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Threonine biosynthetic genes are essential in *Cryptococcus neoformans*

Joanne M. Kingsbury, **John H. McCusker**

Department of Molecular Genetics and Microbiology, Box 3020, Duke University Medical Center, Durham, NC 27710, USA

Summary

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. This study comprises 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 enzymatic steps via the intermediate homoserine, which is also required for methionine synthesis (Fig. 1, reviewed in (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

Corresponding author: John H. McCusker, Department of Molecular Genetics and Microbiology, Box 3020, Duke University Medical Center, Durham, NC 27710, U.S.A., Tel: + 1 919 681 6744. Fax: + 1 919 684 8735. e-mail: mccus001@mc.duke.edu, website: http://www.duke.edu/web/microlabs/mccusker/.

biosynthetic mutants (Arevalo-Rodriguez *et al.*, 2004; Birrell *et al.*, 2001; Birrell *et al.*, 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; Kingsbury *et al.*, 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; Kingsbury *et al.*, 2004b; 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; Kingsbury *et al.*, 2004b; 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: 4.2.3.1) 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 were isogenic with S288c and *C. neoformans* strains were isogenic with H99 (Serotype A Mat α, (Perfect *et al.*, 1993)), and listed in Table 1. One Shot Top10 Chemically Competent *Escherichia coli* (Invitrogen) was used for plasmid propagation. Standard yeast and bacterial media were prepared as described previously (Sambrook *et al.*, 1989; Sherman *et al.*, 1974). Where specified, media was 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 ($CuSO_4$, 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 (Loftus *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 *Cryptococcus neoformans* Serotype A strain H99 sequence. Serotype A *HOM3*, *THR1* and *THR4* occurred in sequence with the NCBI accession numbers AACO02000077.1, AACO02000074.1 and AACO02000068.1, 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 hom3*Δ, *thr1*Δ, and *thr4*Δ strains, using a *C. neoformans* cDNA library. Specifically, *S. cerevisiae* strains YJK2416 (*hom3*Δ *ura3*Δ), YJK1358 (*thr1*Δ *ura3*Δ), and S318 (*thr4*Δ *ura3*Δ), 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 dextrose, as a carbon source. Plasmids that conferred prototrophy were isolated, propagated in *E. coli* DH10B, then analyzed by restriction analysis, and sequenced by the Duke University Cancer Center Sequencing Facility. Plasmids included pJO373 (pYES2.0 + *C. neoformans THR1* cDNA, NCBI accession number EU623435), and pJO378 (pYES2.0 + *C. neoformans THR4* cDNA, NCBI accession number EU635873).

Plasmid and strain construction

In vitro C. neoformans thr4::NAT1, *thr4*::NEO, *thr1*::NAT1, and *hom3*::NAT1 targeting cassettes were constructed using a modified PCR fusion technique (Davidson *et al.*, 2002). To construct the *thr4*::NAT1 and *thr4*::NEO targeting cassettes, the first rounds of PCR amplified 5′ and 3′ *THR4* sequence from H99 genomic DNA (primer pairs ZY125 + JO257, and ZY126 + JO255, respectively), and the NAT1 cassette from pGMC200 (McDade & Cox, 2001) or the NEO cassette from pJAF1 (Fraser *et al.*, 2003) (primers JO254 + JO256). The gel-purified products were used as a template in the final fusion PCR reaction with primers ZY125 + ZY126. To construct the *thr1*::NAT1 targeting construct, the first round of PCR consisted of amplification of 5′ and 3′ *THR1* sequence from H99 genomic DNA (primer pairs JO298 + JO303, and JO302 + JO300, respectively), and the NAT1 cassette from pGMC200 (primers JO301 + JO304), then products were combined for the fusion PCR using primers JO298 + JO300. Construction of the *hom3*::NAT1 cassette consisted of amplification of 5′ and 3′ *HOM3* sequence from H99 genomic DNA (primer pairs JO318 + JO316, and JO320 + JO315, respectively), and NAT1 from pGMC200 (primers JO314 + JO317), followed by fusion of products in the final PCR reaction with primers JO318 + JO320. All gel-purified constructs were cloned into pCR2.1-TOPO (Invitrogen) according to manufacturer's instructions. Plasmids consisted of pJO193 (*thr4*::NAT1), pJO221 (*thr4*::NEO), pJO249 (*thr1*::NAT1), and pJO260 (*hom3*::NAT1).

The NAT1-$P_{CTR4\text{-}1}$ cassette-containing plasmid pJO306 was also created by fusion PCR. The first round of PCR consisted of amplification of NAT1 from pGMC200 (primers JO357 + JO412) and $P_{CTR4.1}$ from template pCTR4.2 (Ory *et al.*, 2004) (primers JO408 + JO409).

The purified products were used as a template in the final fusion PCR reaction using primers JO357 + JO409, and the gel-purified final product was cloned into pCR2.1-TOPO.

In vitro targeting cassettes were constructed to place *HOM3* and *THR1* under control of the *CTR4-1* promoter. To create the NAT1- P_{CTR4-1}-*HOM3* cassette, *HOM3* upstream and 5′ gene sequence were PCR-amplified from H99 genomic DNA (primer pairs JO414 + JO415, and JO416 + JO413), and NAT1- P_{CTR4-1} was amplified from pJO306 (primers JO357 + JO409). The products were combined in the fusion PCR reaction (primers JO413 + JO414), and the resulting product was gel-purified and cloned into pCR2.1-TOPO (pJO310). The first round of PCR for construction of the NAT1- P_{CTR4-1}-*THR1* cassette consisted of amplification of *THR1* upstream and 5′ gene sequence (primer pairs JO359 + JO360, and JO410 + JO362), and NAT1- P_{CTR4-1} from pJO306 (primers JO357 + JO409). The products were used as a template for the final fusion PCR reaction using primers JO359 + JO362, and the resulting product 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- P_{CTR4-1}-*HOM3* and NAT1- P_{CTR4-1}–*THR1* constructs, cells were plated on YPD + sorbitol + BCS and incubated for 2-3 h prior to transformation to allow for expression from P_{CTR4}. Following transformation, plates were incubated for 4 h and cells were replated on YPD + BCS + NAT. Purified transformants were screened from acquisition of auxotrophy on SD + $CuSO_4$ + ascorbic acid plates (P_{CTR4-1}-repressing conditions). The NAT1-P_{CTR4-1}-*THR1* genotype in strain H99-73 and NAT1-P_{CTR4-1}-*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 (Figure 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). 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 2×10^8 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 + $CuSO_4$ + 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 + JO362 (*THR1*), JO223 + JO225 (*GPD*), and JO765 + JO766 (*CTR4*). Probes were labeled with [α-^{32}P]dCTP (Perkin-Elmer) using the RediprimeII Random Prime Labeling System (Amersham Biosciences), according to the manufacturer's instructions. Blots were prehybridized and hybridized in ULTRAhyb buffer (Ambion), and washed according to 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).

All primers used in this study are listed in Table S1 (Supplemental Information).

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, catalyzed 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; Birrell *et al.*, 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 *Cryptococcus 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 thr1*Δ and *thr4*Δ strains respectively (Figure 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 hom3*Δ 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 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 because *HOM3*, *THR1* and *THR4* are 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 $P_{CTR4\text{-}1}$ promoter (Ory *et al.*, 2004), thereby placing the genes under copper-repressible control (Figure 2). Growth of the wildtype (H99), $P_{CTR4\text{-}1}$-*HOM3* (H99-76) and $P_{CTR4\text{-}1}$-*THR1* (H99-73) strains were compared by plating 10-fold spot dilutions of strains that had been pre-grown in YPD + BCS, onto YPD + BCS (promoter-inducing conditions) and YPD + Cu + ascorbic acid (promoter-repressing conditions) (Figure 4). After incubation at 30 °C for three days in promoter-inducing conditions, $P_{CTR4\text{-}1}$-*HOM3* and $P_{CTR4\text{-}1}$-*THR1* strains grew considerably, although less well than the wildtype as judged by colony size, indicating that *HOM3* and *THR1* were expressed at different than normal levels in these strains. However, no colony formation was observed for the $P_{CTR4\text{-}1}$-*HOM3* and $P_{CTR4\text{-}1}$-*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.

Essential phenotype is dependent on nitrogen source and amino acid form

The lack of growth of the $P_{CTR4\text{-}1}$-*HOM3* strain on rich media (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, both when ammonium or proline are the nitrogen source (Nazi *et al.*, 2007; Pascon *et al.*, 2004; Yang *et al.*, 2002). Since both $P_{CTR4\text{-}1}$-*HOM3* and $P_{CTR4\text{-}1}$-*THR1* strains were unable to grow under promoter-repressing conditions, the growth deficiency on

YPD may instead be because *C. neoformans* is unable to satisfy the threonine auxotrophy in this medium.

Growth of the $P_{CTR4\text{-}1}$-*HOM3* and $P_{CTR4\text{-}1}$-*THR1* strains was compared by spot dilutions on minimal media 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 $P_{CTR4\text{-}1}$ repression conditions (Figure 5). While no colony formation of strains was observed in $P_{CTR4\text{-}1}$ 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) media, than on media lacking amino acids. Strains were also unable to grow when SD media 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) media. Furthermore, growth of the $P_{CTR4\text{-}1}$-*THR1* strain required only threonine dipeptides, while the $P_{CTR4\text{-}1}$-*HOM3* strain growth 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 *C. neoformans* auxotrophies (Kingsbury *et al.*, 2004a; Kingsbury *et al.*, 2004b). Combined, these results indicate that *C. neoformans* possesses less amino acid permeases, permeases that have a lower transport velocity, or more are subject to ammonium repression. While YPD medium contains significant levels of peptides, our results indicate that the lack of growth in this media is likely due to nitrogen repression, and/or transport of other peptides out competing 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 *SPE3-lys9* (lysine auxotrophic) and *ilv2* (isoleucine and valine auxotrophic) mutants die in YPD at 37 °C (Kingsbury *et al.*, 2004a; Kingsbury *et al.*, 2004b). We therefore determined whether the incubation temperature affects growth of $P_{CTR4\text{-}1}$-*HOM3* and $P_{CTR4\text{-}1}$-*THR1* strains on YPD. Interestingly, reduction of the incubation temperature to 25 °C allowed the growth of $P_{CTR4\text{-}1}$-*HOM3* strain in repressing conditions, while the $P_{CTR4\text{-}1}$-*THR1* strain was no better able to grow at this temperature than at 30 °C (Figure 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 *C. neoformans*, 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, is toxic to mammalian (Rees *et al.*, 1994) and bacterial cells (Kotre *et al.*, 1973; O'Barr T & 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 *THR1* and *THR4* mutation in *S. cerevisiae*. In *S. cerevisiae*, 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 *C. neoformans*, threonine starvation conditions, such as what Thr1p-inhibited *C. neoformans* 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 $P_{CTR4\text{-}1}$-*HOM3* and $P_{CTR4\text{-}1}$-*THR1* 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 hr incubation of the $P_{CTR4\text{-}1}$-*HOM3*, $P_{CTR4\text{-}1}$-*THR1* and wildtype strains, in promoter-induction and repression conditions, at 25 °C and 37 °C (Figure 6). Following normalization to the *GPD* housekeeping gene, we observed no obvious temperature-dependent changes in *THR1*, *HOM3*, or *CTR4* transcript levels from the $P_{CTR4\text{-}1}$-*HOM3*, $P_{CTR4\text{-}1}$-*THR1* and wildtype 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 in both temperatures, *THR1* and *HOM3* transcription levels in the wildtype did not appear to be enhanced at 37 °C compared with 25 °C. Results also show that *HOM3* and *THR1* transcripts expressed from $P_{CTR4\text{-}1}$-*HOM3* and $P_{CTR4\text{-}1}$-*THR1* strains in induction conditions were at higher than wildtype levels at both 30 °C 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 signaling, are essential in *C. neoformans* but not in *S. cerevisiae* and/or *C. 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 see 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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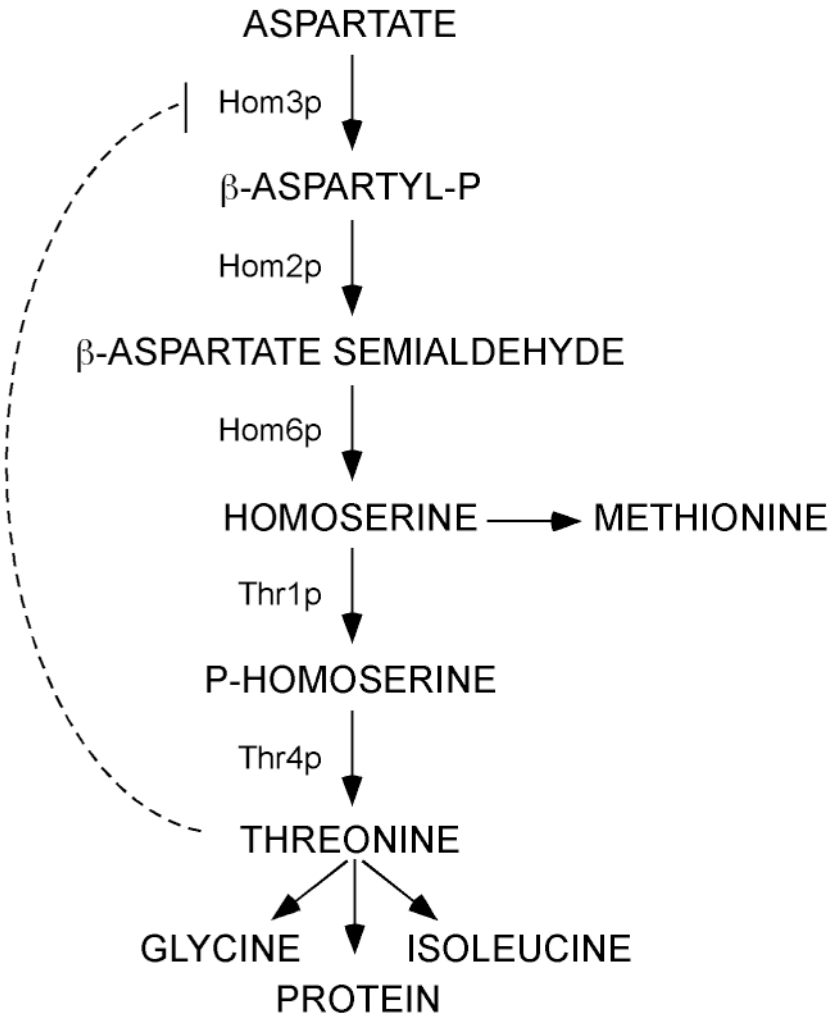

Figure 1.
Fungal threonine biosynthetic pathway.

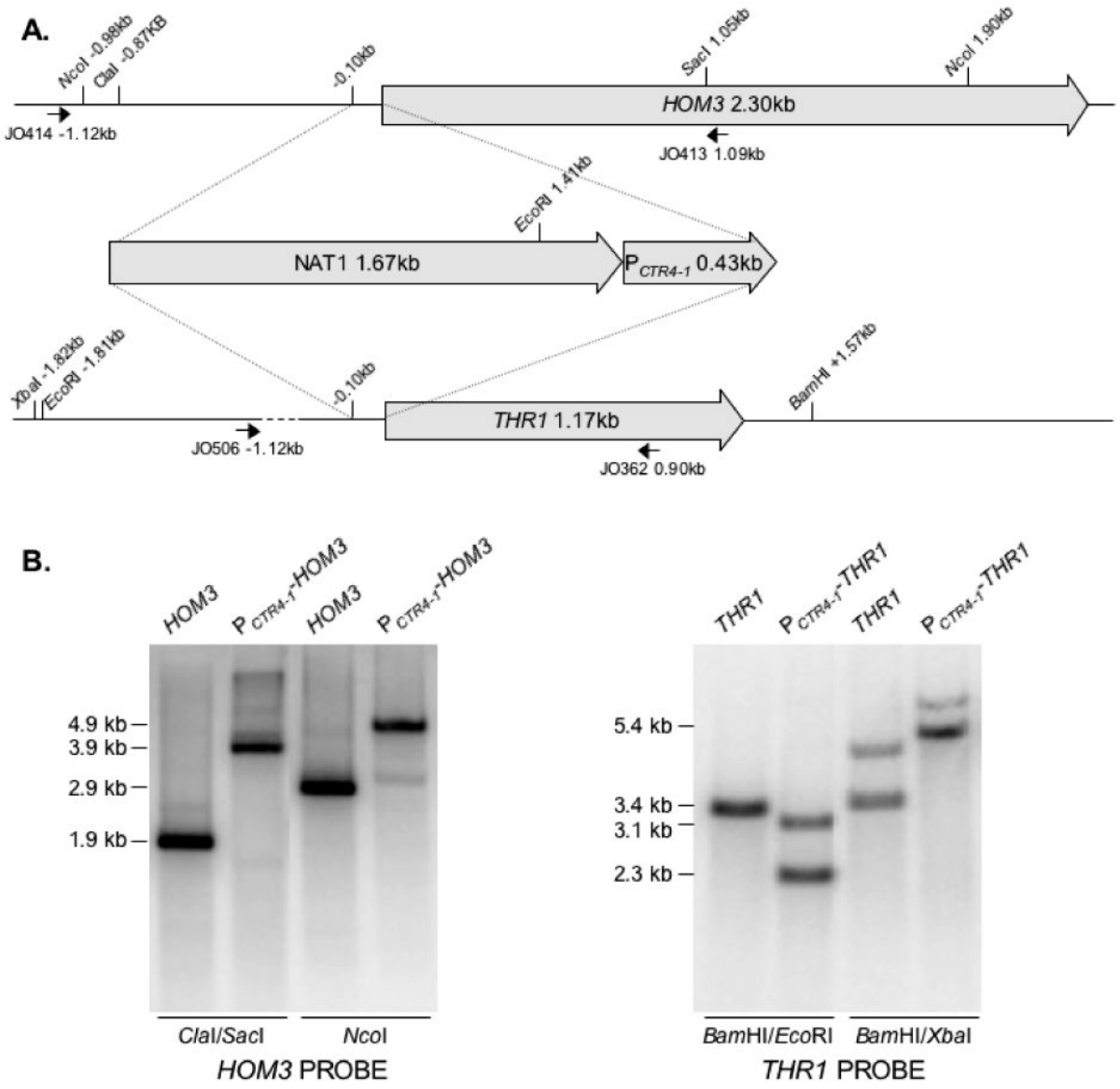

Figure 2.

(A) The *HOM3* and *THR1* genes were placed under the control of the P_{CTR4-1} promoter by inserting the NAT1-P_{CTR4-1} construct immediately upstream of the predicted ORF, as shown in the diagram. (B) Southern hybridization analysis confirming correct positioning of the NAT1-P_{CTR4-1} construct in strains H99-73 (P_{CTR4-1}-*THR1*) and H99-76 (P_{CTR4-1}-*HOM3*). Genomic DNA from strains H99 (wildtype), 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.

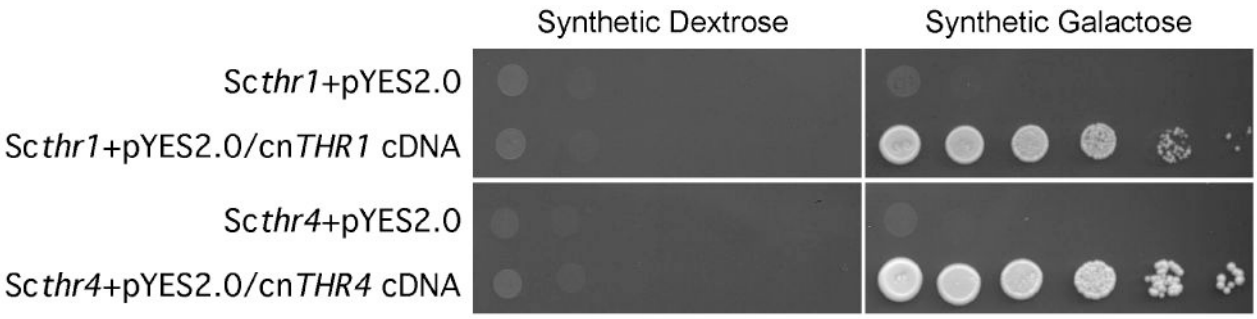

Figure 3.

Functional complementation of *S. cerevisiae thr1* (YJK1358) and *thr4* (S318) strains by *C. neoformans THR1* and *THR4* cDNA. Ten-fold dilutions of YJK1358 transformed with pYES2.0 or pJO373 (pYES2.0/cn*THR1* cDNA), and S318 was transformed with pYES2.0 or pJO378 (pYES2.0/cn*THR4* cDNA), were plated on Synthetic Dextrose or Synthetic Galactose, and incubated at 30 °C for 3 days.

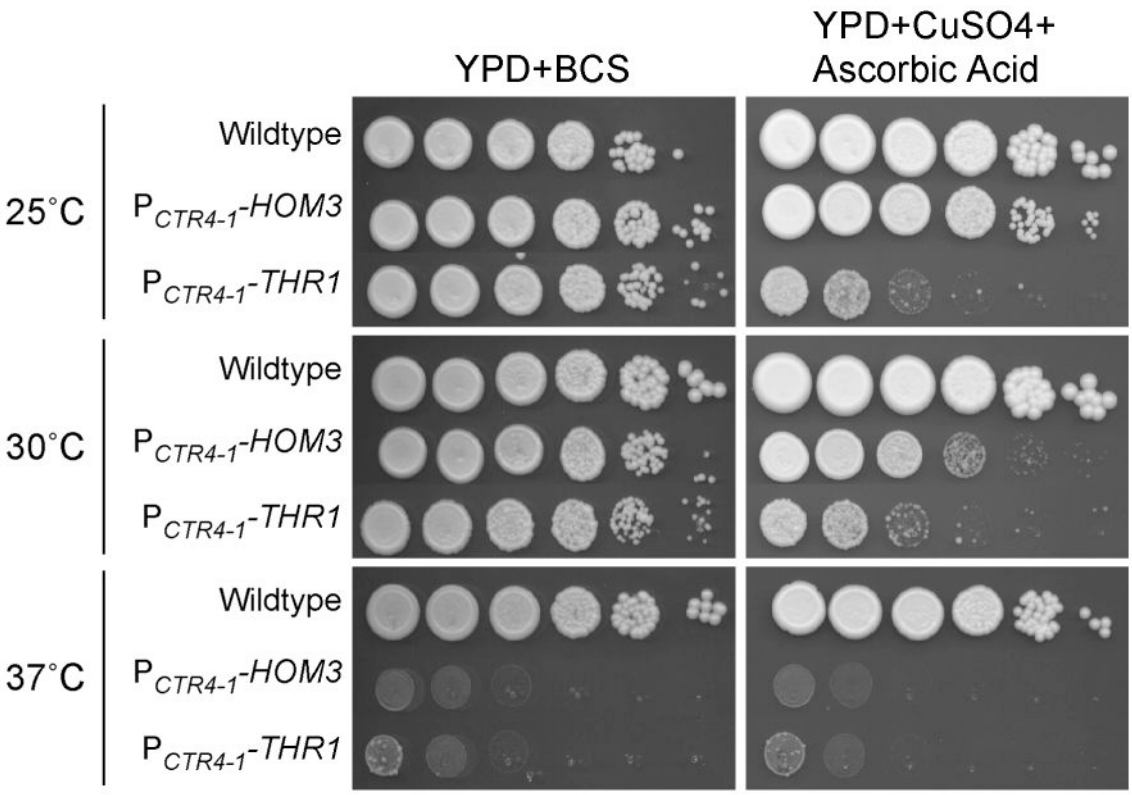

Figure 4.

Temperature-dependent phenotypes of P*CTR4-1*-*HOM3* (H99-76), P*CTR4-1*-*THR1* (H99-73), and the wildtype (H99) strains in gene-repressing ($CuSO_4$ + ascorbic acid) and gene-inducing (BCS) conditions. Ten-fold dilutions of strains were plated and incubated for three days.

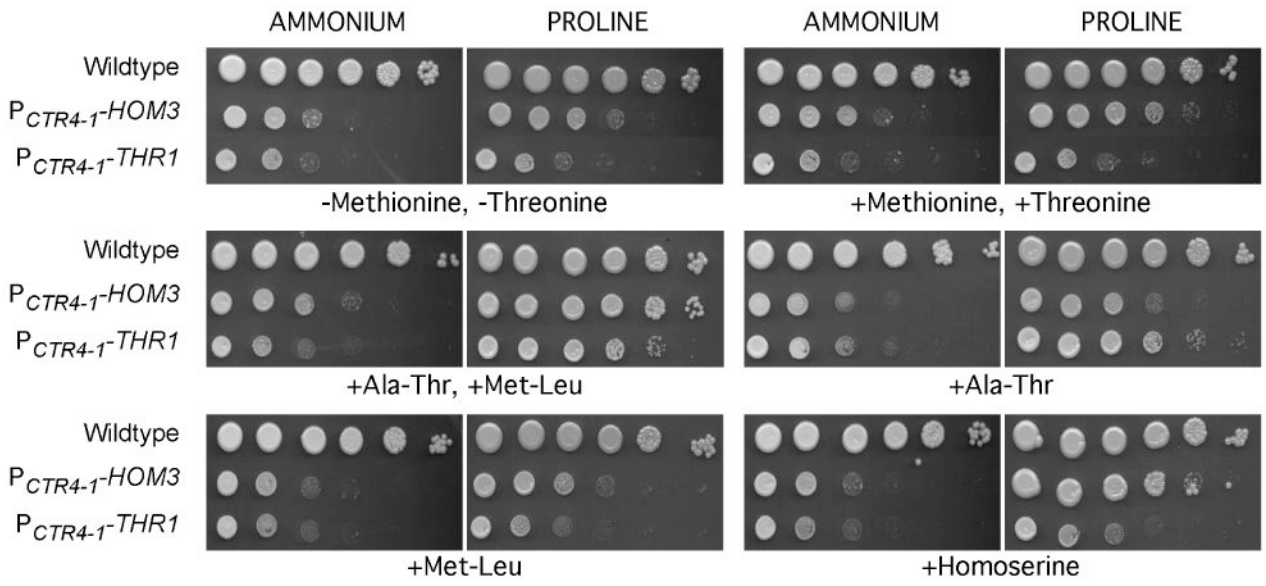

Figure 5.

Auxotrophic supplementation of $P_{CTR4\text{-}1}$-*HOM3* (H99-76) and $P_{CTR4\text{-}1}$-*THR1* (H99-73) strains. Strains were serially diluted ten-fold, and plated on SD+$CuSO_4$+Ascorbic acid or SD(Pro)+$CuSO_4$+Ascorbic acid that was supplemented with various combinations of methionine, threonine, Ala-Thr, Met-Leu or homoserine, as indicated. Plates were incubated for three days at 30 °C.

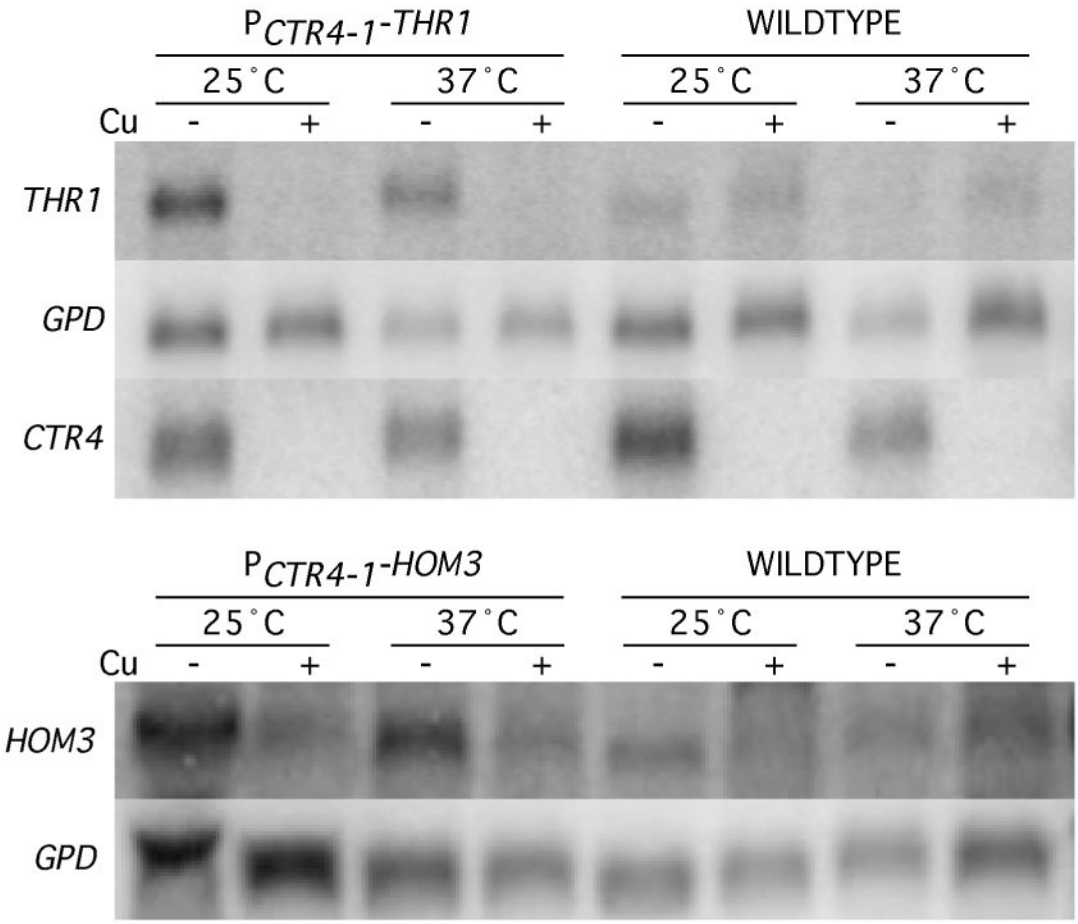

Figure 6.

Northern hybridization analysis comparing *THR1*, *HOM3*, *CTR4* and *GPD* transcript abundance. RNA was prepared from H99 (wildtype), H99-73 (P_{CTR4-1}-*THR1*), and H99-76 (P_{CTR4-1}-*HOM3*) strains, grown in P_{CTR4-1}-repressing (YPD + $CuSO_4$ + ascorbic acid; + Cu) or P_{CTR4-1}-inducing (YPD + BCS; - Cu) conditions, at 25 °C or 37 °C.

Table 1

Strains used in this study

Strain	Genotype	Source
C. neoformans		
H99	Mat α	(Perfect *et al.*, 1993)
H99-73	NAT1-$P_{CTR4\text{-}1}$-*THR1*	This study
H99-76	NAT1-$P_{CTR4\text{-}1}$-*HOM3*	This study
S. cerevisiae		
S157	*ura3*Δ	(Yang *et al.*, 2002)
S318	*ura3*Δ *thr4*Δ::natMX4	This study
YJK1358	*ura3*Δ *thr1*Δ::kan MX4	This study
YJK2416	*ura3*Δ *hom3*Δ::natMX4	This study