Threonine biosynthetic genes are essential in *Cryptococcus neoformans*

Joanne M. Kingsbury and John H. McCusker

We identified and attempted to disrupt the *Cryptococcus neoformans* homoserine and/or threonine biosynthetic genes encoding aspartate kinase (*HOM3*), homoserine kinase (*THR1*) and threonine synthase (*THR4*); however, each gene proved recalcitrant to disruption. By replacing the endogenous promoters of *HOM3* and *THR1* with the copper-repressible *CTR4-1* promoter, we showed that *HOM3* and *THR1* were essential for the growth of *C. neoformans* in rich media, when ammonium was the nitrogen source, or when threonine was supplied as an amino acid instead of a dipeptide. Moreover, the severity of the growth defect associated with *HOM3* or *THR1* repression increased with increasing incubation temperature. We believe this to be the first demonstration of threonine biosynthetic genes being essential in a fungus. The necessity of these genes for *C. neoformans* growth, particularly at physiologically relevant temperatures, makes threonine biosynthetic genes ideal anti-cryptococcal drug targets.

### INTRODUCTION

Amino acid biosynthetic pathways provide attractive candidates for antifungal drug targets since many of these pathways are conserved throughout the fungi and are absent from humans. One such pathway of interest is the threonine biosynthetic pathway, in which threonine is produced from aspartate in five enzymic steps via the intermediate homoserine, which is also required for methionine synthesis (Fig. 1; reviewed by Jones & Fink 1982). In the yeast *Saccharomyces cerevisiae*, this pathway is regulated at the level of transcription by general control (Hinnebusch, 1992; Mountain et al., 1991), and of enzyme activity, particularly by threonine feedback inhibition of aspartate kinase (Hom3p) at the initial step of the pathway (Martin-Rendon et al., 1993; Ramos & Calderon, 1992). In addition to auxotrophy, a number of deleterious phenotypes have been attributed to threonine biosynthetic mutants (Arevalo-Rodriguez et al., 2004; Birrell et al., 2001, 2002; Care et al., 2004; Deutschbauer et al., 2002; Dunn et al., 2006; Enyenihi & Saunders, 2003; Giaever et al., 2002; Roberg et al., 1997), some defects of which, such as temperature sensitivity, salt sensitivity and being petite-negative, could also influence fungal survival *in vivo*. Moreover, the threonine-biosynthetic intermediate homoserine is also required for biosynthesis of methionine, itself a central metabolite, and threonine is required for isoleucine biosynthesis. Significantly, we and others have shown that various fungal methionine (Met2p, Met3p and Met6p) and isoleucine (Ilv2p), as well as threonine (Hom3p), biosynthetic enzymes are required for fungal survival *in vivo* and/or virulence (Kingsbury et al., 2004a, 2006; Nazi et al., 2007; Pascon et al., 2004; Yang et al., 2002).

Amino acid auxotrophy has been shown to be particularly deleterious in the human-pathogenic fungus *Cryptococcus neoformans*. Compared with *S. cerevisiae*, various auxotrophies are less well supplemented by the amino acids for which they are lacking, particularly in the presence of ammonium, suggesting fewer or less active permeases, or a greater proportion subject to nitrogen repression (Kingsbury et al., 2004a, b; Nazi et al., 2007; Pascon et al., 2004). In addition, auxotrophs show defects in known cryptococcal virulence traits such as the ability to proliferate at 37°C, and melanin and capsule production (Kingsbury et al., 2004a, b; Pascon et al., 2004; Yang et al., 2002). We were therefore interested in evaluating the potential of threonine biosynthetic enzymes as anti-cryptococcal targets in *C. neoformans*. Several attempts to disrupt the homoserine and threonine biosynthetic gene *HOM3* (encoding aspartate kinase, EC 2.7.2.4), and the threonine biosynthetic genes *THR1* (encoding homoserine kinase, EC 2.7.1.39) and *THR4* (encoding threonine synthase, EC 2.4.2.3) were unsuccessful. We demonstrate that this is because *HOM3* and *THR1*, and likely other threonine biosynthetic enzymes, are essential for *C. neoformans* growth in most conditions.

### METHODS

**Strains, media and growth conditions.** All *S. cerevisiae* strains used in this study (Table 1) were isogenic with S288c and *C. neoformans*...
strains were isogenic with H99 serotype A Mat α (Perfect et al., 1993). One Shot Top10 Chemically Competent *Escherichia coli* (Invitrogen) was used for plasmid propagation. Standard yeast and bacterial media were used (Sherman et al., 1979; Sambrook et al., 1989). Where specified, media were supplemented with nourseothricin (Nat; 100 μg ml⁻¹; Hans Knöll Institute für Naturstoff-Forschung, Jena, Germany), geneticin (200 μg ml⁻¹; Life Technologies), proline (1 g l⁻¹), sorbitol (1 M), bathocuproinedisulfonic acid (BCS; 200 μM), cupric sulfate (CuSO₄·25 μM), ascorbic acid (1 mM), threonine (2.5 mM), homoserine (2.5 mM), methionine (0.13 mM), Ala-Thr (2.5 mM) and Met-Leu (0.13 mM).

Identification of *C. neoformans* serotype A HOM3, THR1 and THR4. The predicted *C. neoformans* HOM3, THR1 and THR4 genes were initially identified from a *C. neoformans* serotype D strain JEC21 database that had been annotated by a genome-wide BLAST search (Lofus et al., 2005). NCBI accession numbers for the predicted Hom3p, Thr1p and Thr4p included XP_572658, XP_572893 and XP_568789, respectively. Sequences were then BLASTed against the *C. neoformans* serotype A strain H99 sequence. Serotype A HOM3, THR1 and THR4 occurred in sequence with the NCBI accession numbers AAC00200077.1, AAC002000074.1 and AAC002000068.1, respectively.

We also attempted to isolate the *C. neoformans* HOM3, THR1 and THR4 cDNAs by complementation of the methionine and/or threonine auxotrophies of *S. cerevisiae* hom3Δ, thr4Δ and thr4A strains, using a *C. neoformans* cDNA library. Specifically, *S. cerevisiae* strains YJK2416 (hom3Δ ura3Δ), YJK1358 (thr4Δ ura3Δ) and S318 (thr4A ura3Δ) were transformed by lithium acetate-mediated transformation (Gietz et al., 1995), with a library that contained *C. neoformans* H99 cDNAs under the control of the *S. cerevisiae* GAL1 promoter in the pYES2.0 vector (Invitrogen) (Suvarna et al., 2000). Ura⁺ transformants were screened for the acquisition of methionine and/or threonine prototrophy in the presence of galactose, but not glucose, as a carbon source. Plasmids that conferred prototrophy were isolated, propagated in *E. coli* DH10B, then analysed by restriction analysis, and sequenced by the Duke University Cancer Center Sequencing Facility. Plasmids included pJO373 (pYES2.0 + *C. neoformans* THR4 cDNA, NCBI accession number EU623435), and pJO378 (pYES2.0 + *C. neoformans* THR4 cDNA, NCBI accession number EU635873).

**Table 1. Strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tr>
<td><em>C. neoformans</em></td>
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<tr>
<td>H99</td>
<td>Matα</td>
<td>Perfect et al. (1993)</td>
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<tr>
<td>H99-73</td>
<td>NAT1-PCTR4-1::THR1</td>
<td>This study</td>
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<tr>
<td>H99-76</td>
<td>NAT1-PCTR4-1::HOM3</td>
<td>This study</td>
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<tr>
<td><em>S. cerevisiae</em></td>
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<tr>
<td>S157</td>
<td>ura3Δ</td>
<td>Yang et al. (2002)</td>
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<td>S318</td>
<td>ura3Δ thr4Δ::natMX4</td>
<td>This study</td>
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<tr>
<td>YJK1358</td>
<td>thr4Δ::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>YJK2416</td>
<td>ura3Δ hom3Δ::natMX4</td>
<td>This study</td>
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was cloned into pCR2.1-TOPO (pJO308). All plasmid constructions were confirmed by restriction digestion and PCR analyses.

The targeting cassettes were PCR-amplified from their respective plasmids, and introduced into strain H99 by biolistic transformation (Toffaletti et al., 1993). For transformation with the thr4::NAT1, thr4::NEO, thr1::NAT1, and hom3::NAT1 constructs, transformation was performed on YPD + sorbitol, and after a 4 h incubation, cells were scraped off plates and spread on YPD + NAT or G418 plates to select for transformants. Transformants were purified and plated on SD to screen for acquisition of auxotrophy. For transformation with the NAT1-PCTR4-1-HOM3 and NAT1-PCTR4-1-THR1 constructs, cells were plated on YPD + sorbitol + BCS and incubated for 2–3 h prior to transformation to allow for expression from PCTR4. Following transformation, plates were incubated for 4 h and cells were replated on YPD + BCS + NAT. Purified transformants were screened for acquisition of auxotrophy on SD + CuSO4 + ascorbic acid plates (PCTR4-1-repressing conditions). The NAT1-PCTR4-1-THR1 genotype in strain H99-73 and NAT1-PCTR4-1-HOM3 genotype in strain H99-76 was confirmed by PCR (primer pairs JO281 + JO300 and JO506 + JO280 for H99-73, and JO281 + JO320 and JO505 + JO280 for H99-76) and Southern hybridization analysis (Fig. 2).

The HOM3, THR1 and THR4 genes were replaced in the S. cerevisiae S157 strain by the natMX4 or kanMX4 cassettes, using PCR-mediated gene disruption (Goldstein & McCusker, 1999; Wach et al., 1994).

**Fig. 2.** (a) The HOM3 and THR1 genes were placed under the control of the PCTR4-1 promoter by inserting the NAT1-PCTR4-1 construct immediately upstream of the predicted ORF, as shown in the diagram. (b) Southern hybridization analysis confirming correct positioning of the NAT1-PCTR4-1 construct in strains H99-73 (PCTR4-1-THR1) and H99-76 (PCTR4-1-HOM3). Genomic DNA from strains H99 (wild-type), H99-73 and H99-76 was digested with the restriction enzymes indicated, and blots were hybridized as indicated with a HOM3 or THR1 DNA probe, amplified using primer pairs JO414 + JO413 and JO506 + JO362, respectively.
Gene deletions were confirmed by PCR, and by acquisition of methionine and/or threonine auxotrophy.

**Manipulation of nucleic acids.** Plasmid DNA from *E. coli* was extracted using the QIAprep Spin Miniprep kit (Qiagen), according to the manufacturer's instructions. Extraction of plasmid DNA from *S. cerevisiae*, and genomic DNA from *C. neoformans* for PCR analysis, was performed as described previously (Hoffman & Winston, 1987). Genomic DNA from *C. neoformans* for Southern hybridization analysis was isolated as described previously (Yang et al., 2002), 2 µg of which was digested with various restriction enzymes, separated by electrophoresis on a 0.75 % (w/v) agarose gel, denatured and transferred to a nylon membrane (Roche), as described previously (Sambrook et al., 1989).

RNA for Northern analyses was prepared from cells that had first been grown to a density of approximately 2 × 10⁸ cells ml⁻¹, in 50 ml YPD + BCS. Cells were harvested, washed twice in sterile water, then split four ways and incubated with shaking in 50 ml YPD + BCS or YPD + CuSO₄ + ascorbic acid, at 25 °C or 37 °C. Following incubation for 5 h, RNA was isolated as described previously (Yang et al., 2002). Each sample was prepared in duplicate, and 10 µg duplicates of each preparation were separated in a 1 % (w/v) agarose-formaldehyde gel, and transferred to a nylon membrane.

Probes for Southern and Northern hybridizations were prepared from gel-purified PCR products. Specifically, probes for Southern hybridizations were amplified using primer pairs JO413 + JO414 (*HOM3*) and JO362 + JO506 (*THR1*). Primer pairs for amplification of Northern hybridization probes included JO770 + JO772 (*HOM3*), JO298 + JO363 (*THR1*), JO223 + JO225 (*GPD*) and JO765 + JO766 (*CTR4*) (see Table S1 for primer sequences). Probes were labelled with [α-³²P]dCTP (Perkin-Elmer) using the Rediprimell Random Prime Labelling System (Amersham Biosciences), according to the manufacturer's instructions. Blots were prehybridized and hybridized in ULTRAhyb buffer (Ambion), and washed according to the manufacturer's instructions. Membrane signal was visualized using a Typhoon 9200 Variable Mode Imager (Molecular Dynamics), and band signal intensity was quantified using ImageQuaNT 5.2 software (Molecular Dynamics).

**RESULTS AND DISCUSSION**

**Identification of *HOM3*, *THR1* and *THR4* in *C. neoformans***

Given the absence of the threonine biosynthetic pathway in humans (Payne & Loomis, 2006) and the avirulence or inability to survive *in vivo* of various amino acid auxotrophs, we were interested in assessing the potential of threonine biosynthetic enzymes as antifungal drug targets in the human-pathogenic fungus *C. neoformans*. In particular, we focused on, first, the aspartate kinase (encoded by *HOM3*), the initial and key feedback regulatory enzyme of the pathway. The avirulence of other cryptococcal methionine auxotrophs (Nazi et al., 2007; Pascon et al., 2004; Yang et al., 2002) indicates that *C. neoformans* is unable to supplement this auxotrophy in the *in vivo* environment, thus we reasoned that the combined threonine and methionine auxotrophies of *hom3* mutants should be even more detrimental to survival *in vivo* and/or virulence. Moreover, *HOM3* is required for the *in vivo* survival of *S. cerevisiae* (Kingsbury et al., 2006). We were also interested in the final two steps of threonine biosynthesis, catalysed by homoserine kinase (encoded by *THR1*) and threonine synthase (encoded by *THR4*). Mutation of these genes in *S. cerevisiae* results in a plethora of deleterious phenotypes in addition to auxotrophy (Birrell et al., 2001, 2002; Care et al., 2004; Deutschbauer et al., 2002; Dunn et al., 2006; Enyenihi & Saunders, 2003; Giaever et al., 2002; Roberg et al., 1997), which may also influence *in vivo* survival and/or virulence.

We identified the putative *C. neoformans* H99 *HOM3*, *THR1* and *THR4* genes from the *C. neoformans* serotype A strain H99 through sequence similarity with the respective predicted ORFs in serotype D. Furthermore, cDNAs matching the predicted *THR1* and *THR4* genes were isolated from a *C. neoformans* H99 cDNA library based on the ability to confer threonine prototrophy to *S. cerevisiae thr1Δ* and *thr4Δ* strains, respectively (Fig. 3), thus verifying that the identified genes encoded the predicted enzyme activities. We were unable to isolate the *HOM3* cDNA by complementation of a *S. cerevisiae hom3Δ* strain, however, likely due to under-representation of the *HOM3* cDNA in the library. Consistent with this, we were unable to PCR-amplify the *HOM3* cDNA from the library DNA. The *C. neoformans* H99 *HOM3*, *THR1* and *THR4* genes were predicted to contain six, two and five introns, respectively. The predicted *C. neoformans* *Hom3p*, *Thr1p* and *Thr4p* sequences were highly similar to the corresponding

![Fig. 3. Functional complementation of *S. cerevisiae thr1* (YJK1358) and *thr4* (S318) strains by *C. neoformans* *THR1* and *THR4* cDNA. Tenfold dilutions of YJK1358 transformed with pYES2.0 or pJO373 (pYES2.0/cn*THR1* cDNA), and S318 transformed with pYES2.0 or pJO378 (pYES2.0/cn*THR4* cDNA), were plated on SD (synthetic dextrose) or synthetic galactose (SGal) medium, and incubated at 30 °C for 3 days.](image-url)
proteins in *S. cerevisiae*, with approximately 50, 56 and 50% amino acid identity, respectively.

**C. neoformans HOM3, THR1 and THR4 are recalcitrant to disruption**

In order to study the phenotypes of *C. neoformans* hom3, thr1 and thr4 mutants, we attempted to disrupt HOM3, THR1 and THR4 in the serotype A strain H99, using hom3::NAT1, thr1::NAT1, thr4::NAT1 and thr4::NEO targeting cassettes. However, no auxotrophic mutants were obtained after screening 151 transformants for HOM3 disruption, 89 transformants for THR1 disruption, and 469 transformants for THR4 disruption. An inability to disrupt these genes may be due to HOM3, THR1 and THR4 being essential for *C. neoformans* growth.

**C. neoformans HOM3 and THR1 are essential**

To determine whether threonine biosynthetic genes are essential in *C. neoformans*, we replaced the endogenous promoters of HOM3 and THR1 with the P<sub>CTR4-1</sub> promoter (Ory et al., 2004), thereby placing the genes under copper-repressible control (Fig. 2). Growth of the wild-type (H99), P<sub>CTR4-1</sub>-HOM3 (H99-76) and P<sub>CTR4-1</sub>-THR1 (H99-73) strains was compared by plating 10-fold dilutions of strains, pre-grown in YPD + BCS, onto YPD + BCS (promoter-inducing conditions) and YPD + CuSO<sub>4</sub> + ascorbic acid (promoter-repressing conditions) (Fig. 4). After incubation at 30°C for 3 days in promoter-inducing conditions, P<sub>CTR4-1</sub>-HOM3 and P<sub>CTR4-1</sub>-THR1 strains grew considerably, although less well than the wild-type as judged by colony size, indicating that HOM3 and THR1 were expressed at different from normal level in these strains. However, no colony formation was observed for the P<sub>CTR4-1</sub>-HOM3 and P<sub>CTR4-1</sub>-THR1 strains in promoter-repressing conditions. Since the yeast extract and Bacto peptone in YPD contains significant levels of threonine and methionine for supplementation of Thr and Met auxotrophies (Difco Manual, 11th edition), these results indicate that THR1 and HOM3 are essential in *C. neoformans*, even in the presence of abundant threonine and methionine.

**The essential phenotype is dependent on nitrogen source and amino acid form**

The lack of growth of the P<sub>CTR4-1</sub>-HOM3 strain on rich medium (YPD) in promoter-repressing conditions is likely not due to an inability of *C. neoformans* to supplement the methionine auxotrophy of this strain, since *C. neoformans* met2, met3 and met6 mutants all grow on YPD, and their methionine auxotrophy can be supplemented by methionine or methionine dipeptides, when either ammonium or proline is the nitrogen source (Nazi et al., 2007; Pascon et al., 2004; Yang et al., 2002). Since both P<sub>CTR4-1</sub>-HOM3 and P<sub>CTR4-1</sub>-THR1 strains were unable to grow under promoter-repressing conditions, the growth deficiency on YPD may instead be due to *C. neoformans* being unable to satisfy the threonine auxotrophy in this medium.

Growth of the P<sub>CTR4-1</sub>-HOM3 and P<sub>CTR4-1</sub>-THR1 strains was compared by spot dilutions on minimal medium containing ammonium (SD) or proline [SD(Pro)] as the nitrogen source, supplemented with various combinations of homoserine, methionine and threonine amino acids or dipeptides, in P<sub>CTR4-1</sub>-repression conditions (Fig. 5). While no colony formation of strains was observed in P<sub>CTR4-1</sub>-repression conditions in the absence of amino acid supplements, residual growth similar to that present on YPD in repressing conditions was observed, which we attribute to a basal level of gene expression still occurring, and/or utilization of cell reserves accumulated during the pre-growth in gene-expressing conditions. Strains grew no better when the amino acids methionine and threonine were added to either SD or SD(Pro) medium than on

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**Fig. 4.** Temperature-dependent phenotypes of P<sub>CTR4-1</sub>-HOM3 (H99-76), P<sub>CTR4-1</sub>-THR1 (H99-73) and the wild-type (H99) strains in gene-repressing (CuSO<sub>4</sub> + ascorbic acid) and gene-inducing (BCS) conditions. Tenfold dilutions of strains were plated and incubated for 3 days.
media lacking amino acids. Strains were also unable to grow when SD medium was supplemented with threonine and methionine dipeptides, but growth was enhanced above background levels when the threonine and methionine dipeptides were added to SD(Pro) medium. Furthermore, growth of the P<sup>CTR4</sup>-1-<i>HOM3</i> strain required only threonine dipeptides, while growth of the P<sup>CTR4</sup>-1-<i>HOM3</i> strain required both methionine and threonine dipeptides, or homoserine, providing further evidence that these genes indeed confer homoserine kinase and aspartate kinase activities, respectively.

The demonstration that threonine dipeptides, but not amino acids, supplement cryptococcal threonine auxotrophy, and only in the presence of proline, but not ammonium, as a sole nitrogen source, is similar to results observed previously for supplementation of other <i>C. neoformans</i> auxotrophies (Kingsbury et al., 2004a, b). Combined, these results indicate that <i>C. neoformans</i> possesses fewer amino acid permeases, permeases that have a lower transport velocity, or more permeases that are subject to ammonium repression. Although YPD medium contains significant levels of peptides, our results indicate that the lack of growth in this medium is likely due to nitrogen repression, and/or transport of other peptides outcompeting transport of threonine-containing peptides.

### Temperature determines severity of phenotype

While the inability of a cryptococcal amino acid auxotroph to grow on YPD at 30 °C has not been previously demonstrated, it has been shown that <i>SPE3-lys9</i> (lysine auxotrophic) and <i>ilv2</i> (isoleucine and valine auxotrophic) mutants die in YPD at 37 °C (Kingsbury et al., 2004a, b). We therefore determined whether the incubation temperature affects growth of P<sup>CTR4</sup>-1-<i>HOM3</i> and P<sup>CTR4</sup>-1-<i>THR1</i> strains on YPD. Interestingly, reduction of the incubation temperature to 25 °C allowed the growth of the P<sup>CTR4</sup>-1-<i>HOM3</i> strain in repressing conditions, while the P<sup>CTR4</sup>-1-<i>THR1</i> strain was no better able to grow at this temperature than at 30 °C (Fig. 4).

Differences in the growth phenotype between the two strains could be attributable to either homoserine kinase having a role in addition to threonine biosynthesis in <i>C. neoformans</i>, or the block in the biosynthetic pathway caused by inhibition of homoserine kinase leading to the accumulation of a toxic intermediate. Consistent with this, elevated levels of the intermediate predicted to accumulate, homoserine, are toxic to mammalian (Rees et al., 1994) and bacterial cells (Kotre et al., 1973; O‘Barr & Everett, 1971). Moreover, the accumulation of a toxic intermediate has been hypothesized to be responsible for toxic effects associated with other amino acid biosynthetic mutants (Arevalo-Rodriguez et al., 2004; Kingsbury et al., 2004a; Pascon et al., 2004; Suliman et al., 2007), and may explain deleterious phenotypes associated with <i>THR1</i> and <i>THR4</i> mutation in <i>S. cerevisiae</i>. In <i>S. cerevisiae</i>, the threonine pathway is regulated positively in response to threonine starvation, by upregulation of gene transcription and eliminating feedback inhibition (Hinnebusch, 1992; Martin-Rendon et al., 1993; Mountain et al., 1991; Ramos & Calderon, 1992). If the pathway is similarly regulated in <i>C. neoformans</i>, threonine starvation conditions, such as what Thr1p-inhibited <i>C. neoformans</i> would likely encounter in vivo, should result in increased flux through the threonine biosynthetic pathway, thus increased toxic intermediate accumulation, and hence intensified growth defects.

We also compared the growth of P<sup>CTR4</sup>-1-<i>HOM3</i> and P<sup>CTR4</sup>-1-<i>THR1</i> strains at 37 °C. While some background growth of both strains was still present at 30 °C in repressing conditions, growth was completely eliminated at
37 °C in repressing conditions. Surprisingly, growth was also eliminated in induction conditions, which may indicate that there is a greater difference between expression from the CTR4-1 promoter and endogenous THR1 and HOM3 expression at 37 °C, compared with 30 °C. To examine this, we compared HOM3, THR1 and CTR4 transcript levels following a 5 h incubation of the P_{CTR4-1}HOM3, P_{CTR4-1}THR1 and wild-type strains, in promoter-induction and repression conditions, at 25 and 37 °C (Fig. 6). Following normalization to the GPD housekeeping gene, we observed no obvious temperature-dependent changes in THR1, HOM3 or CTR4 transcript levels from the P_{CTR4-1}HOM3, P_{CTR4-1}THR1 and wild-type strains, respectively, grown in promoter-induction conditions; thus the CTR4 (and CTR4-1) promoter is not regulated by temperature. Furthermore, although HOM3 transcript levels were barely detectable at both temperatures, THR1 and HOM3 transcription levels in the wild-type did not appear to be enhanced at 37 °C compared with 25 °C. Results also show that HOM3 and THR1 transcripts expressed from P_{CTR4-1}HOM3 and P_{CTR4-1}THR1 strains in induction conditions were at higher than wild-type levels at both 30 and 37 °C. One possible explanation for the lack of growth in induction conditions is that higher expression of these genes might result in growth impairment by perturbing metabolic flux, interfering with general cell metabolism, which may be accentuated at higher temperatures. Given our SPE3-lys9 and ilv2 findings, the increased severity of growth defects at 37 °C in repressing conditions may be due to decreased threonine transport at this temperature.

Given the different niche occupation and evolutionary distance between S. cerevisiae, an ascomycete, and C. neoformans, a basidiomycete, it is not surprising to see differences in gene requirement between species; for example, the fatty acid synthesis genes FAS1 and FAS2, and RAM1 required for signalling, are essential in C. neoformans but not in S. cerevisiae and/or Candida albicans (Chayakulkeeree et al., 2007; Vallim et al., 2004). However, given the highly conserved nature of the threonine biosynthetic pathway between fungi, it is surprising to find that threonine biosynthetic genes are essential in C. neoformans, and to our knowledge, this is the first documented case of threonine biosynthetic gene necessity in fungi. The essential nature of these genes, particularly at 37 °C, makes aspartate kinase and homoserine kinase excellent candidates for anti-cryptococcal drug targets.

**ACKNOWLEDGEMENTS**

The authors would like to thank Dr Zhonghui Yang for some strain construction, Dr Joseph Heitman for comments on the manuscript, and Drs James Fraser and Tamara Doering for the generous gifts of plasmids pJAF1 and pCTR4-2, respectively. We also thank Dr Brian Wong for the C. neoformans cDNA library, which was a gift to the Duke University Mycology Research Unit. This study was funded by the National Institutes of Health R01 grant GM070541 and R21 grant AI070247.

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Edited by: J. F. Ernst