at 3 and 5 days, respectively. In contrast, infection with the *ura4* mutant strain did not result in host killing in this assay, statistically similar to PBS treatment ($p<0.05$ between mutant and wild-type). (Figure 6). Therefore, we conclude that the *ura4* mutant is attenuated for virulence at both permissive and restrictive temperatures for its growth.

3.9. Pyrimidine *de novo* and salvage pathway genes respond to high temperature growth

We have demonstrated that several of the major virulence phenotypes are affected by both *ura4* and *ura5* mutations. Therefore, we analyzed the transcriptional pattern of the genes involved in the pyrimidine biosynthetic and salvage pathways by real time PCR in complex medium (YPD) and synthetic medium (YNB) at 30°C and 37°C. Figures 7A and 8A show that the expression pattern for genes in both pathways does not change substantially in rich medium (YPD) regardless of the temperature. All the genes tested, in both *de novo* and salvage pathways, were induced when incubated in YNB medium not supplemented with uracil at 37°C compared to 30°C (Figure 7B and 8B, respectively). The highest levels of induction in these experiments are for *URA4, URA2 and URA1*, which have 4.6-, 6.5- and 2.9-fold increases in expression after 2 hours at 37°C in YNB. In the salvage pathway there is a 5-fold increase in *CDD1* expression at 37°C in YNB and a 2-fold increase for *URK1* and *URH1*. These results indicate that there is increased transcript abundance for multiple genes in the pyrimidine *de novo* biosynthesis and also the salvage pathway during growth at human physiological temperature in poor nutritional condition, which is a situation likely encountered during the host infection.

We also tested the role of the *ura4* mutation on the transcriptional induction of other genes in these pathways. *URA2* and *URA3* are 2.5- and 1.8-fold induced, respectively, in complex medium (YPD) at 37°C in the *ura4* mutant compared to YPD medium at 30°C. Under the same conditions we observed no change in expression for *URA1*, *URA5* and *URA6* (Figure 7C).

A pronounced shift in expression pattern of the genes in the *de novo* biosynthetic pathway was observed when the *ura4* mutant strain was cultivated in synthetic medium (YNB) at the restrictive temperature (37°C): all 5 genes tested are down-regulated at 37°C (Figure 7D). *URA1*, *URA2* and *URA6* had a 20% reduction of their expression on YNB at 37°C compared to 30°C. *URA3* and *URA5* genes also had their transcription pattern dramatically repressed (40% and 20%) compared to YNB at 30°C (Figure 8D).

Comparatively, all 5 genes (*URA1*, *URA2*, *URA3*, *URA5* and *URA6*) were induced in the wild type strain incubated in YNB at 37°C (2.9-, 4.6-, 1.8-, 2.0- and 2.8-fold respectively, Figure 7B). These data suggest that the dihydroorotase gene may play a regulatory role in the pyrimidine biosynthetic pathway where the lack of its function impairs the transcriptional response necessary during heat stress. No significant alterations in transcript abundance were noted for the genes in the uracil salvage pathway in the *ura4* mutant background, at either temperature, or in either rich or poor growth media (Figure 8C and D). Taken together these observations suggest that dihydroorotase may act as a regulatory point

in both the *de novo* and the salvage pathways, being necessary to respond to the stresses encountered at high temperatures with nutrient depletion.

4. Discussion

Insertional mutagenesis mediated by *Agrobacterium tumefaciens* in *C. neoformans* has been a useful tool for gene function discovery and analysis (Idnurm *et al.*, 2004; Idnurm and Heitman, 2005; Walton *et al.*, 2005; 2006; Feretzaki and Heitman, 2013; Lee *et al.*, 2013). In this paper, this technique was employed to identify genes involved in high temperature growth, an important virulence factor for this yeast. Among the transformants unable to grow at high temperature, we identified one that has a truncated copy of the *URA4* gene. This enzyme, which is involved in the third step on the pyrimidine pathway, catalyzes the conversion of carbamoyl-L-aspartate to dihydroorotate. The role of the *C. neoformans* pyrimidine biosynthetic pathway in high temperature growth was confirmed with independently generated *ura4* mutants, as well as by documenting temperature-restricted growth of the *ura5* mutant strain, with a defect in a separate gene in this pathway. Transcriptional profiling data also support the correlation between pyrimidine availability and heat stress, since both biosynthetic and salvage pathways are highly induced at 37°C. Another relevant finding was the role of this pathway in general stress tolerance. Moreover, strains with loss of pyrimidine biosynthesis had attenuated virulence.

Previously, *C. albicans URA3* has been used as a selectable marker (URA-blaster), generating over time a large mutant collections (Fonzi and Irwin, 1993). Brand *et al.* (2004) reported a genome position effect upon *URA3* expression: lower *URA3* transcriptional levels determined by location correlated with attenuated virulence. In *C. neoformans* the *URA5* gene was used to assist molecular genetic manipulation of this fungus as a selectable marker for transformation (Edman and Kwon-Chung, 1990; Varma *et al* 1992). The *in vitro* phenotypes of this mutant strain were not explored in depth. The *ura5* mutant was noted to be highly attenuated in a murine model of systemic cryptococcosis, although this strain eventually caused lethal disease in mice (Varma *et al.*, 1992). This finding is similar to the studies presented here: although the *ura4* mutant caused no mortality after 40 days of infection, the strain was still viable and recoverable from infected tissue at the end of the experiment.

In similar studies, an adenine auxotrophic mutant was recovered from a patient with systemic cryptococcosis (Schiappa *et al.*, 2002). Prior studies had suggested that defects in adenine biosynthesis resulted in profound virulence defects in a rabbit model of cryptococcal meningitis (Perfect *et al.*, 1993). It is possible that different microenvironments in the host may offer different nutrient availability for these strains. Therefore, an adenine auxotrophic strain may be able to grow in the bone marrow, a tissue in which adenine is not predicted to be a limiting nutrient given the high cell turnover at this site. However, the same auxotrophic strain may be unable to grow in cerebral spinal fluid, which is more nutrient-poor.

Other studies have suggested that the analysis of metabolic pathways has the potential to identify targets for novel therapeutic interventions for cryptococcal disease. *C. neoformans*

mutants with defects in methionine, threonine, isoleucine, valine and lysine biosynthesis display reduced growth rates, high temperature growth defects, sensitivity to the inhibitory effects of antifungal drugs and reduced virulence (Yang *et al.*, 2002; Pascon *et al.*, 2004; Kingsbury *et al.*, 2004; Kingsbury *et al.*, 2004a; Kingsbury and McCusker, 2008; Kingsbury and McCusker, 2010; Kingsbury and McCusker, 2010a; Kingsbury and McCusker, 2010b). Therefore, defects in metabolic pathways may offer more attenuation in survival than merely inducing specific nutritional auxotrophies.

Recently, the purine biosynthetic pathway in *C. neoformans* was studied as a novel target for antifungal intervention (Morrow *et al.*, 2012). Defects in *de novo* purine biosynthesis, and not purine salvage, resulted in strains with growth defects and reduced virulence. Moreover, fungal-specific features were identified in the predicted structure of the inosine monophosphate dehydrogenase enzyme involved in guanine nucleotide synthesis. Together, these studies suggest that nucleic acid synthesis pathways offer potential, unexplored targets for intervention in treating fungal diseases.

Reduced growth caused by dihydroorotase defects have been reported in other organisms. For example, in *Salmonella typhimurium* lack of *pyrC* (*URA4* homolog) prevents the conversion of carbamoyl aspartate to dihydroorotate, leading to the accumulation of toxic intermediaries, which may cause growth reduction (Turnbough and Bochner, 1985). In *S. cerevisiae* the *ura4* mutant has reduced vegetative growth at 30°C (Yoshikawa *et al.*, 2011). In a clinical isolate of *S. cerevisiae*, lack of the Ura3 protein led to a reduced rate of growth for the mutant and also renders a strain with attenuated virulence in a murine model of infection (Goldstein and McCusker, 2001). In the parasite *Trypanosoma brucei* it was found that alterations in pyrimidine biosynthesis yielded strains with low infectivity in animal model (Ali *et al.* 2013). Also in *Toxoplasma gondii*, strains with a uracil auxotrophy elicit protective immunity to subsequent infection (Fox and Bzik, 2010). Moreover, Hegewald *et al* (2013) demonstrated that dihydroorotate dehydrogenase (URA1) is a relevant drug target for 1-hydroxyquuinolones in *T. gondii*. Taken together, this work implicates the importance of the pyrimidine biosynthetic pathways in the virulence of multiple microbial pathogens, suggesting that inhibitors of gene products within this pathway could help to treat diverse infections, perhaps in association with other therapeutic drugs. We showed that lack of functional Ura4 in *C. neoformans* affects other virulence factors and reduced stress response; however, at this point our results do not allow us to explain the mechanism of this interference, and new experiments such as global gene expression under pyrimidine depletion could be performed to shed some light on this issue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1A

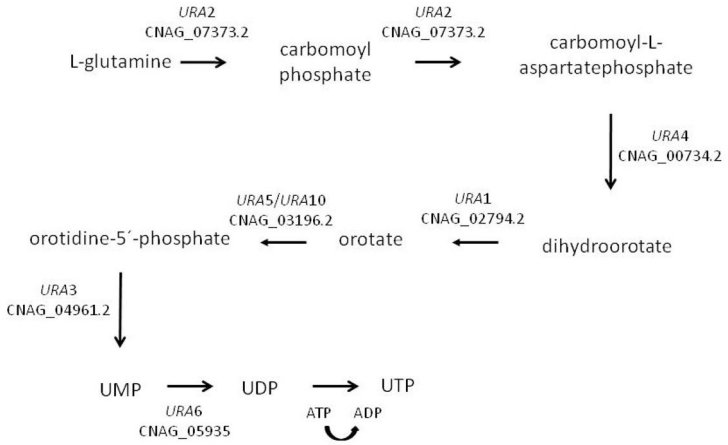


Figure 1B

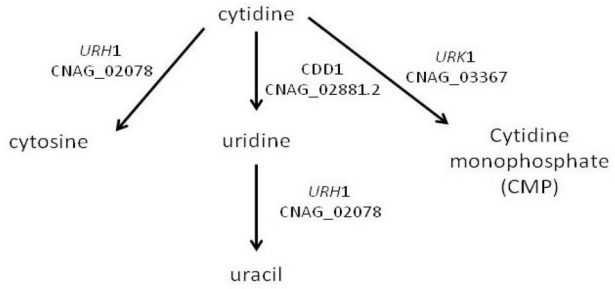


Figure 1.

Representation of the pymidine ribonucleotide *de novo* biosynthetic (A) and salvage (B) pathways. (Adapted from http://www.yeastgenome.org/).

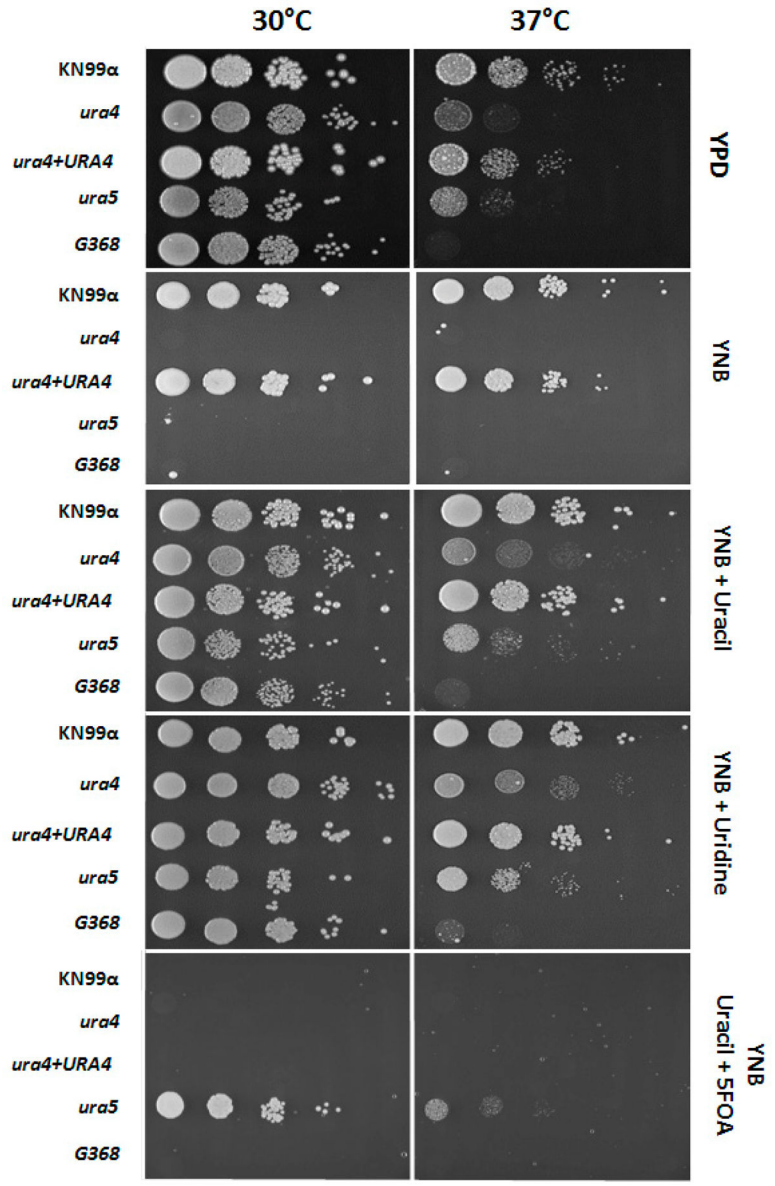


Figure 2.

High temperature growth and auxotrophic analysis. Wild type (KN99α), *ura4*::Neo^R (FGC003), reconstituted strain *ura4*Δ+*URA4* (FGC003R1), *ura*5 mutant and the original insertional mutant G368 were incubated on the following media at 30°C and 37°C for 48 hours: YPD; YNB supplemented with 2% glucose, 5 g/L ammonium sulfate, and amino acids (YNB), with or without addition of uracil (20μM) or uridine (20μM); and 5-FOA (5-fluoroorotic acid) medium (10 mg/mL). From left to right the amount of cells inoculated in each spot is: 1×10^4, 1×10^3, 1×10^2, 1×10^1 and 1 cell.

Figure 3A

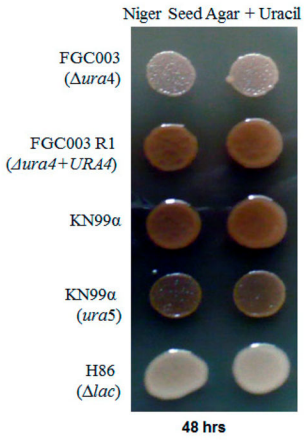
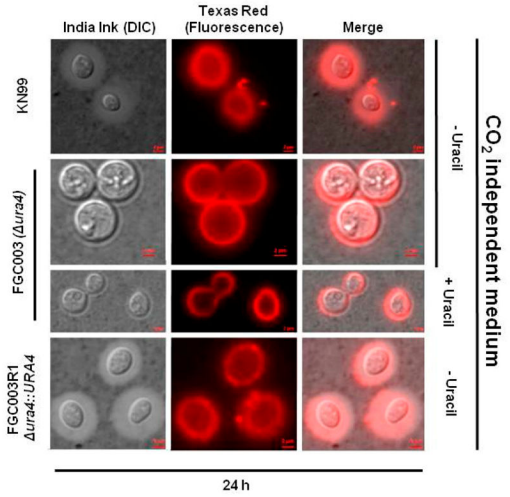


Figure 3B



colored

Figure 3.

Melanin and capsule production in *ura4* mutant. (A) 1×10^5 cells were spotted on Niger Seed Agar medium with or without uracil (20μM) and incubated at 30°C for up to 96 hours. Melanin production is represented by a dark brown pigment. Strains used are: Wild type (KN99α), *ura4*::Neo^R (FGC003), reconstituted strain *ura4+URA4* (FGC003R1), *ura*5 mutant and H86 (laccase-deficient mutant) as negative control. (B) Capsule production detected by India ink staining and DIC microscopy in bright field, immune fluorescence microscopy and both images merged. Medium used was CO_2-independent medium with and without uracil. Image captures were performed after 24 hours post-capsule induction. Strains used: Wild type (KN99α), *ura4*::Neo^R (FGC003), reconstituted strain *ura4*Δ*+URA4* (FGC003R1). Refer to Material and Methods for immunofluorescence details.

Figure 4A

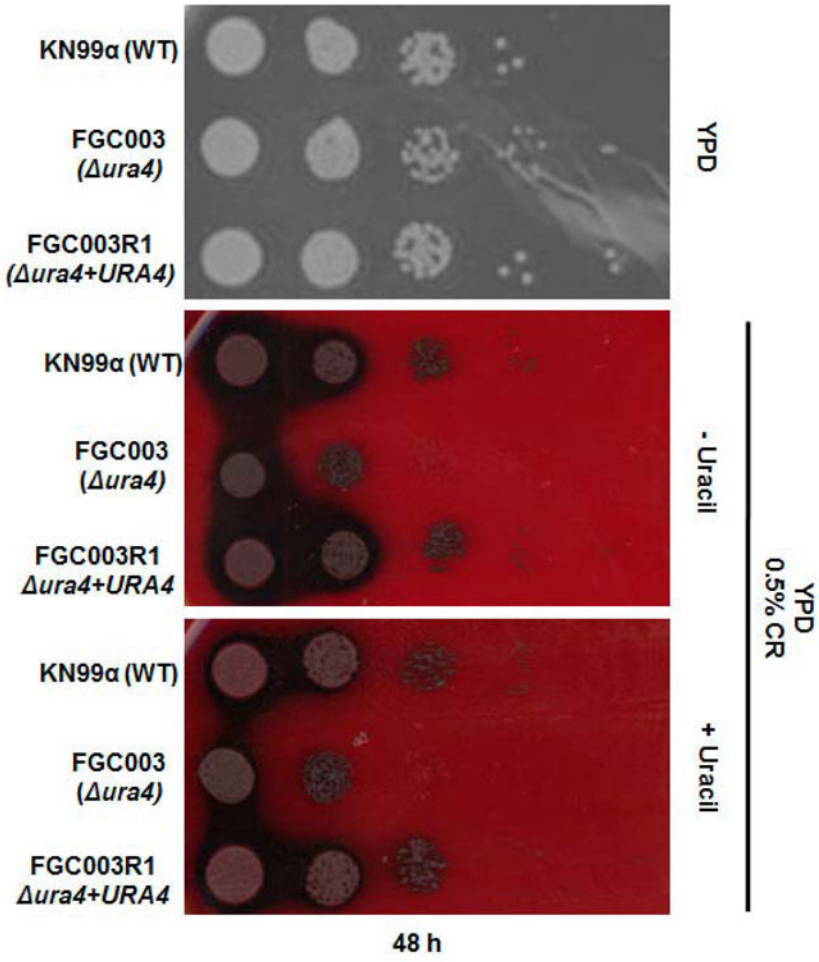


Figure 4B

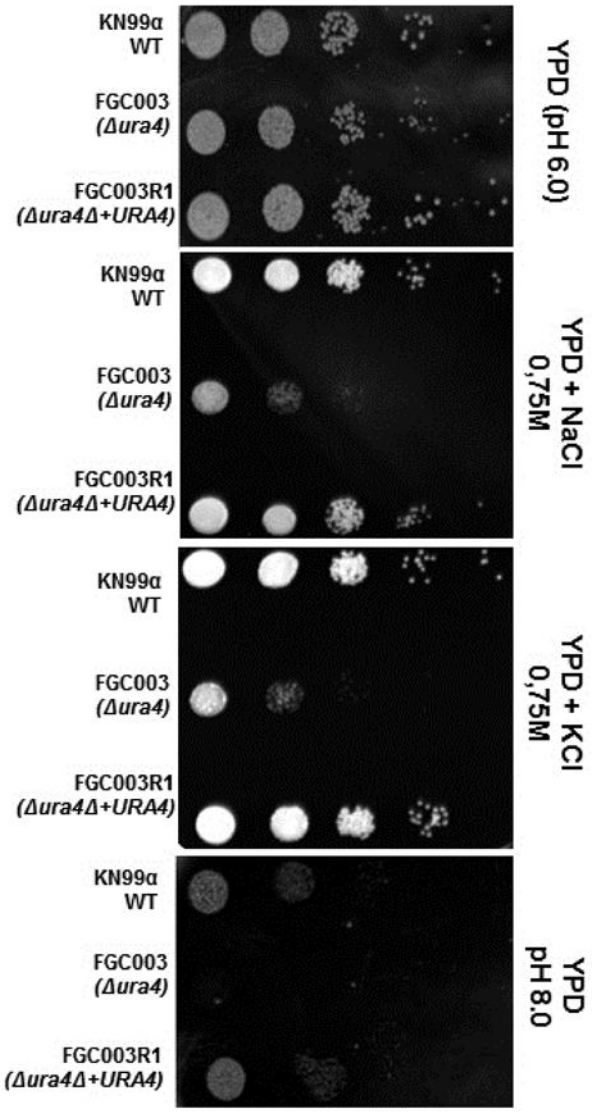


Figure 4.

The *ura4* mutation renders cells susceptible to multi stress. Dilutions (10^4 to 1 cell) of wild type (KN99α), *ura4*::Neo^R (FGC003) and reconstituted strain *ura4*Δ+*URA4* (FGC003R1) were spotted on (A) YPD, YPD + 0.5% Congo Red and YPD + 0.5% Congo Red + Uracil and (B) YPD, YPD + 0.75 M NaCl, YPD + 0.75 M KCl and YPD (pH 8.0). Plates were incubated at 30°C from 48 to 72 hours.

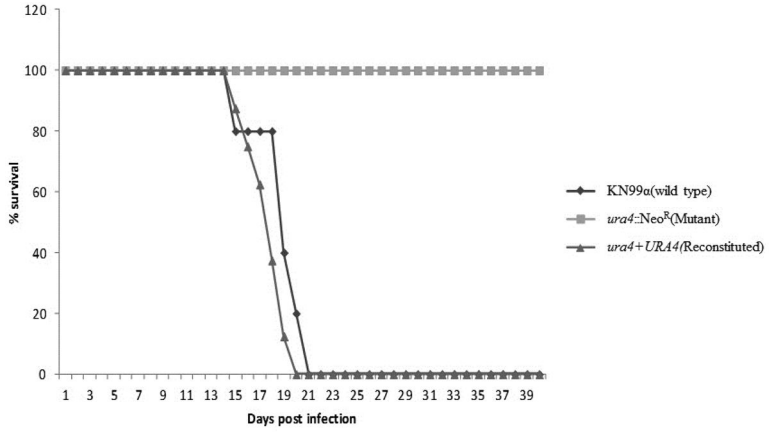


Figure 5.

Virulence test. Groups of ten 4- to 6-week-old 57BL/6 mice were infected by nasal inhalation with wild type (KN99α), *ura4*::Neo^R (FGC003) and reconstituted strain *ura4*Δ*::URA4* (FGC003R1). Survival was followed during the course of the infection. P values were <0.001 for the comparisons between *ura4*::Neo^R (FGC003), and wild type and *ura4*Δ*+URA4* (FGC003R1). Five mice were inoculated with the wild type (KN99α).

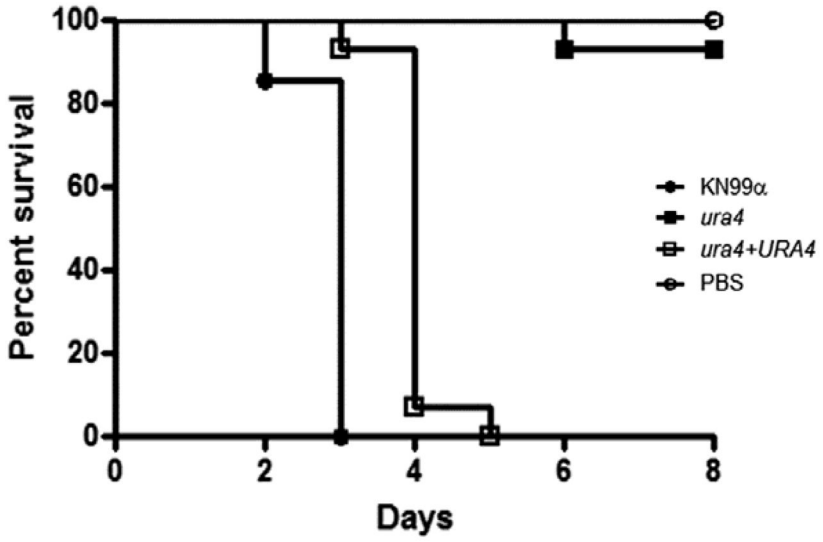


Figure 6.

C. neoformans ura4 mutant is avirulent in an invertebrate infection model at 30°C. *Galleria mellonella* larvae were inoculated with either the wild type (KN99α), *ura4*::Neo^R (FGC003) or reconstituted strain *ura4*Δ+*URA4* (FGC003R1). The infected larvae were monitored for survival

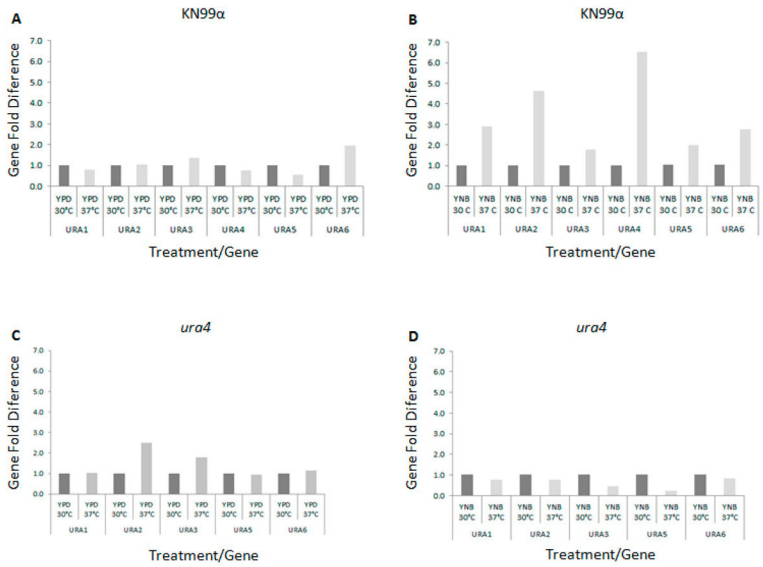


Figure 7.

Real time PCR. Expression pattern of the pyrimidine *de novo* pathway genes *URA1*, *2*, *3*, *4*, *5* and *6* in the wild type strain KN99 cultivated in (A) rich medium YPD and (B) synthetic medium YNB (no uracil added) and the mutant strain *ura4* cultivated in (C) rich medium YPD and (D) synthetic medium YNB (no uracil added) at 30°C and 37°C.

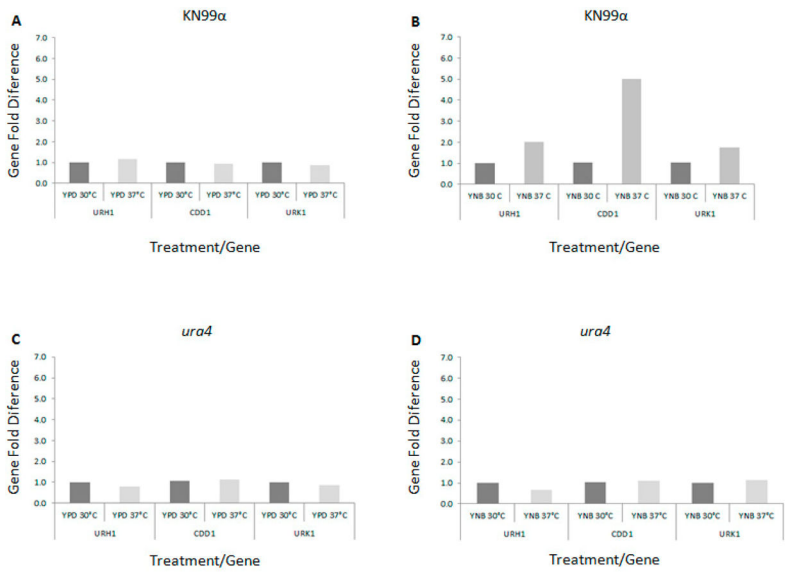


Figure 8.

Real time PCR. Expression pattern of the pyrimidine salvage pathway genes *CDD1*, *URK1*, *URH1* in the wild type strain KN99 cultivated in (A) rich medium YPD and (B) synthetic medium YNB (no uracil added) and the mutant strain *ura4* cultivated in (C) rich medium YPD and (D) synthetic medium YNB (no uracil added) at 30°C and 37°C.