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Fungal Homoserine Kinase (*thr1*Δ) Mutants Are Attenuated in Virulence and Die Rapidly upon Threonine Starvation and Serum Incubation▽†

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The fungally conserved subset of amino acid biosynthetic enzymes not present in humans offer exciting potential as an unexploited class of antifungal drug targets. Since threonine biosynthesis is essential in *Cryptococcus neoformans*, we further explored the potential of threonine biosynthetic enzymes as antifungal drug targets by determining the survival in mice of *Saccharomyces cerevisiae* homoserine kinase (*thr1*Δ) and threonine synthase (*thr4*Δ) mutants. In striking contrast to aspartate kinase (*hom3*Δ) mutants, *S. cerevisiae thr1*Δ and *thr4*Δ mutants were severely depleted after only 4 h *in vivo*. Similarly, *Candida albicans thr1*Δ mutants, but not *hom3*Δ mutants, were significantly attenuated in virulence. Consistent with the *in vivo* phenotypes, *S. cerevisiae thr1*Δ and *thr4*Δ mutants as well as *C. albicans thr1*Δ mutants were extremely serum sensitive. In both species, serum sensitivity was suppressed by the addition of threonine, a feedback inhibitor of Hom3p. Because mutation of the *HOM3* and *HOM6* genes, required for the production of the toxic pathway intermediate homoserine, also suppressed serum sensitivity, we hypothesize that serum sensitivity is a consequence of homoserine accumulation. Serum survival is critical for dissemination, an important virulence determinant: thus, together with the essential nature of *C. neoformans* threonine synthesis, the cross-species serum sensitivity of *thr1*Δ mutants makes the fungus-specific Thr1p, and likely Thr4p, ideal antifungal drug targets.

Fungal infections are an increasingly significant cause of human disease and morbidity due to an expanding immunocompromised population. However, only four main classes of broad-spectrum antifungal drugs are currently available (polyenes, azoles, echinocandins, and 5-fluorocytosine), which target only three cellular components: the cell membrane, cell wall, and nucleotide biosynthesis (55). Compared with the identification of antibacterial drug targets, an obstacle to antifungal drug target identification is the eukaryotic nature of both the fungal pathogen and the host, ensuring a considerably higher degree of conserved genes and pathways. Since a subset of amino acid biosynthetic pathways are not present in humans (46), yet are conserved in fungi, and many are required for survival *in vivo* and/or virulence (22, 31, 35, 36, 45, 58), various amino acid biosynthetic enzymes are an attractive, unexploited class of antifungal drug targets.

The threonine biosynthetic pathway is of particular interest for antifungal drug targets. Threonine is produced from aspartate, via the intermediate homoserine, in a series of five enzymatic steps, initiated by aspartate kinase (Hom3p). Homoserine is converted to threonine by the sequential actions of homoserine kinase (Thr1p) and threonine synthase (Thr4p). Threonine synthesis is regulated by induction of pathway genes via the general control pathway in response to amino acid starvation (26, 43) and by feedback regulation of aspartate kinase when threonine is abundant (41, 48). Homoserine and threonine are intermediates in the synthesis of methionine and isoleucine, respectively, and we have found that various fungal methionine and isoleucine auxotrophs are unable to survive *in vivo* and/or are avirulent (31, 36, 45, 58). Myriad phenotypes in addition to auxotrophy have been associated with *Saccharomyces cerevisiae* threonine biosynthetic mutants, particularly *thr1*Δ or *thr4*Δ mutants (2, 8, 14–16, 20, 33, 51) as a result of toxic homoserine accumulation (33), many phenotypes of which may also affect the ability of these mutants to survive *in vivo*.

Since we find that *S. cerevisiae hom3*Δ mutants are unable to survive *in vivo* (31) and that *C. neoformans hom3*Δ and *thr1*Δ mutations are lethal (34), we further investigated the potential of threonine biosynthetic enzymes as antifungal drug targets by examining the *in vivo* survival of *thr1*Δ and *thr4*Δ mutants constructed in a clinically derived *S. cerevisiae* strain. Given the severe survival defects of these mutants after only 4 h *in vivo*, we extended our investigations to *Candida albicans*, a more clinically relevant pathogen, and observed that *C. albicans thr1*Δ mutants had attenuated virulence. Consistent with the *in vivo* defects, we demonstrated that *S. cerevisiae thr1*Δ, *thr4*Δ, and *C. albicans thr1*Δ strains were serum sensitive. We explored the basis of the serum sensitivity and show that low serum threonine concentrations and the accumulation of the biosynthetic intermediate homoserine are key to the rapid death of *thr1*Δ and *thr4*Δ mutants in serum.

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MATERIALS AND METHODS

Strains, media, and growth conditions. All strains used in this study are listed in Table 1. *S. cerevisiae* strains were isogenic with clinically derived YJM145 (42), and *C. albicans* strains were isogenic with strain SC5314 (21). Unless otherwise specified, all strains described are diploid and homozygous for the gene disruption described. Standard culture media included yeast extract-peptone-dextrose

TABLE 1. Strains used in this study

Strain	Genotype	Reference
S. cerevisiae		
YAG40	*HO*/*ho*Δ::hphMX4	22
YAG129	*MAT***a**/*MAT*α *gal2*/*gal2 HO*/*HO*	22
YJK369b	*thr4*Δ::natMX4/*thr4*Δ::natMX4	This study
YJK391b	*thr4*Δ::kanMX4/*thr4*Δ::kanMX4	This study
YJK456	*THR4*/*thr4*Δ::kanMX4	This study
YJK461	*THR4*/*thr4*Δ::natMX4	This study
YJK487a	*hom3*Δ::kanMX4/*hom3*Δ::kanMX4	31
YJK495a	*hom3*Δ::natMX4/*hom3*Δ::natMX4	31
YJK498a	*thr1*Δ::kanMX4/*thr1*Δ::kanMX4	This study
YJK506a	*thr1*Δ::natMX4/*thr1*Δ::natMX4	This study
YJK547	*THR1*/*thr1*Δ::kanMX4	This study
YJK677	*hom3*Δ::kanMX4/*hom3*Δ::kanMX4 *thr4*Δ::natMX4/*thr4*Δ::natMX4	This study
YJK682	*hom3*Δ::kanMX4/*hom3*Δ::kanMX4 *thr1*Δ::natMX4/*thr1*Δ::natMX4	This study
YJK862	*hom6*Δ::kanMX4/*hom6*Δ::kanMX4	This study
YJK948	*hom6*Δ::kanMX4/*hom6*Δ::kanMX4 *thr1*Δ::natMX4/*thr1*Δ::natMX4	This study
YJK1078	*thr1*Δ::natMX4/*thr1*Δ::natMX4 *gcn4*Δ::kanMX4/*gcn4*Δ::kanMX4	This study
YJK1379	*hom6*Δ::kanMX4/*hom6*Δ::kanMX4 *thr4*Δ::natMX4/*thr4*Δ::natMX4	This study
C. albicans		
SC5314	Wild type	21
CJK18	*HOM3*/*hom3*Δ::*SAT1*	This study
CJK22	*THR1*/*thr1*Δ::*SAT1*	This study
CJK26	*HOM3*/*hom3*Δ::*FRT*	This study
CJK30	*THR1*/*thr1*Δ::*FRT*	This study
CJK35, CJK37	*thr1*Δ::*SAT1*/*thr1*Δ::*FRT*	This study
CJK41, CJK43	*hom3*Δ::*SAT1*/*hom3*Δ::*FRT*	This study
CJK59	*THR1*/*thr1*Δ::*SAT1* (*THR1*-complemented strain)	This study
CJK61	*HOM3*/*hom3*Δ::*SAT1* (*HOM3*-complemented strain)	This study
CJK77	*hom3*Δ::*FRT*/*hom3*Δ::*FRT thr1*Δ::*SAT1*/*thr1*Δ::*FRT*	This study

(YPD) and synthetic dextrose (SD), which were prepared as described previously (54). Dulbecco's modified Eagle's medium (DMEM) (with dextrose, L-glutamine, and sodium pyruvate; Mediatech, Inc., catalog no. 10-013-CV) was supplemented with $NaHCO_3$ (22 mM) and HEPES (50 mM) or Na MOPS (morpholinepropanesulfonic acid; 25 mM) or, alternatively, made as specified by Mediatech, Inc., but with various components omitted. RPMI 1640 (with L-glutamine and $NaHCO_3$) was obtained from Sigma (catalog no. R8758). Where specified, media were supplemented with L-methionine (20 μg ml^{-1}), maltose (20 mg ml^{-1}), nourseothricin (Nat; 100 μg ml^{-1} for *S. cerevisiae* selection and 200 μg/ml for *C. albicans* selection; Hans Knöll Institute für Naturstoff-Forschung, Jena, Germany), hygromycin B (300 μg ml^{-1}; Calbiochem) and Geneticin (200 μg ml^{-1}; Life Technologies). Unless specified otherwise, L-threonine was added to SD at a concentration of 300 μg ml^{-1}.

Gene deletions. *S. cerevisiae* genes were replaced with natMX4, kanMX4, or hphMX4 cassettes by PCR-mediated gene deletion (23, 56). Since the YJM145 background is diploid and homothallic, transformants were sporulated at 30°C and tetrads were dissected to achieve homozygous gene deletions. To construct multiple deletions in a strain, separate strains containing deletions with different drug markers were sporulated and crossed, and diploids were selected by acquisition of multiple drug resistance. Strains were sporulated and dissected to obtain strains with multiple homozygous deletions. To complement mutant strains with wild-type alleles, strains were transformed with a PCR product containing the wild-type gene and flanking sequence. Gene deletions and mutant complementation were confirmed by PCR and by acquisition or loss of a phenotype.

Genes were disrupted in *C. albicans* using a similar PCR-mediated strategy, in which the *SAT1* flipper cassette (50) was amplified using primers that contained at their 5′ ends 60 bp of sequence homologous to the region immediately flanking the gene of interest. Strains were transformed with the gene-targeting *SAT1* PCR product by electroporation (50), and Nat-resistant transformants were purified and verified by PCR analysis. Transformants were grown for 2 h in YP medium containing maltose [YP(maltose)] to induce FLP-mediated excision of the *SAT1* cassette, leaving an FLP recombination target (*FRT*) site. Nat-sensitive strains then underwent a second round of transformation to disrupt the second allele. To complement strains with a wild-type gene, homozygous deletion strains were transformed with the gene of interest, amplified using primers homologous to sequence upstream and downstream of the deleted region. Transformants were selected by reversion to prototrophy, and transformants in which the wild-type allele had replaced a disrupted allele were chosen; thus, the introduced gene was expressed from its original chromosomal location. Gene disruptions and mutant complementation were verified by PCR, phenotype where available, and Southern hybridization analysis (see Fig. S1 in the supplemental material).

All primers used in this study are listed in Table S1 in the supplemental material.

Manipulation of nucleic acids. DNA was extracted from *S. cerevisiae* and *C. albicans* strains for PCR and Southern hybridization analyses, as described previously (27). To confirm *C. albicans* gene deletions by Southern hybridization analysis, 2 μg of genomic DNA was digested with various restriction enzymes, separated by agarose gel electrophoresis, denatured, and transferred to a nylon membrane (Roche) as described previously (52). Southern hybridization probes were prepared from PCR products (agarose gel purified using the QIAquick gel extraction kit; Qiagen) and labeled with [α-^{32}P]dCTP (Perkin-Elmer) using the RediprimeII random prime labeling system (Amersham Biosciences), as described by the manufacturer. Prehybridization and hybridization were performed in ULTRAhyb buffer (Ambion), blots were washed according to the manufacturer's instructions, and hybridized bands were visualized using a Typhoon 9200 variable mode imager (Molecular Dynamics).

Serum treatments and sensitivity assays. To test *S. cerevisiae* for serum sensitivity, strains were typically grown overnight in YPD, washed twice in sterile distilled water, and then added to 1 or 3 ml fetal bovine serum (FBS; Sigma catalog no. F2442) at a concentration of approximately 1×10^6 cells/ml. The volume of FBS depended on whether cell viability was estimated by spot dilution or cells were plated for absolute numbers. Strains were either added to independent serum tubes, or differently marked strains (typically the wild-type, YAG40, and two differently marked strains with the same gene disrupted) were competed in the same tube. Serum was incubated at 37°C, and at various time points, aliquots were removed, serially diluted, and plated to selective media to determine cell viability. To provide an approximate estimate of cell viability, cultures were diluted 10-fold and 5-μl spots were plated. To determine absolute numbers, 100-μl aliquots of appropriate dilutions were plated at least in duplicate. To test *C. albicans* serum survival, the assay was essentially the same, but strains were incubated in serum separately, and the incubation temperature was

30°C to reduce hyphal formation. Survival in other media was assayed similarly. Typically, experiments were performed with two individually constructed strains with the same gene disrupted to ensure reproducibility of results.

Serum was treated in various ways to remove individual components. Serum and YPD pH was adjusted using HCl or KOH, following the addition of 0.05 mM HEPES buffer. Serum was delipidated by using PHM-L Liposorb (Calbiochem) according to the manufacturer's instructions. To remove proteins, serum was filtered through a <3-kDa Centricon centrifugal filter and then heat inactivated by incubation at 56°C for 30 min, followed by an overnight digestion with proteinase K (InVitrogen) (50 μg ml^{-1}) at 37°C. Calcium and iron were chelated by the addition of 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA; 4 mM) or deferoxamine mesylate (25 μg ml^{-1}), respectively. Other divalent cations were removed by incubation of serum with 5% (wt/vol) Chelex-100 (Sigma) at 4°C for 3 h. Catalase (200 μg ml^{-1}) was added and incubated for 3 h at 25°C to remove serum peroxide. Where specified, amino acids were added to serum at the following concentrations: L-serine (250 μg ml^{-1}), L-threonine (400 μg ml^{-1}), L-methionine (170 μg ml^{-1}), L-isoleucine (120 μg ml^{-1}), L-glycine (230 μg ml^{-1}), and L-valine (410 μg ml^{-1}). Survival in human serum (Sigma catalog no. H4522) was also tested.

Experimental mouse infections. The *in vivo* survival of *S. cerevisiae* strains was compared following infection by lateral tail vein injection of 4-week-old male CD-1 mice (outbred, immune competent; Charles River Laboratories), as described previously (10, 22). For each gene disruption tested, 15 mice were injected with 2×10^7 CFU of an equal ratio of one reference strain (YAG40) and two experimental strains, each containing different markers disrupting the same gene, that had been grown to mid-log phase in YPD medium, washed in sterile phosphate-buffered saline (PBS), and resuspended in PBS. To determine the exact proportion of each strain present, the inocula were diluted and plated to selective media. At times 4 h, 1 week, and 2 weeks postinfection, five mice per time point were euthanized by CO_2 inhalation. Since the brain is the predominant organ inhabited by *S. cerevisiae* in CD-1 mice (10), the brains were recovered, homogenized in 5 ml PBS supplemented with 100 μg ml^{-1} ampicillin and streptomycin, pelleted by centrifugation (700 × *g* for 10 min), resuspended in 1 to 2 ml PBS, and plated to selective media to determine the relative numbers of each strain present. Results at each time point ($t = x$) were expressed as competitive index (CI) values (7, 9), a measure of (experimental strain/reference strain)$_{t = x}$/(experimental strain/reference strain)$_{t = 0}$.

To test virulence of *C. albicans* strains, 7-week-old male CD-1 mice were given a lateral tail vein injection of 1×10^6 cells suspended in PBS buffer. Mice were observed twice daily, and animals that appeared moribund (>15% loss of body weight, lethargic, or not accessing food) were sacrificed. Mice that remained healthy throughout the course of the experiment were euthanized after 28 days, and their organs were recovered, homogenized in 5 ml PBS plus ampicillin plus streptomycin, and then plated to plates containing YPD plus nourseothricin (YPD + Nat) to determine if the infection had been cleared. Mouse survival data were analyzed using the Kaplan-Meier test.

Mice were fed *ad libitum* for the course of the experiments. All animal experiments met with institutional guidelines and were approved by the Institutional Animal Care and Use Committee.

MIC assays. To determine the MICs of fluconazole (Pfizer), 5-fluorocytosine (Sigma), amphotericin B (Gibco), and caspofungin (Merck), 10-μl volumes of a 2-fold dilution series of each drug were dispensed into the wells of a flat-bottomed microdilution plate (Corning). Subsequently, 90-μl volumes of cells resuspended in 1.1 × YPD or SD plus methionine plus threonine (YPD + Met + Thr) to a concentration of approximately 2×10^3 CFU ml^{-1} were added to the microdilution plate wells containing the drug dilutions, and the microdilution plates were incubated for 2 days at 30°C. Experiments were typically performed in triplicate on two independent mutants. MICs were defined as the minimum concentration of drug that inhibited growth to ≤20% of the OD_{600} measurement of the no-drug control, following background correction (MIC_{80}). To determine minimum fungicidal concentrations (MFCs), the entire contents of wells containing no visible growth were plated on YPD. The MFC was defined as the lowest concentration of drug that resulted in a 95% reduction in CFU from the initial inoculums (MFC_{95}).

RESULTS

***S. cerevisiae thr1*Δ and *thr4*Δ mutants are very rapidly eliminated *in vivo*.** Having determined that *S. cerevisiae hom3*Δ mutants are eliminated by 1 week *in vivo* (31), we decided to assess the *in vivo* survival requirement of other threonine biosynthetic genes. The survival in the brains of mice of two differently marked *thr1*Δ (YJK498a and YJK506a) and *thr4*Δ (YJK369b and YJK391b) strains, constructed in a clinically derived *S. cerevisiae* strain, was compared with that of the wild-type strain (YAG40). Like the *hom3*Δ mutants, the *thr1*Δ and *thr4*Δ mutants were unrecoverable after 1 week *in vivo*, with average CI values of <0.0009 (*thr1*Δ mutants) and <0.0015 (*thr4*Δ mutants) (Fig. 1). However, in contrast to the *hom3*Δ mutants, the *thr1*Δ and *thr4*Δ mutants were severely attenuated after only 4 h *in vivo*, with average CI values of 0.032 and 0.045, respectively. Complementation of the mutants with the wild-type *THR1* (YJK547) and *THR4* (YJK456 and YJK461) genes restored survival to wild-type levels (average CI values of 0.91 and 0.76 after 2 weeks, respectively); thus, *in vivo* survival was indeed dependent upon *THR1* and *THR4*.

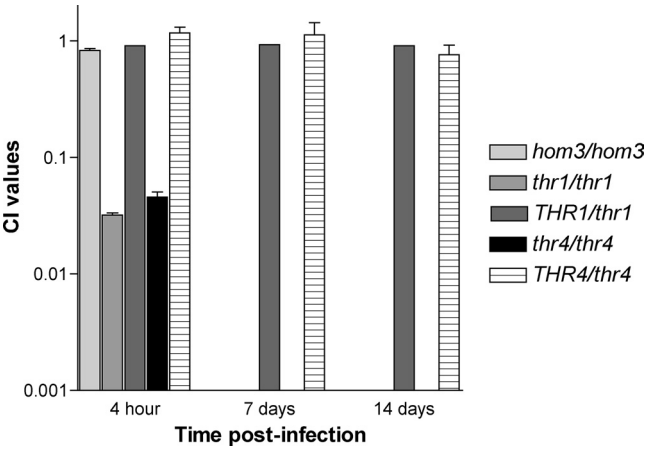

FIG. 1. Survival of *S. cerevisiae thr1*Δ and *thr4*Δ strains *in vivo*. Results were the average of experiments with two independent mutants with the same gene deleted. *hom3*Δ mutant results were previously published (31) and were included as a comparison. CI values = (experimental strain/reference strain)$_{t = x}$/(experimental strain/reference strain)$_{t = 0}$.

***C. albicans thr1*Δ mutants have attenuated virulence.** Given the severe *in vivo* survival defects of *S. cerevisiae* threonine pathway mutants and the essential nature of these genes in *C. neoformans* (34), we investigated the virulence phenotypes of *hom3*Δ and *thr1*Δ mutants constructed in the highly clinically relevant pathogen *C. albicans*. We chose to focus on *C. albicans thr1*Δ rather than *thr4*Δ mutants since *S. cerevisiae thr1*Δ mutants have phenotypes similar to, to more severe than, those of *thr4*Δ mutants (33). The mice infected with the *hom3*Δ mutant (CJK41) survived a similar length of time to those infected with the wild type (SC5314) and the *HOM3*-complemented mutant (CJK61), with mean survival times of 4.9 ± 3.8, 3.1 ± 0.3, and 3.6 ± 1.1 days, respectively (Fig. 2). In contrast, the virulence of the *thr1*Δ (CJK35) mutant was considerably attenuated compared with the wild type ($P < 0.0001$), with 60% of the infected mice still surviving after 28 days. *thr1*Δ mutants were still recovered from the kidneys of all of the surviving mice; therefore, mice had not cleared the infection. The attenuated virulence of the *thr1*Δ mutant was confirmed to be due to the absence of *THR1*, as the *THR1*-complemented strain (CJK59) had similar virulence to the wild type (mean survival time of 1.7 ± 1.0 days).

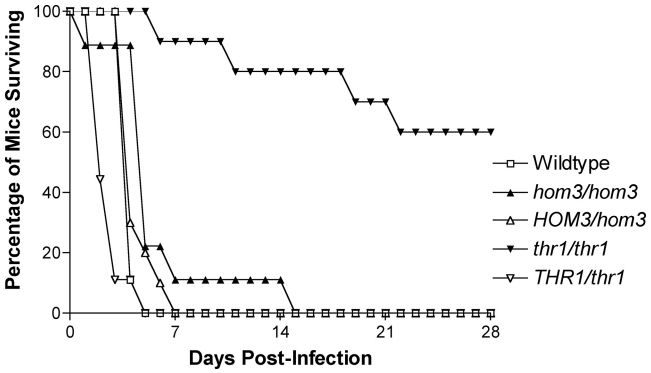

FIG. 2. Virulence phenotypes of *C. albicans* threonine pathway mutants.

***thr1*Δ and *thr4*Δ mutants are extremely serum sensitive.** The considerable difference in survival between the *S. cerevisiae thr1*Δ and *thr4*Δ mutants and the wild type and *hom3*Δ mutants after only 4 h *in vivo* was far too high to be attributable to differences in growth rate and was not due to auxotrophy *per se*. The results instead indicate that the *in vivo* environment is more toxic to *thr1*Δ and *thr4*Δ mutants. Since an initial stage in this experimental infection process, and likely most natural infections, involves transit through the bloodstream, we investigated whether *S. cerevisiae thr1*Δ and *thr4*Δ mutants were serum sensitive. Two each of the *hom3*Δ, *thr1*Δ, and *thr4*Δ mutants were incubated with the wild type in fetal bovine serum (FBS) at 37°C and then serially diluted and plated to selective media to determine survival. As judged by semiquantitative spot dilution assays (Fig. 3A), the wild-type viability increased over time and *hom3*Δ mutant viability remained at the inoculated level, while *thr1*Δ and *thr4*Δ mutant numbers declined dramatically, with an approximately 100- to 1,000-fold reduction after 4 h. Since *thr1*Δ (and *thr4*Δ) mutants were already highly depleted after 4 h, a separate competition experiment to determine how rapidly mutants are killed in serum compared overall numbers of the wild type (YAG40) and two differently marked *thr1*Δ mutants (YJK498a and YJK506a) surviving serum incubation at earlier time points. As can be seen in Fig. 3B, *thr1*Δ mutant viability declined rapidly, with average reductions from input values of 8-fold, 279-fold, 863-fold, and 2,309-fold, following 1, 2, 3, or 4 h of incubation, respectively.

To investigate whether serum sensitivity is a conserved phenomenon of *thr1*Δ mutants, the serum survival rates of *C. albicans*, *thr1*Δ (CJK35 and CJK37), *hom3*Δ (CJK41 and CJK43), and wild-type (SC5314) strains were also compared. All strains were incubated at 30°C to minimize hyphal formation, but microscopic examination at 4 h revealed that the wild-type and the *hom3*Δ mutants still produced some hyphae. Since even low levels of hyphal formation in serum by the wild-type and *hom3*Δ mutants resulted in some clumping in serum, their overall cell numbers were greater than those represented by spot dilution. However, no hyphal formation was observed for the *thr1*Δ mutant. While the *C. albicans* wild-type and *hom3*Δ mutant numbers increased over time, *thr1*Δ mutants were found to also be extremely serum sensitive, with an approximately 100- to 1,000-fold reduction in viability after 4 h (Fig. 3A).

Serum toxicity is due to low threonine levels. Microscopic examination of serum-incubated cells showed that *S. cerevisiae thr1*Δ mutants were predominantly unbudded cells, thus ruling out cell clumping as an explanation for low numbers of recoverable colonies. Therefore, to identify the toxic feature of serum, *S. cerevisiae thr1*Δ mutants were incubated in serum that had been treated in various ways to remove serum components, individually and in combination, and viability at different time points was estimated by spot dilution on YPD. Reduction of incubation temperature to 30°C had no influence on *S. cerevisiae thr1*Δ mutant survival, nor did serum pH, serum source (with FBS and human serum tested), proteins, peroxide, lipids, iron, or other trivalent and divalent ions other than calcium (see Table S2 in the supplemental material). Chelation of calcium resulted in an approximately 10-fold increase in *S. cerevisiae thr1*Δ mutant survival after 4 h but had no benefit at later time points (Fig. 4). The calcium effect was serum specific, as addition of $CaCl_2$ at levels equivalent to calcium in serum (3.1 mM) had no effect on the viability of *S. cerevisiae thr1*Δ mutants in SD + Met + Thr (data not shown).

Interestingly, incubation in two defined serum-substitute media, DMEM (plus Na MOPS plus $NaHCO_3$) and RPMI 1640, was also toxic to *S. cerevisiae thr1*Δ mutants, with an approximately 10-fold reduction in RPMI 1640 and a 100- to 1,000-fold reduction in DMEM after 4 h (see Fig. S2 in the supplemental material). Therefore, the *thr1*Δ-inhibitory feature of serum is not serum specific. Various versions of DMEM were prepared that were deficient in each inorganic salt individually or in combination, amino acids, or vitamins. Aside from a slight protective effect after 4 h in the absence of calcium chloride, consistent with calcium chelation in serum, the *S. cerevisiae thr1*Δ mutant died at a similar level in all of the other medium formulations.

The results implied that rather than the inhibitory feature being something that was present in serum and defined serum substitute media, it was in fact the absence or smaller amount of a compound. Since amino acids are present in serum and tissue culture media at levels lower than those added to yeast minimal media (54), threonine, and the threonine-related amino acids serine, glycine, methionine, isoleucine, and valine were added individually to serum at 10-fold-higher concentrations than those present in mouse serum (11). Interestingly, addition of threonine (Fig. 4), but no other amino acid tested (see Table S2 in the supplemental material), completely suppressed *S. cerevisiae thr1*Δ mutant serum sensitivity. Similarly, when *S. cerevisiae thr1*Δ mutants were incubated in SD + Met + low Thr (39 $\mu g\ ml^{-1}$; equivalent to threonine levels in mouse serum [11]) medium, viability was reduced at least 100-fold after 8 h of incubation. In addition, when threonine was completely omitted from the medium, *S. cerevisiae thr1*Δ mutant numbers were reduced at least 100-fold after 4 h of incubation (Fig. 5). Consistent with serum results, incubation for 8 h in threonine-deficient medium had no effect on *hom3*Δ mutant viability. Therefore, the principal feature of serum toxic to *S. cerevisiae thr1*Δ mutants is the low threonine concentration.

Similar experiments were also performed to ascertain why *C. albicans thr1*Δ mutants were serum sensitive. In contrast to *S.*

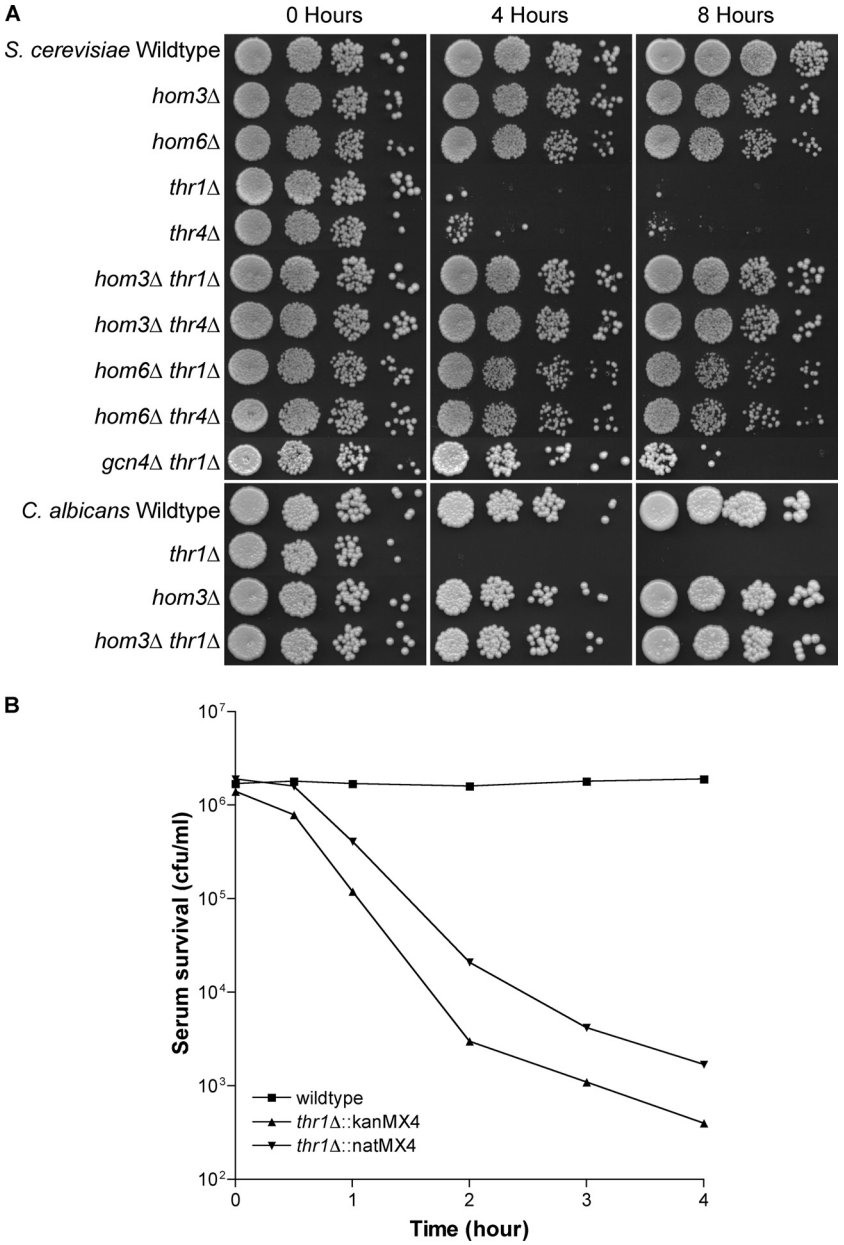

FIG. 3. (A) Serum sensitivity of *S. cerevisiae* and *C. albicans* threonine pathway mutants and the *S. cerevisiae thr1*Δ *gcn4*Δ mutant. Strains were incubated in serum for the time designated, and 5-μl volumes of 10-fold dilutions were plated on YPD medium. (B) Time course for serum killing of *S. cerevisiae thr1*Δ mutants.

*cerevisiae thr1*Δ mutants, calcium chelation had no effect on *C. albicans thr1*Δ mutant survival (see Table S2 in the supplemental material). Also contrasting with the *S. cerevisiae thr1*Δ results, buffering the serum pH to 7.5 or below substantially ameliorated *C. albicans thr1*Δ serum sensitivity, with an at least 100-fold increase in survival after 4 h (Fig. 4). The *C. albicans thr1*Δ mutants were observed to form hyphae in serum only when pH levels were conducive to survival. The pH sensitivity of *C. albicans thr1*Δ mutants was medium dependent, since there was no cytocidal effect when mutants were incubated in YPD medium at pH 8 (see Fig. S3 in the supplemental material). Importantly, as with the *S. cerevisiae thr1*Δ mutant, *C. albicans thr1*Δ mutant serum sensitivity could be completely overcome by threonine addition (Fig. 4), and mutants were similarly sensitive to incubation in minimal medium lacking threonine (Fig. 5).

Serum sensitivity is a consequence of intermediate accumulation. The cidal nature of serum and threonine starvation on *thr1*Δ mutants in both *S. cerevisiae* and *C. albicans* is not merely a consequence of threonine auxotrophy since these phenotypes are not observed in *hom3*Δ mutants. Furthermore, we have shown that *S. cerevisiae thr1*Δ and *thr4*Δ as well as *C. albicans thr1*Δ mutants are sensitive to exogenous homoserine, and the starvation-cidal phenotype of *S. cerevisiae thr1*Δ and *thr4*Δ mu-

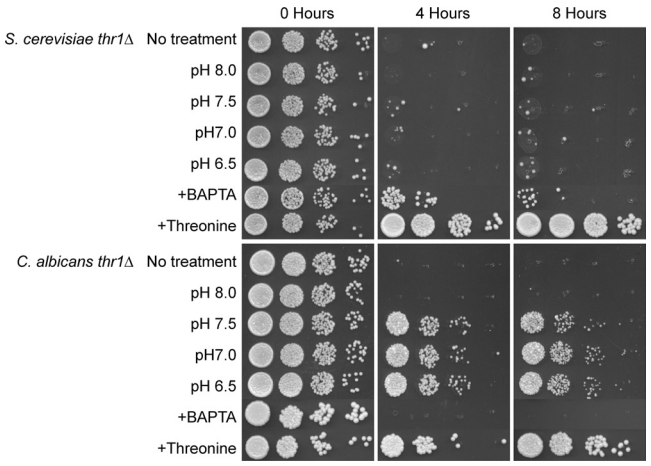

FIG. 4. Effect of pH, calcium chelation, and threonine addition on serum sensitivity of *S. cerevisiae* and *C. albicans thr1*Δ mutants. Strains were incubated in serum for the time designated, and 5-μl volumes of 10-fold dilutions were plated onto YPD.

tants is due to the inability to detoxify homoserine (33). To investigate whether the *S. cerevisiae thr1*Δ and *thr4*Δ and *C. albicans thr1*Δ serum and threonine starvation sensitivity are also due to intermediate accumulation, we compared the serum and threonine starvation survival phenotypes of *S. cerevisiae hom6*Δ (YJK862), *hom3*Δ *thr1*Δ (YJK682), *hom3*Δ *thr4*Δ (YJK677), *hom6*Δ *thr1*Δ (YJK948), *hom6*Δ *thr4*Δ (YJK1379), and *C. albicans hom3*Δ *thr1*Δ (CJK77) mutants. As observed for the *S. cerevisiae thr1*Δ and *thr4*Δ threonine starvation sensitivity (33), the *C. albicans* threonine starvation sensitivity and the serum sensitivity in both species were suppressed by disruption of *HOM3* and/or *HOM6*, indicating that these phenotypes are also due to the accumulation of a toxic intermediate (Fig. 3A and 5A). Because mutation of genes required for the production of the intermediate homoserine blocks serum and threonine starvation sensitivity and mutation of the genes required for homoserine conversion to threonine confers serum and threonine starvation sensitivity, we predict that homoserine is the toxic intermediate accumulating.

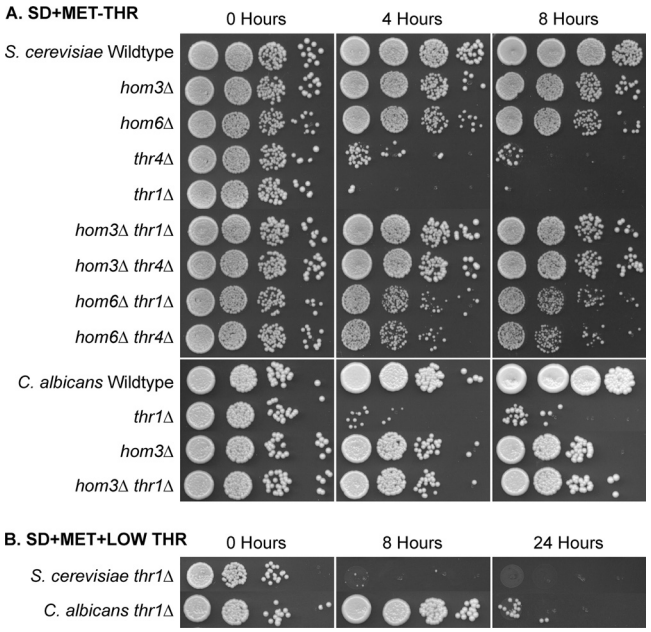

FIG. 5. Threonine starvation is cytocidal for *S. cerevisiae* and *C. albicans thr1*Δ mutants. Strains were incubated in SD + Met or SD + Met + low Thr (39 μg ml^{-1}; equivalent to serum levels), and at the designated times, aliquots were removed and dilutions plated to YPD.

TABLE 2. MIC_{80}s of 5-fluorocytosine

Strain genotype	MIC_{80} of 5-fluorocytosine on medium:	
	SD + Met + Thr (ng ml^{-1})	YPD (μg ml^{-1})
S. cerevisiae		
Wild type	10–20	250
*hom3*Δ	10	250
*thr1*Δ	10	62.5
*thr4*Δ	10	62.5
*hom3*Δ *thr1*Δ	10	250
*hom3*Δ *thr4*Δ	10	250
C. albicans		
Wild type	15.6	>250
*hom3*Δ	15.6	>250
*thr1*Δ	3.9	7.81
*hom3*Δ *thr1*Δ	15.6	>250

Conditions that increase the flux through the threonine biosynthetic pathway, such as the induction of general control upon amino acid starvation, are toxic to *thr1*Δ mutants due to increased homoserine production (33). We investigated whether disruption of *GCN4*, encoding the master regulator of general control (26), influenced *S. cerevisiae thr1*Δ mutant serum survival. Although *gcn4*Δ *thr1*Δ (YJK1078) mutants were reduced in viability over time, survival was substantially higher than that of *thr1*Δ mutants at early time points, with an approximately 100-fold increased survival after 4 h of serum incubation and 10-fold increased survival after 8 h, as estimated by spot dilution (Fig. 3A). Therefore, results are consistent with low threonine levels in serum contributing to *thr1*Δ mutant serum sensitivity by increasing homoserine production through the elicitation of general control.

Drug sensitivity of threonine auxotrophs. To test if Thr1p or Thr4p inhibitors would have synergistic action with other classes of antifungal drugs, the MICs of 5-fluorocytosine and the MICs and MFCs of fluconazole, amphotericin B, and caspofungin were determined for *S. cerevisiae* and *C. albicans* threonine pathway mutants in SD + Met + Thr and YPD media. Mutation of threonine pathway genes had little to no effect on fluconazole or caspofungin sensitivity (data not shown). While *C. albicans* strains were equally sensitive to amphotericin B, *S. cerevisiae thr1*Δ and *thr4*Δ strains were modestly (2-fold) more sensitive to amphotericin B in YPD, but not in SD + Met + Thr medium. Interestingly, while *S. cerevisiae thr1*Δ and *thr4*Δ strains were equally sensitive to 5-fluorocytosine in SD + Met + Thr medium, relative to the wild type, *hom3*Δ, and *hom3*Δ *thr1*Δ and *hom3 thr4*Δ double mutants, *thr1*Δ and *thr4*Δ mutants were at least four times more sensitive to 5-fluorocytosine in YPD (Table 2). Similarly, relative to the wild type, *hom3*Δ, and *hom3*Δ *thr1*Δ mutants, *C. albicans thr1*Δ mutants were four times more sensitive to 5-fluorocytosine in

SD + Met + Thr medium and at least 30-fold more sensitive in YPD. In both species, since *hom3*Δ blocked the 5-fluorocytosine sensitivity of *thr1*Δ and *thr4*Δ mutants, sensitivity is a consequence of toxic intermediate accumulation. Therefore, even in the presence of abundant exogenous threonine, intermediate accumulation has deleterious, clinically relevant effects.

DISCUSSION

The ability to survive in serum represents a crucial aspect for fungal virulence since travel through the bloodstream is necessary for fungal dissemination and systemic infection, and many components and conditions present in the bloodstream likely mimic those found in other body niches occupied during fungal infection. However, serum is a hostile environment for fungal growth due to various components of innate immunity, high pH, ionic composition, and the low concentrations of many nutrients. Consequently, we observed only modest serum proliferation by wild-type *S. cerevisiae*, a rarely observed pathogen. Conversely, the robustly pathogenic *C. albicans* proliferates profusely in serum and undergoes a yeast-to-hypha morphological transition upon serum exposure, enabling tissue invasion and escape from the bloodstream, which is mediated by a number of environmental cues, such as pH and temperature (reviewed in references 6, 24, and 39). Not surprisingly, the loss of the ability to survive in serum, mediated by disruption of the calcium-activated phosphatase calcineurin, correlates with attenuated virulence in both *C. albicans* and *Aspergillus fumigatus* (5, 13). Despite the importance of serum survival as a virulence factor, much of what is known about the genetic requirements for fungal proliferation and survival in serum is implied through fungal transcriptional responses observed following serum exposure (18, 19). Consistent with a commonly observed lack of correlation between transcription and phenotype (for example, see reference 2), threonine biosynthetic pathway genes were not identified in these screens.

Since some threonine biosynthetic mutants have deleterious phenotypes in addition to auxotrophy (2, 8, 14–16, 20, 33, 51), *S. cerevisiae hom3*Δ mutants do not survive *in vivo* (31), and *C. neoformans* threonine biosynthetic genes are essential (34), we were interested in further exploring the potential of threonine biosynthetic enzymes as antifungal drug targets. To this end, we demonstrated that *S. cerevisiae thr1*Δ and *thr4*Δ mutants were not only unable to survive *in vivo*, they were also highly attenuated after only 4 h postinfection. Consistent with this attenuation, serum incubation for the same period of time resulted in a severe decline in viability of *thr1*Δ and *thr4*Δ, but not *hom3*Δ, mutants. Furthermore, while disruption of *HOM3* in the clinically relevant pathogen *C. albicans* did not influence virulence, *C. albicans thr1*Δ mutants were considerably attenuated in virulence and were also serum sensitive.

Calcium was shown to cause the serum sensitivity of *C. albicans* calcineurin mutants (4), and we also identified calcium as a minor contributor to the serum- and DMEM-mediated killing of *S. cerevisiae thr1*Δ mutants. While *S. cerevisiae thr4*Δ mutants have been reported to be sensitive to calcium chloride at higher concentrations (0.7 M in YPD) (8), we observed no effect on *thr1*Δ mutant growth when calcium was added to SD + Met + Thr at levels equivalent to those found in serum (9.1 to 9.5 mg 100 ml^{-1}) (11). Similar results were observed by Blankenship et al. (4), who attributed differences to the presence of a possible calcium chelator in YPD, reducing the effective calcium concentration, or different metabolic rates in the different types of media affecting the calcium response.

High pH was determined to be a major contributor to *C. albicans thr1*Δ serum lethality. At pHs greater than 7.5, *thr1*Δ mutants were also deficient in germ tube formation, a process that occurs soon after serum exposure; thus, *thr1*Δ mutants that managed to survive serum exposure would also be deficient in tissue invasion, a potential clinically relevant benefit of Thr1p inhibitors. The pH of mouse serum can range from 7.3 to 7.55 (11), thus straddling the pH limit to which *C. albicans thr1*Δ mutants survive in serum and form hyphae, which might explain why these mutants are not completely eradicated *in vivo*, compared with *S. cerevisiae thr1*Δ mutants. Any localized pH drop caused by fungal metabolism within a microcolony that managed to establish *in vivo*, could also provide a protective effect. While lowering serum pH did not support *S. cerevisiae thr1*Δ mutant survival in serum, *thr1* mutants have been reported to be pH sensitive (20), and indeed, we observed that incubation of *thr1*Δ mutants in YPD at pHs above 7 was cidal. Thus, pH could be cidal in combination with an additional component of serum for *S. cerevisiae thr1*Δ mutants.

The major feature of serum demonstrated to be toxic to both *S. cerevisiae* and *C. albicans thr1*Δ mutants was its low threonine concentration (25 to 40 μg ml^{-1}) (11, 12), which equates to approximately one-tenth of the concentration typically added to yeast minimal media (54). Since *hom3*Δ and *hom6*Δ mutants are not serum sensitive, and disruption of *HOM3* or *HOM6* blocks *thr1*Δ and *thr4*Δ mutant serum sensitivity, toxicity is not a consequence of altered expression of neighboring genes, threonine auxotrophy, or the loss of a second function of Thr1p or Thr4p; rather, we hypothesize that serum sensitivity is due to the accumulation of the biosynthetic intermediate homoserine, which we find to be toxic (33). Our model predicts that the low levels of threonine present in serum reduce threonine feedback repression of Hom3p (41, 48), increase transcription of threonine pathway genes by the induction of general control (26, 43), and hence increase pathway flux and homoserine formation. While the threonine biosynthetic intermediate β-aspartate semialdehyde has also been shown to be toxic (1), we find that *hom6*Δ mutants are not serum sensitive and *hom6*Δ suppresses *thr1*Δ mutant serum sensitivity; therefore, we ruled out a role for this intermediate in the serum sensitivity of *thr1*Δ and *thr4*Δ strains. Consistent with our toxic homoserine accumulation hypothesis, there is precedence for homoserine toxicity in mammalian (49) and bacterial (37, 44) cells. Increased homoserine levels, even in the presence of exogenous threonine, may enhance the sensitivity of *thr1*Δ and *thr4*Δ strains to various stresses, such as calcium and 5-fluorocytosine, and explain why *C. neoformans THR1* repression is more deleterious than *HOM3* repression (34). Furthermore, we determined that both endogenously produced homoserine and exogenously added homoserine are toxic in both *S. cerevisiae thr1*Δ and *thr4*Δ mutants and *C. albicans thr1*Δ mutants, and this toxicity contributes to various other phenotypes described for *S. cerevisiae thr1*Δ and *thr4*Δ mutants, including pH sensitivity, likely by acting as a threonine analog (33). One hypothesis is that homoserine may act as

a toxic threonine analog, inhibiting or misincorporating in processes that utilize threonine as a substrate.

The extreme survival defects of *S. cerevisiae thr1*Δ and *thr4*Δ mutants *in vivo*, the inhibition of hyphal formation, the attenuated survival of *C. albicans thr1*Δ mutants, and the essentiality of these genes in *C. neoformans* (34) all validate the potential of Thr1p and Thr4p as antifungal drug targets. Several inhibitors, including phosphohomoserine analogs (17) and the phosphohomoserine analog-containing peptides rhizocticin and plumbemycin (38, 40), have been identified that target bacterial threonine synthase; however, the therapeutic efficacy of each is limited since many peptides are subject to serum protease digestion (30, 47), various phosphohomoserine analogs are also *N*-methyl-D-aspartate (NMDA) receptor antagonists (57), and we found no inhibition of yeast by D,L-*threo*-β-hydroxyaspartic acid or D,L-2-amino-5-phosphonovaleric acid (data not shown). Nonetheless, the rapidity and profound lethality observed upon exposure of *thr1*Δ and *thr4*Δ mutants to serum and threonine starvation conditions indicate that Thr1p and Thr4p inhibitors would be fungicidal *in vivo*. Such a fungicidal effect would be advantageous over fungistatic agents since fungistatic agents require immune function to clear existing fungi from the body so that the infection does not recrudesce upon drug removal, and many fungal infections occur in immunocompromised individuals. Since both *S. cerevisiae* and *C. albicans thr1*Δ are sensitive to other inhibitors of amino acid biosynthesis, likely due to general control-mediated increased flux through the threonine biosynthetic pathway that results in increased homoserine production (33), we predict that inhibitors of Thr1p (and Thr4p) would have a therapeutically beneficial synergistic action with inhibitors of other amino acid biosynthetic pathways. For example, *thr1*Δ mutants are hypersensitive to the herbicide sulfometuron methyl (33), which targets the isoleucine and valine biosynthetic enzyme acetolactate synthase, also an interesting drug target, since it is also required for fungal virulence and/or survival *in vivo* (31, 32, 36). An additional virtue for targeting Thr1p and Thr4p is the demonstration that *thr1*Δ and *thr4*Δ mutants are hypersensitive to the clinically relevant drug 5-fluorocytosine, even under conditions of high threonine. Since 5-fluorocytosine is converted into 5-fluorouracil, which inhibits RNA and DNA metabolism (25, 28, 29, 53), and *thr1*Δ mutants are hypersensitive to DNA-damaging agents (2, 3), 5-fluorocytosine-induced DNA damage may explain the observed hypersensitivity. Thus, novel Thr1p or Thr4p inhibitors would have great potential as sole (*C. neoformans*) and combination (*C. albicans*) therapies.

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