Homoserine Toxicity in *Saccharomyces cerevisiae* and *Candida albicans* Homoserine Kinase (*thr1Δ*) Mutants

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Received 22 February 2010/Accepted 10 March 2010

In addition to threonine auxotrophy, mutation of the *Saccharomyces cerevisiae* threonine biosynthetic genes *THRI* (encoding homoserine kinase) and *THR4* (encoding threonine synthase) results in a plethora of other phenotypes. We investigated the basis for these other phenotypes and found that they are dependent on the toxic biosynthetic intermediate homoserine. Moreover, homoserine is also toxic for *Candida albicans thr1Δ* mutants. Since increasing levels of threonine, but not other amino acids, overcome the homoserine toxicity of *thr1Δ* mutants, homoserine may act as a toxic threonine analog. Homoserine-mediated lethality of *thr1Δ* mutants is blocked by cycloheximide, consistent with a role for protein synthesis in this lethality. We identified various proteasome and ubiquitin pathway components that either when mutated or present in high copy numbers suppressed the *thr1Δ* mutant homoserine toxicity. Since the *doa4Δ* and proteasome mutants identified have reduced ubiquitin- and/or proteasome-mediated proteolysis, the degradation of a particular protein or subset of proteins likely contributes to homoserine toxicity.

The enzymatic reactions and regulation of the threonine biosynthetic pathway, which have attracted interest as a set of potential antifungal drug targets (11, 48–50), have been studied extensively in the yeast *Saccharomyces cerevisiae* (46). Yeast synthesizes threonine from aspartate in five enzymatic steps via the intermediate homoserine, which is also required for methionine synthesis (Fig. 1) (reviewed in reference 46). Homoserine is converted to threonine by the sequential actions of homoserine kinase (Thr1p) and threonine synthase (Thr4p). The threonine pathway is regulated at various steps. A majority of the pathway genes are regulated at the transcriptional level by general control in response to amino acid starvation (34, 64). The pathway is also regulated at the level of enzyme activity, most critically by threonine feedback inhibition of aspartate kinase (Hom3p), the initial step of the pathway (56, 73), which requires an interaction between Hom3p and the *FPR1*-encoded FK506-binding protein, FKBP12 (1).

In addition to threonine auxotrophy, a number of phenotypes have been observed in homoserine kinase (*thr1*) and/or threonine synthase (*thr4*) mutants, including being petite negative (10) and having sensitivity to cold (78), UV radiation (5, 78), and the *ho* mutant (51) (encodes threonine synthase) (*THR4*). Mutants have been observed in *hom3* (encoding aspartate kinase) (*HOM3*), *hom5* (encoding homoserine kinase), *thr4* (encoding threonine synthase), and the *hsp82* (encoding FK506-binding protein, FKBP12 (1). Yeast synthesizes threonine from aspartate in five enzymatic steps via the intermediate homoserine, which is also required for methionine synthesis (Fig. 1) (reviewed in reference 46). Homoserine is converted to threonine by the sequential actions of homoserine kinase (Thr1p) and threonine synthase (Thr4p). The threonine pathway is regulated at various steps. A majority of the pathway genes are regulated at the transcriptional level by general control in response to amino acid starvation (34, 64). The pathway is also regulated at the level of enzyme activity, most critically by threonine feedback inhibition of aspartate kinase (Hom3p), the initial step of the pathway (56, 73), which requires an interaction between Hom3p and the *FPR1*-encoded FK506-binding protein, FKBP12 (1).

In addition to threonine auxotrophy, a number of phenotypes have been observed in homoserine kinase (*thr1*) and/or threonine synthase (*thr4*) mutants, including being petite negative (10) and having sensitivity to cold (78), UV radiation (5, 6), ionizing radiation, cisplatin, hydrogen peroxide (5), pH 7 (21), caffeine, sodium chloride, manganese chloride, calcium chloride (7), and HSP90 inhibitors (57, 97), and showing synthetic lethality with *hsp82Δ* (68), as well as defects in sporulation (9, 14) and fluid-phase endocytosis (7). In this study, we also identified *thr1Δ* and *thr4Δ* mutants as being sensitive to high temperatures and, in the accompanying study, as starvation-cidal (49). We investigated the cause of these non-threonine auxotrophic phenotypes of *thr1* and *thr4* mutants by comparing phenotypes of various single- and double-threonine pathway mutants and determined that these phenotypes are the consequence of toxic intermediate accumulation. We present evidence that the toxic intermediate is homoserine, which acts as a threonine analog, and test different theories to explain homoserine toxicity. Our results demonstrate a role for the ubiquitin pathway and proteasome in homoserine toxicity.

**MATERIALS AND METHODS**

**Strains, plasmids, media, and growth conditions.** The *S. cerevisiae* strains used in this study are isogenic with the HO strains YM145 (58) and Y5S (60) or the *hho28* (63) background, as noted; *Candida albicans* strains are isogenic with SC5314 (23). All yeast strains and plasmids used in this study are listed in Table S1 in the supplemental material. *Excherichia coli* strains consisted of DH10B (Gibco BRL) and POP2136 (K-12 background; New England Biolabs). Standard bacterial and yeast culture media included Luria-Bertani (LB), yeast extract-peptone-dextrose (YPD), yeast extract-peptone-galactose (YPGd), and synthetic dextrose (SD) or SD with proline (0.1% wt/vol) as the nitrogen source [SD/proline], and were prepared as described previously (83, 86). Yeast extract-peptone-maltose [YP/maltose] was prepared as for YPD, except that maltose (2% wt/vol) replaced dextrose. Where specified, medium was supplemented with 1-amino acids (56), nourseothricin (Nat; 100 µg ml⁻¹ for *S. cerevisiae* selection and 200 µg ml⁻¹ for *C. albicans* selection; Hans Knöll Institut für Naturstoff-Forschung, Jena, Germany), hygromycin B (Hyg; 300 µg ml⁻¹; Calbiochem), or G418 (Geneticin; 200 µg ml⁻¹; Life Technologies). Other supplements were added as indicated in the text. *S. cerevisiae* and *C. albicans* cultures were incubated at 30°C, and *E. coli* cultures were incubated at 37°C, unless specified otherwise.YPD medium buffered to pH 8 contained 50 mM HEPES, and the pH was adjusted prior to autoclaving using potassium hydroxide. Salts were added to media prior to autoclaving, except calcium chloride, which was added after autoclaving.

**Manipulation of DNA.** DNA for PCR and Southern analyses was extracted from *S. cerevisiae*, *C. albicans*, and *E. coli* as described previously (36), and plasmid DNA was isolated from *E. coli* using the QIAprep spin miniprep kit (Qiagen) according to the manufacturer’s instructions. For Southern analysis, DNA (2 µg) was digested with restriction enzyme regimes that discriminated the various wild-type (WT) and disruption genotypes, separated on a 1% (wt/vol) agarose gel, denatured, and transferred to a nylon membrane (Roche) by capillary transfer (83). PCR-amplified hybridization probes were purified following agarose gel electrophoresis using the QIAquick gel extraction kit (Qiagen) and labeled with [α-32P]dCTP (Perkin-Elmer) using the RediprimeII random prime labeling system (Amersham Biosciences), according to the manufacturer’s instructions. Prehybridizations and hybridizations were carried out in ULTRAhyb...
buffer (Ambion), and membranes were washed as recommended by the manufacturer. Hybridized bands were visualized using a Typhoon 9200 variable mode imager (Molecular Dynamics).

All primers used in this study are listed in Table S2 in the supplemental material. All sequencing was performed by the Duke University Cancer Center Sequencing Facility.

Gene deletion and strain and plasmid construction. To disrupt genes in C. albicans, strains were transformed by electroporation with the SAT1 flipper PCR product, amplified from the plasmid pPS5A (77) using primers that contained at their 5′ termini 60 bp of sequence homologous to sequence flanking the gene of interest. Nat-resistant transformants were purified and verified by PCR analysis. Transformants were then cultured in YP(maltose) to induce FLP-mediated excision of the SAT1 cassette, leaving an FLP recombination target (FRT). Nat-sensitive Δ/Δ strains then underwent a second round of transformation to disrupt the second allele. Transformants were confirmed by phenotype where available, PCR analysis, and Southern hybridization analysis.

S. cerevisiae gene deletions were constructed by replacing genes with natMX4, kanMX4, or hphMX4 cassettes by PCR-mediated gene deletion (25, 94). Transformation of diploid strains resulted in heterozygous gene deletions; thus, transformants were sporulated at 30°C, tetrads were dissected, and drug-resistant segregants were selected. When two or more gene deletions were made in one strain, deletions were typically made using different drug markers, sporulated strains were crossed, and diploids were selected by acquisition of multiple drug resistance. Strains were sporulated and tetrads were dissected to obtain strains with multiple homozygous deletions. Strain construction was confirmed by phenotype, where possible, and by PCR analysis.

To create the HOM3ΔG1355A C1356T allele encoding feedback-resistant Hom3p with a G452D substitution (56), a strain was first constructed containing an insertion of the kanMX4 cassette within both HOM3 alleles, deleting nucleotide 1355. This strain was transformed (22) to threonine and methionine prototrophy using a mutagenic oligonucleotide that was homologous to sequences 5′ and 3′ of the kanMX4 insertion and containing the G1355A and C1356T substitutions. Thr′Met′ transformants were sporulated and dissected to obtain homozygous HOM3ΔG1355A C1356T segregants. The presence or absence of the introduced mutation was confirmed by PCR using primers homologous at the immediate 3′ end to the mutated sequence or wild-type sequence, respectively. In addition, the G1355A and C1356T substitutions introduced a Cia1 site, which could be detected following PCR amplification of HOM3 sequence and subsequent restriction endonuclease digestion by Cia1.

To place genes under PTEFC-regulated control, 100 bp of promoter sequence immediately upstream of the start codon of the gene of interest was replaced by DNA containing hphMX4-PTEFC by PCR-mediated deletion. hphMX4-PTEFC DNA was amplified from plasmid pAG107 using primers homologous to hphMX4-PTEFC 5′ (termini) and the HOM3 promoter sequence or the start of the HOM3 open reading frame (ORF) (5′ termini). pAG107 was constructed by replacing the kanMX6 cassette in pFA6a-kanMX6-PGAL1 (55) with an hphMX4-containing BglII-SpeI fragment from pAG32 (25) and is available from EUROSCARF (University of Frankfurt, Germany). The PCR product was introduced into yeast, and Hg-resistant transformants were selected. Strain construction was confirmed by PCR and by galactose dependence for methionine and threonine prototrophy in homozygous hphMX4-PTEFC::HOM3 strains. The stc1 (suppressor of thr4 cold sensitivity) mutant was isolated in the Y55 background as follows. Spores of Y55-1040 were plated onto YPD and incubated for 1 month at 3°C to select for cold sensitivity (Cs′) mutants. A Cs′ Thr′stc1 thr4 thr4 mutant was sporulated and crossed with spores of an isogenic HJ02 strain, which was sporulated and dissected. The resulting segregants were tested for threonine auxotrophy, cold sensitivity, and sensitivity to 0.75 M NaCl and 1.5 M KCl. The thr4 and stc1 mutations were unlinked. The stc1 mutation did not suppress the threonine auxotrophy of thr4 but did suppress the thr4 cold sensitivity and 0.75 M NaCl sensitivity phenotypes. In both thr4 and THR4 backgrounds, the stc1 mutation conferred sensitivity to 1.5 M KCl.

To identify the stc1-complementing gene plasmid pJO304 (YCp50) backbone with genomic DNA insert cloned at the BamHI site (79), various deletions were made and their ability to complement stc1 was determined (see Fig. S1 in the supplemental material). To construct plasmid pJO316, pJO304 was digested with SnaBI and religated according to the ligase manufacturer’s instructions (Invitrogen), resulting in a 4.86-kb deletion. Plasmid pJO317 was constructed following the digestion of pJO304 with Swal and religation, resulting in a 7.91-kb deletion. Plasmid pJO318 contained an 8.33-kb deletion following the digestion of pJO304 with XbaI and subsequent ligation. Plasmid pJO318 contained a precise replacement of DOA4 with natMX4 following transformation of YJK1054 with a dou4::natMX4 PCR targeting product. The plasmids pJO335-pJO340 and pJO348-pJO350 were identified as high-copy suppressors of the thr4Δ mutant homozygous threonine toxicity and contain 5-10 kb insertions within the Sall restriction site on plasmid pRS426 (see Table S1 in the supplemental material). To identify the gene responsible for homoserine toxicity suppression in each plasmid, various deletions were made and the plasmid was tested for the ability to suppress the thr4Δ homozygous threonine toxicity (see Fig. S2 in the supplemental material). Plasmid pJO335 was digested with BamHI and HindIII individually and religated to create plasmids pJO341 and pJO342, containing 3.35-kb and 4.20-kb deletions, respectively. pJO336 was digested with HindIII and SpeI individually and religated, resulting in plasmids pJO343 and pJO344 with 0.72-kb and 4.20-kb deletions, respectively. pJO345 contained a 1.27-kb deletion following the digestion of pJO338 with SpeI and subsequent religation. pJO347 resulted from deletion of a 1.29-kb SacI fragment from pJO339, pJO401, pJO402, pJO403, and pJO404 contained a precise replacement by hphMX4 of either the entire NTR2 sequence, bp 1 to 575 of NTR2, the entire ALY1 sequence, or bp 1 to 1411 of ALY1, respectively, constructed by transformation of YJK1386 with PCR targeting constructs. All constructs were confirmed by restriction digest analysis.

The plasmids pJO334 and pJO368 were constructed by cloning S. cerevisiae HTH1 or E. coli thrRS, respectively, under the control of the TEF promoter and terminator, replacing the hph gene in pBN05, which was derived from pAG26 (hphMX4 CEN URA3) (25) by replacement of the URA3 ORF by NAT1 (available from EUROSCARF, University of Frankfurt, Germany). THSI was amplified from strain S157 DNA, and thrRS was amplified from E. coli (pOP2136 (isoenic with E. coli K-12 [New England Biolabs]) using primers that were homologous at their 5′ ends to the 3′ region of the TEF promoter or 5′ region of the TEF terminator. PCR products and Nat-digested pBN05 (cuts twice within hphMX4) were introduced into strain YJK738 or S94, and transformants in which the plasmid has been gap repaired (65) by pJO365 or thrRS (pJO367 and pJO368) PCR product or reannealed (pJO365; hph partial deletion) were selected by acquisition of Nat resistance. Hg-sensitive plasmids were isolated, introduced into E. coli DH10B (87) for amplification, purified, and then confirmed by PCR and restriction endonuclease digestion analysis.

Sensitivity assays. For each sensitivity assay, two independent mutants containing the same gene deletion were typically tested. Sensitivity to peroxide was assayed by resuspending approximately 10^7 CFU ml ^(-1) of cells, which had been cultured for approximately 16 h in YPD, into 3 ml YPD containing tert-butyl-peroxide (Sigma; 0.1% [vol/vol]). Cultures were incubated with aeration at 30°C for 2 h, and survival was assessed following plating of 5-ml volumes of 10-fold spot dilutions of aliquots, removed before and after incubation.

Sensitivities to FK506 (LC Laboratories), l-homoserine (Sigma or Novabiochem), sulfometuron methyl (SM; Chem Service), and 3-amino-1,2,4-triazole (3-AT; Sigma) were determined in triplicate using the MIC assay (MIC_int), as described previously (51). FK506 and homoserine MICs were performed in YPD medium and incubated for 1 day, while SM and 3-AT MICs were performed in SD medium supplemented with threonine and methionine (SD + Thr + Met), and incubated for 2 days. FK506 was prepared as 10Χ working concentrations in
9% (vol/vol) ethanol–1% (vol/vol) Tween 20, and the other drugs were diluted in water.

To test UVC sensitivity, 5-μl volumes of 10-fold dilutions were spotted onto YPD plates. Plates were irradiated 200 J/m² with UV (254 nm) using a Stratalinker UV Cross-linker (Stratagene) and then incubated in the dark at 30°C for 2 days.

Sensitivity to sodium chloride (0.7 M), lithium chloride (0.05 M), and calcium chloride (0.25 M) at pH 8 in 41°C, 6°C, or 0°C was assayed by plating 5-μl volumes from a 10-fold dilution series of cultures (starting at approximately 10⁸ CFU/ml) onto the desired YPD-based medium and/or incubation at the temperature specified.

To perform threonine starvation assays, strains that had been grown overnight in YPD and washed twice in sterile water were inoculated into 5 ml SD or SD + Met to a concentration of approximately 10⁶ CFU/ml. Aliquots were removed at various time points, and viable counts were either estimated by plating 5-μl aliquots of specific dilutions onto 10-fold spot dilutions, or 100-μl aliquots of 10-fold serial dilutions were plated for more precise estimates. When overall numbers were determined, experiments were performed in triplicate.

Cycloheximide suppression of homoserine toxicity. To test whether cycloheximide suppresses homoserine toxicity in thr1Δ mutants, thr1Δ mutants were grown overnight in minimal medium (SD) with 10× regular threonine concentrations (3 mg ml⁻¹, to reduce flux through threonine pathway by feedback inhibition and hence reduce homoserine accumulation). Cells were added to SD + Thr (10×) medium containing 0.1, 0.1, 1, or 10 μg ml⁻¹ cycloheximide (approximately 1 × 10⁻⁴ cells ml⁻¹) and incubated with aeration for 2 h at 30°C. Cells were pelleted, washed twice with water, and resuspended in an equivalent volume of SD + Thr (0.1× or 0.03 mg ml⁻¹) medium containing cycloheximide, at levels used previously. Cultures were split, and homoserine (1 mg ml⁻¹) was added to one sample of each duplicate. Cultures were incubated at 30°C with aeration, aliquots were removed at times 0, 3, 6, and 24 h following homoserine addition, and 5-μl volumes of 10-fold dilutions were plated.

**RESULTS**

Phenotypes of threonine pathway mutants. To determine whether thr1 and thr4 mutant phenotypes are caused by (i) threonine auxotrophy, (ii) THR1 and/or THR4 encoding an activity additional to threonine biosynthesis, or (iii) the accumulation of a toxic intermediate caused by pathway blockage, the phenotypes of threonine (and methionine) biosynthetic mutants were compared for various conditions reported to be inhibitory to thr1 and/or thr4 mutants (5–7, 21, 49, 78), as well as a variety of other phenotypes described below.

Unless specified otherwise, experiments were performed in the YJM145 background. In this background, thr1Δ (YJK498a and YJK506a) and thr4Δ (YJK369b and YJK391b) mutants were not sensitive to CaCl₂ (0.25 M), and thr1Δ mutants were not petite negative, since mlp1Δ thr1Δ segregrants from sporulated and dissected MIP1/MLP1 THR1/thr1Δ strains were viable. However, thr1Δ mutants were more sensitive than the wild-type (YAG129), met2Δ (YJK561 and YJK565), and hom3Δ (YJK487a and YJK495a) mutants to sodium chloride (0.7 M), lithium chloride (0.05 M), and pH 8, incubation at 41°C and 6°C, threonine starvation, tert-butylperoxide, caffeine, and UVC (Table 1). The thr1Δ mutants were also more sensitive than hom6Δ (YJK862 and YJK865) mutants to pH 8, 41°C, UVC, lithium chloride, and threonine starvation. Similarly, thr4Δ mutants were sensitive to each condition that inhibited thr1Δ mutants, although to a lesser degree in some instances. Therefore, since the thr1Δ and thr4Δ mutant phenotypes differ from those observed for hom3Δ and hom6Δ mutants, these phenotypes are not due to threonine auxotrophy alone.

To determine whether phenotypes result from Thr1p and Thr4p having an additional, non-threonine biosynthetic role or from toxic intermediate accumulation caused by pathway blockage, hom3Δ thr1Δ (YJK671 and YJK682), hom3Δ thr4Δ (YJK677 and YJK680), hom6Δ thr1Δ (YJK539 and YJK548), and hom6Δ thr4Δ (YJK1380 and YJK1381) double mutants were constructed, and their phenotypes were compared with those of single mutants. For every phenotype tested, the phenotypes of the hom3Δ thr or hom6Δ thr double mutants were identical to the phenotypes of single hom3Δ or hom6Δ mutants, respectively (Table 1). Therefore, since the hom3Δ and hom6Δ mutations suppressed thr1Δ and thr4Δ phenotypes, the deleterious phenotypes of thr1Δ and thr4Δ mutants are likely caused by the accumulation of a toxic intermediate, such as homoserine (Fig. 1).

**Exogenous homoserine is toxic to thr1Δ and thr4Δ mutants.** If endogenously produced homoserine is toxic, with the homoserine metabolizing enzymes acting as major (Thr1p and Thr4p) and minor (Met2p) homoserine detoxification pathways (Fig. 1), then exogenous homoserine should be toxic to thr1Δ and thr4Δ mutants. While no inhibition of hom3Δ, hom6Δ, met2Δ, or wild-type strains by homoserine was observed on YPD plates (1 mg ml⁻¹ homoserine; Fig. 2) or YPD broth (MIC₅₀ >2 mg ml⁻¹), consistent with homoserine being toxic unless detoxified by conversion into threonine, thr1Δ, thr4Δ, and thr1Δ met2Δ strains were extremely sensitive to exogenous homoserine (Fig. 2; MIC₅₀, 0.125 mg ml⁻¹). Although hom6Δ thr1Δ and hom6Δ thr4Δ strains were unable to...
Mutations in \textit{HOM3} that abolish the threonine-mediated feedback regulation of aspartate kinase (Hom3p) result in overproduction of threonine and homoserine (16, 17, 56, 73, 74). A diploid strain was constructed that was heterozygous for a feedback-resistant (FBR) \textit{HOM3} allele and \textit{THRI} deletion (YJK1595; \textit{HOM3}^{FBR}/\textit{hom3}::kanMX4 \textit{THRI}/\textit{thr1}Δ::natMX4). The strain was sporulated, tetrads were dissected, and resulting viable colonies were replica plated to YPD + Nat and YPD + G418 media to determine spore genotypes. In 45 tetrads dissected, all \textit{thr1}Δ \textit{HOM3}^{FBR} segregants were inviable (Fig. 3A). Therefore, \textit{thr1}Δ is synthetic lethal with \textit{HOM3}^{FBR}.

Since an interaction between \textit{Hom3p} and FKBP12 (encoded by \textit{FPR1}) is required for feedback regulation of the threonine pathway, disruption of \textit{FPR1} results in increased pathway flux (1). Consistent with the effect of \textit{fpr1}Δ on pathway flux, following the sporation and dissection of 82 tetrads of \textit{THRI}/\textit{thr1}Δ \textit{FPR1}/\textit{fpr1}Δ strains (YJK1592 and YJK1793; Fig. 3B), no \textit{thr1}Δ \textit{fpr1}Δ segregants were recovered. Therefore, \textit{thr1}Δ is also synthetically lethal with \textit{fpr1}Δ.

\textit{HOM3} and \textit{HOM3}^{FBR} were also placed under the control of the \textit{GAL} promoter (\textit{P}_{\text{\textit{GAL},HOM3}} and \textit{P}_{\text{\textit{GAL},HOM3}^{FBR}}) in both \textit{thr1}Δ and \textit{thr4}Δ backgrounds, and growth was compared on media with either dextrose (YPD, \textit{P}_{\text{\textit{GAL}}\text{OFF}}) or galactose (YPGal, \textit{P}_{\text{\textit{GAL}}\text{ON}}) as the carbon source. Strains grew as well as the wild type on YPD, but were severely inhibited compared with the wild-type on YPGal (Fig. 3C). The degree of growth inhibition for strains corresponded with the predicted levels of homoserine accumulated by each strain: \textit{THRI} \textit{ME2} (no inhibition) \textit{> P}_{\text{\textit{GAL},HOM3}} \textit{THRI} \textit{Δ} \textit{> P}_{\text{\textit{GAL},HOM3}} \textit{THRI} \textit{Δ} \textit{> P}_{\text{\textit{GAL},HOM3}^{FBR}} \textit{THRI} \textit{Δ} \textit{> P}_{\text{\textit{GAL},HOM3}^{FBR}} \textit{THRI} \textit{Δ} \textit{> P}_{\text{\textit{GAL},HOM3}^{FBR}} \textit{THRI} \textit{Δ} \textit{> P}_{\text{\textit{GAL},HOM3}^{FBR}} \textit{THRI} \textit{Δ} \textit{> P}_{\text{\textit{GAL},HOM3}^{FBR}} \textit{THRI} \textit{Δ} \textit{> P}_{\text{\textit{GAL},HOM3}^{FBR}} \textit{THRI} \textit{Δ} \textit{> P}_{\text{\textit{GAL},HOM3}^{FBR}} \textit{THRI} \textit{Δ} \textit{> P}_{\text{\textit{GAL},HOM3}^{FBR}} \textit{THRI} \textit{Δ} \textit{> P}_{\text{\textit{GAL},HOM3}^{FBR}} \textit{THRI} \textit{Δ} \textit{> P}_{\text{\textit{GAL},HOM3}^{FBR}} \textit{THRI} \textit{Δ} \textit{> P}_{\text{\textit{GAL},HOM3}^{FBR}} \textit{THRI} \textit{Δ} \textit{> P}_{\text{\textit{GAL},HOM3}^{FBR}} \textit{THRI} \textit{Δ} \textit{> P}_{\text{\textit{GAL},HOM3}^{FBR}} \textit{THRI} \textit{Δ} \textit{> P}_{\text{\textit{GAL},HOM3}^{FBR}} \textit{THRI} \textit{Δ}

Finally, since the Hom3p-FKBP12 interaction is blocked by the immunosuppressant drug FK506 (1), we tested the FK506 sensitivity of strains. Consistent with Hillenmeyer et al. (33), \textit{thr1}Δ mutants (YJK498a and YJK506a) were found to be more sensitive than the wild-type (YAG129), \textit{hom3}Δ (YJK487a), and \textit{hom3}Δ \textit{thr1}Δ (YJK671) strains to FK506 in a MIC assay at 37°C. Specifically, \textit{MIC}_{\text{50}} values were >100 \textit{μg ml}^{-1} for wild-type, \textit{hom3}Δ, and \textit{hom3}Δ \textit{thr1}Δ strains, compared with 12.5 to 25 \textit{μg ml}^{-1} for \textit{thr1}Δ strains (Table 2). Taken together, these results demonstrate increased toxicity to \textit{thr1}Δ strains as a consequence of increasing the flux through the threonine bio-

\begin{table}[h]
\centering
\caption{MIC\textsubscript{50}s of drugs that increase threonine pathway flux\textsuperscript{a}}
\begin{tabular}{llll}
\hline
\textbf{Strain genotype} & \textbf{MIC\textsubscript{50} of:} & \textbf{FK506 (μg ml}^{-1}\textbf{)} & \textbf{3-AT (mg ml}^{-1}\textbf{)} & \textbf{SM (μg ml}^{-1}\textbf{)} \\
\hline
\textit{S. cerevisiae} & & & & \\
\textit{Wild type} & >100 & 5 & 5 \\
\textit{hom3}Δ & >100 & 0.63 & 5 \\
\textit{thr1}Δ & 12.5–25 & 0.02–0.04 & 1.25 \\
\textit{hom3}Δ \textit{thr1}Δ & >100 & 0.63 & 5 \\
\hline
\textit{C. albicans} & & & & \\
\textit{Wild type} & >100 & 0.02 & 12.5 \\
\textit{hom3}Δ & >100 & 0.02 & 3.13 \\
\textit{thr1}Δ & >100 & 0.02 & 0.39 \\
\textit{hom3}Δ \textit{thr1}Δ & >100 & 0.04 & 3.13 \\
\hline
\end{tabular}
\textsuperscript{a}FK506 MIC\textsubscript{50} assays were determined in YPD following incubation for 1 day, and sulfometuron methyl (SM) and 3-amino-1,2,4-triazole (3AT) MIC\textsubscript{50} assays were performed in SD + Thr + Met, following incubation for 2 days.
\end{table}
Homoserine is also toxic for \textit{C. albicans} \textit{thr1}Δ mutants. To investigate whether homoserine accumulation is also toxic in \textit{C. albicans}, we first tested if \textit{C. albicans} \textit{thr1}Δ mutants were sensitive to exogenous homoserine. Like \textit{S. cerevisiae}, we first tested if investigating whether homoserine accumulation is also toxic in \textit{C. albicans} \textit{thr1}Δ mutants was completely inhibited by exogenous homoserine. Like \textit{S. cerevisiae}, \textit{C. albicans} \textit{thr1}Δ mutants were no more sensitive to 3-AT than the wild type, the \textit{thr1}Δ mutants were 32 times more sensitive than the \textit{hom3}Δ and \textit{hom3}Δ \textit{thr1}Δ strains. Therefore, as with \textit{S. cerevisiae}, the SM hypersensitivity of \textit{C. albicans} \textit{thr1}Δ mutants is consistent with toxic intermediate accumulation.

The mechanism of \textit{C. albicans} \textit{Hom3p} feedback regulation differs from that of \textit{S. cerevisiae}. We tested the effect of FKBP12 and FK506, which regulate threonine feedback inhibition of \textit{S. cerevisiae} Hom3p (1), on \textit{C. albicans} \textit{thr1}Δ mutants. \textit{C. albicans} homolog of \textit{S. cerevisiae} Fpr1p/FKBP12, with which it shares 56% amino acid identity, is Rbp1p. To determine if the mechanism of feedback regulation of \textit{C. albicans} Hom3p functions in the same manner as in \textit{S. cerevisiae}, we attempted to construct a \textit{thr1}Δ \textit{rbp1}Δ mutant. Interestingly, in contrast to the \textit{S. cerevisiae} \textit{thr1}Δ \textit{fpr1}Δ mutant synthetic lethality, the \textit{C. albicans} \textit{thr1}Δ \textit{rbp1}Δ mutant (CJIK06) was viable and only modestly (2-fold) more sensitive than the \textit{thr1}Δ mutant to homoserine in a MIC\textsubscript{90} assay (MIC\textsubscript{90} values were 0.25 and 0.5 mg ml\textsuperscript{-1} homoserine, respectively, after 1 day of incubation in YPD at 30°C). Consistent with this, we determined that \textit{C. albicans} \textit{thr1}Δ mutants were not hypersensitive to FK506 following incubation at 30°C or 37°C for 1 day; the MIC\textsubscript{90} values for all strains were >100 μg ml\textsuperscript{-1} (Table 2). The lack of an effect of administration of FK506 and little effect of disruption of \textit{RBP1} on \textit{C. albicans} \textit{thr1}Δ mutants suggest that either the \textit{C. albicans} threonine pathway flux is controlled by a Hom3p feedback-independent mechanism, such as phosphatase degradation of aspartyl phosphate, or the threonine feedback mechanism of \textit{C. albicans} Hom3p differs from that of \textit{S. cerevisiae} in that it does not involve Rbp1p.

Does homoserine act as a serine or threonine analog? Since homoserine is structurally similar to serine and threonine, one model to explain the homoserine toxicity is that homoserine
may act as an analog, either inhibiting or being metabolized by a reaction normally utilizing threonine or serine as a substrate. If homoserine is acting as a threonine or serine analog, increasing levels of exogenous threonine or serine should counteract the homoserine toxicity. We therefore compared the growth rates of the \( P_{\text{GAL}} \cdot \text{HOM3} \) and \( P_{\text{GAL}} \cdot \text{HOM3}^{\text{FB}} \) thr1Δ and/or met2Δ homoserine-accumulating strains (YJK1004, YJK1007, YJK1010, and YJK1012) on YPGal plates containing a filter disc to which 50 μl of the following amino acids had been added individually: leucine (10 mg ml\(^{-1}\)), lysine (15 mg ml\(^{-1}\)), histidine (20 mg ml\(^{-1}\)), methionine (20 mg ml\(^{-1}\)), glycine (60 mg ml\(^{-1}\)), isoleucine (6 mg ml\(^{-1}\)), valine (30 mg ml\(^{-1}\)), tryptophan (10 mg ml\(^{-1}\)), d,l-homocysteine (10 mg ml\(^{-1}\)), serine (60 mg ml\(^{-1}\)), or threonine (60 mg ml\(^{-1}\)). The only amino acids in which elevated levels affected growth were serine, which further inhibited growth, possibly by competing for threonine uptake, and threonine, which substantially enhanced growth (Fig. 4). To eliminate the possibility that threonine suppression of homoserine-mediated inhibition was due to uptake of threonine competing with reuptake of homoserine that may have been excreted, the growth of homoserine-accumulating strains was also compared when 50 μl of the dipeptides Ala-Thr or Thr-Ala (50 mg ml\(^{-1}\)) was added, and similar suppression was observed. Since the suppression by threonine was observed in strains in which \textit{HOM3} expression was regulated by \( P_{\text{GAL}} \), and not general control and that were resistant to Hom3p feedback inhibition, the threonine suppression of toxicity was not due to a reduction in flux through the threonine pathway. Instead, since both threonine and the threonine-containing dipeptides overcame the growth suppression caused by homoserine, the results are consistent with homoserine acting as a toxic threonine analog.

If homoserine toxicity is caused by inhibition of a reaction that utilizes threonine as a substrate, disruption of the gene whose product is inhibited by homoserine would result in the same phenotypes in a \textit{THRI} background as those associated with homoserine accumulation in a \textit{thr1}Δ background. Therefore, to test this hypothesis, we disrupted three genes whose products metabolize threonine (\textit{CHA1}, \textit{ILV1}, and \textit{GLY1}) in a \textit{THRI} background and compared homoserine accumulation-associated phenotypes (sensitivity to 41°C, 8°C, NaCl, LiCl, and pH 8). While several single gene deletions resulted in some of the same phenotypes as \textit{thr1}Δ mutants, none of the mutants was sensitive to all conditions (Table 3). Thus, homoserine is likely not acting as an inhibitor of Cha1p, Ilv1p, or Gly1p.

If homoserine toxicity is mediated by its metabolism in a reaction for which threonine is a substrate, disruption of the gene whose product is metabolizing homoserine would suppress the homoserine toxicity and phenotypes associated with homoserine accumulation of \textit{thr1}Δ mutants. However, we find that the \textit{cha1}Δ mutation did not suppress the \textit{thr1}Δ homoserine toxicity or any of the homoserine accumulation-dependent phenotypes. The phenotypes of the \textit{gly1}Δ \textit{thr1}Δ and \textit{ilv1}Δ \textit{thr1}Δ strains could not be tested due to synthetic lethality, which was suppressed by \textit{hom3}Δ, consistent with \textit{gly1}Δ and \textit{ilv1}Δ increasing threonine biosynthetic pathway flux by general control. However, \textit{hom3}Δ \textit{gly1}Δ \textit{thr1}Δ and \textit{hom3}Δ \textit{ilv1}Δ \textit{thr1}Δ strains were as sensitive to exogenous homoserine as a \textit{hom3}Δ background.

### Table 3. Phenotypes of various threonine-metabolizing mutants in \textit{THRI} and \textit{thr1}Δ backgrounds

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Homoserine</th>
<th>41°C</th>
<th>8°C</th>
<th>pH 8</th>
<th>NaCl (0.7 M)</th>
<th>LiCl (0.05 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\textit{thr1}</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\textit{thr1 hom3}</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\textit{cha1}</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\textit{cha1 thr1}</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\textit{ilv1}</td>
<td>+</td>
<td>+ /-</td>
<td>+ /-</td>
<td>+ /-</td>
<td>+ /-</td>
<td>+ /-</td>
</tr>
<tr>
<td>\textit{gly1}</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\textit{gly1 thr1 hom3}</td>
<td>+/-</td>
<td>NT(^{*})</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

\(^{*}\) NT, not tested.
**Does homoserine inhibit or replace threonine incorporation in translation?** The final process using threonine as a substrate involves translation. If homoserine inhibits the threonyl-tRNA synthetase Ths1p, overexpression of THS1 should alleviate thr1Δ homoserine toxicity. The homoserine sensitivity of the *S. cerevisiae* YJM145 background strains YJK1301, YJK1302 (P_GAL1/THS1/THS1), YJK1319, and YJK1321 (P_GAL1/THS1/THS1 thr1Δ/thr1Δ), were compared in P_GAL1 inducing and repressing conditions. In addition, the homoserine sensitivity of S288c background strains YJK726 (thr1Δ), YJK2380 (thr1Δ P_GAL1/THS1), and YJK2376 [thr1Δ P_GAL1/THS1 plus pJO364 (CEN THS1MX4)] were compared under inducing conditions.

In all cases, overexpression of THS1 had no influence on homoserine toxicity of thr1Δ mutants; thus, homoserine likely does not inhibit Ths1p.

The toxic effects of homoserine may be due to its misacylation into proteins in the place of threonine, via mis-acylation by the threonyl-tRNA synthetase, Ths1p. Since the *E. coli* threonyl-tRNA synthetase, encoded by thrRS, does not mis-acylate homoserine (30), to test if homoserine toxicity is due to aminoacylation by *S. cerevisiae* Ths1p, we investigated whether the homoserine toxicity of *S. cerevisiae* thr1Δ mutants could be suppressed by expression of the *E. coli* thrRS instead of THS1. First, we tested the functionality of the thrRS gene product in *S. cerevisiae*. The growth rates of S288c background thr1Δ P_GAL1/THS1 strains containing low-copy-number plasmids expressing hph (pJO365 in control strain YJK2403), THS1 (pJO364 in strain YJK2376), or thrRS (pJO367 and pJO368 in strains YJK2405 and YJK2412, respectively) from the constitutive TEF promoter were compared on YPD and YPGal. The thr1Δ P_GAL1/THS1 control strain (YJK2403) did not grow on YPD; therefore, P_GAL1/THS1 is indeed not expressed in YPD. Since strains containing the thrRS-expressing plasmids grew on YPD (i.e., chromosomal P_GAL1/THS1 OFF), *E. coli* thrRS encodes a functional threonyl-tRNA synthetase in *S. cerevisiae*. The homoserine MIC50 values were then compared in both YPD and YPGal. We found no difference between the homoserine sensitivity of thr1Δ P_GAL1/THS1 strains expressing plasmid-borne THS1 versus thrRS in YPD (MIC50 values were 500 μg ml⁻¹) or YPGal (MIC50 values of 1,000 μg ml⁻¹). Therefore, our results indicate that the toxic effects of homoserine do not involve, or do not solely involve, mis-acylation by the threonyl-tRNA synthetase Ths1p.

If homoserine toxicity is due to its incorporation into proteins in place of threonine, treatment with the translation inhibitor cycloheximide should suppress the homoserine toxicity of thr1Δ mutants. To test this hypothesis, a thr1Δ strain, YJK498a, was first incubated for 2 h in SD + Thr (10×, 3 mg ml⁻¹) with various amounts of cycloheximide (0 to 10 μg ml⁻¹) and in the absence of exogenous homoserine, over which time, thr1Δ mutant cell viability was unchanged from initial levels for all samples (Fig. 5). Cells were then transferred to SD + Thr (0.1× at 0.03 mg ml⁻¹) containing the same cycloheximide concentrations as the preincubation step, cultures were split, and homoserine was added to half of the samples. Following 3 h of incubation, while all other samples remained at input levels, there was an approximate 10,000-fold reduction in thr1Δ mutant levels in the sample containing homoserine without cycloheximide. Similarly, following 6 h of incubation, there was a 10,000-fold reduction in thr1Δ mutant levels in the sample without homoserine or cycloheximide (probably reflecting starvation), as well as a further decline in levels for the sample containing homoserine without cycloheximide. However, the presence of cycloheximide blocked killing in all samples, with increasing protection correlating with increasing levels of cycloheximide. The results indicate a requirement for protein synthesis for homoserine toxicity, consistent with homoserine toxicity being caused by its incorporation into proteins.

**Identification of high-copy suppressors of the homoserine toxicity.** To better understand homoserine toxicity, we isolated high-copy suppressors of thr1Δ mutant homoserine toxicity. Strain YJK928 (thr1Δ ura3Δ) was transformed with a URA3-marked 2μm high-copy-number yeast genomic library containing 5- to 10-kb insertions within the Sall restriction site on plasmid pRS426 (constructed by C. Alarcon). Ura+ transformants were selected, replica plated to YPD + homoserine (1 mg ml⁻¹), and homoserine-resistant transformants were screened for threonine prototrophy to eliminate clones containing the THR1 gene. Plasmids were isolated from threonine-auxotrophic, homoserine-resistant transformants and reintroduced into naïve YJK928 to confirm the plasmid dependence of the homoserine-resistant phenotype. From a total of approximately 30,000 transformants screened, we isolated eight plasmids belonging to five different complementation groups. Following subcloning, and ruling out genes designated by the *Saccharomyces* Genome Database (www.yeastgenome.org) as dubious ORFs, we determined that the suppressing genes were...
First, we investigated the effect of reduced proteasome function by overexpression of \( \text{ALY1} \) (pJO335; strain YJK1386), \( \text{HUA1} \) (pJO338; strain YJK1388), \( \text{SSH4} \) (pJO339; strain YJK1389), and \( \text{TAT1} \) (pJO340; strain YJK1517). Other strains included YJK498a (\( \text{thr1} \)), YJK1074 (\( \text{doa4} \)), and YJK1077 (\( \text{thr1 doa4} \)).

**Synthetic pma1-114 doa4, thr1Δ pma1-114 and thr1Δ doa4 phenotypes.** In the S. cerevisiae Y55 background, a mutation designated \( \text{stc1} \) was isolated that suppressed \( \text{thr4} \) cold sensitivity and partially suppressed the homoserine toxicity of \( \text{thr4} \) mutants (Materials and Methods). Interestingly, we find that the \( \text{stc1} \), \( \text{thr4} \), and \( \text{thr1} \) mutations all have synthetic phenotypes in combination with a mutant allele of the plasma membrane Pma1p ATPase gene, \( \text{pma1-114} \) (61), which results in depolarization of cellular membrane potential (71). Compared with \( \text{thr4} \), \( \text{thr1Δ} \), or \( \text{pma1-114} \) mutants, \( \text{thr4} \) \( \text{pma1-114} \) and \( \text{thr1Δ pma1-114} \) strains were hypersensitive to growth on YPD + KCl (1.5 M) or glycerol (2.5 M), and these phenotypes were homoserine dependent, since sensitivity was suppressed by \( \text{hom3Δ} \) (data not shown). Furthermore, relative to \( \text{pma1-114} \) strains and \( \text{stc1} \) strains, \( \text{pma1-114 stc1} \) strains were more temperature sensitive at 37°C and sensitive to growth on YPD + 1.5 M KCl, a phenotype that we exploited to identify \( \text{STC1} \).

To clone \( \text{STC1} \), we introduced a wild-type yeast genomic library containing 15- to 20-kb genomic inserts housed in a YCp50 \( \text{URA3} \)-marked centromeric vector (79) into an \( \text{stc1 pma1-114} \) strain (YJK1018) and screened for complementation of the \( \text{KC1} \) sensitivity. From approximately 25,000 transformants, two \( \text{KC1} \)-resistant transformants were isolated; following restriction digest analysis, both plasmids were determined to contain identical 15.2-kb inserts (pJO304). The complementing plasmid conferred \( \text{KC1} \) and high-temperature resistance when introduced into a naïve \( \text{stc1 pma1-114} \) strain. By deleting various regions of the complementing plasmid, the complementing gene was determined to be \( \text{DOA4} \) (see Fig. S1 in the supplemental material). Furthermore, a \( \text{doa4Δ pma1-114} \) strain (YJK1060) exhibited the same \( \text{KC1} \) and temperature sensitivity observed for \( \text{stc1 pma1-114} \) strains. Finally, \( \text{doa4Δ} \) disruption in the YJM145 background \( \text{thr1} \) strain (YJK1077) partially suppressed the \( \text{thr1Δ} \)-dependent \( \text{KC1} \) and homoserine sensitivity (Fig. 6A). \( \text{DOA4} \) encodes a ubiquitin isopeptidase that is important for recycling ubiquitin from proteasome-bound ubiquitinated intermediates (90). The synthetic \( \text{doa4Δ pma1-114} \) phenotype is consistent with previous findings that the fate and sorting of another mutant Pma1p protein are regulated by ubiquitination (26, 72). Importantly, the suppression of \( \text{thr1Δ} \) phenotypes by \( \text{doa4Δ} \) further implicates the ubiquitin pathway in mediating the \( \text{thr1Δ} \) mutant homoserine toxicity.

To investigate whether \( \text{doa4Δ} \) may suppress \( \text{thr1Δ} \) phenotypes by mechanisms distinct from enhanced stability of various amino acid permeases (18, 27) that might increase threonine uptake, we studied the effect of \( \text{doa4Δ} \) on \( \text{thr1Δ} \) starvation in SD. Interestingly, while the viability of a \( \text{thr1Δ} \) strain (YJK498a) was reduced on average 66-fold after 4 h of starvation and 479-fold after 8 h of starvation, an isogenic \( \text{doa4Δ} \) \( \text{thr1Δ} \) strain (YJK1077) remained at approximately input levels after 8 h, with a reduction in viability of only 1.5-fold (Fig. 6B). Therefore, while increased amino acid import may play a role in the \( \text{doa4Δ} \) suppression of \( \text{thr1Δ} \) phenotypes, suppression also occurs by an alternative mechanism.

**Role for proteasome function in homoserine toxicity.** The \( \text{doa4Δ} \) suppression of \( \text{thr1Δ} \) phenotypes suggests that reduction of ubiquitin-mediated turnover of a protein, a subset of proteins, or all proteins benefits \( \text{thr1Δ} \) survival. Since ubiquitinated proteins may be degraded by the proteasome, we investigated a role for the proteasome in \( \text{thr1Δ} \) toxic phenotypes. The ATPases encoded by \( \text{RPT6} \) (\( \text{CRL3} \)) and \( \text{RPT4} \) (\( \text{CRL13} \)), and a proteolytic enzyme encoded by \( \text{PRE3} \) (\( \text{CRL21} \)) comprise three subunits of the proteasome (24, 28, 37, 80, 82), all of which are required for maximal proteasome-dependent proteolysis (20, 81). First, we investigated the effect of reduced proteasome function on the homoserine sensitivity of \( \text{thr1Δ} \) mutants, using the Y55 background—the background in which the \( \text{crf} \) (cycloheximide-resistant, ts-lethal) mutants were isolated and described (59, 60). Experiments with the \( \text{crf} \) strains were performed at the semipermissive temperature of 30°C. Significantly, \( \text{thr1Δ} \) strains

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**FIG. 6.** (A) Suppression of the \( \text{thr1Δ} \) mutant homoserine toxicity by \( \text{doa4Δ} \) and overexpression of \( \text{ALY1} \) (pJO335; strain YJK1386), \( \text{HUA1} \) (pJO338; strain YJK1388), \( \text{SSH4} \) (pJO339; strain YJK1389), and \( \text{TAT1} \) (pJO340; strain YJK1517). Other strains included YJK498a (\( \text{thr1Δ} \)), YJK1074 (\( \text{doa4Δ} \)), and YJK1077 (\( \text{thr1Δ doa4Δ} \)). (B) Viability following starvation in SD for \( \text{thr1Δ} \) and \( \text{thr1Δ doa4Δ} \) strains.
types and determined that rather than being due to a general consequence of threonine auxotrophy or the loss of a secondary function of these enzymes, the phenotypes are caused by the toxic intermediate homoserine. Homoserine was also toxic for C. albicans thr1Δ mutants. Our findings are consistent with the growth impairment previously observed in homoserine-overproducing yeast, which was attributed to substantial restructuring of the metabolic flux or accumulation of metabolites, such as threonine or homoserine, interfering with general cell metabolism (16). Our results are also consistent with homoserine toxicity in mammalian cells, which is suppressed by coexpression of bacterial homoserine kinase and threonine synthase, which simultaneously synthesize threonine and detoxify homoserine (75).

In S. cerevisiae, threonine feedback regulation is mediated by an interaction between aspartate kinase and the FPR1 gene product, FKB12, which is inhibited by FK506 binding FKBP12, resulting in increased flux through the pathway (1). Consistent with this, S. cerevisiae thr1Δ mutation was synthetic lethal in combination with fpr1Δ, and thr1Δ mutants were hypersensitive to FK506. Interestingly, however, we found no observable phenotype upon disruption of the orthologous gene in C. albicans, RBP1, in combination with thr1Δ mutation, and C. albicans thr1Δ mutants were not hypersensitive to FK506. Therefore, despite similarities in S. cerevisiae Fpr1p and C. albicans Rbp1p functions (8), C. albicans Hom3p function may be controlled less by threonine feedback regulation than the S. cerevisiae Hom3p, or C. albicans Hom3p employs a different binding partner to mediate the feedback regulation. It is also possible that C. albicans threonine pathway flux could be controlled by an alternative mechanism, such as phosphatase degradation of aspartyl phosphate, or C. albicans Hom2p may have a weaker affinity for aspartyl phosphate than S. cerevisiae Hom2p.

Intermediate accumulation has been demonstrated to result in toxic effects in various other amino acid biosynthetic mutants. The essential nature of methionine synthase (MET7) in C. albicans (89) and the drug sensitivity of C. neoformans met6 mutants (67) have been attributed to the accumulation of homocysteine, which is structurally similar to homoserine and has been reported to interfere with purine and sterol biosynthesis in Schizosaccharomyces pombe and S. cerevisiae, respectively (19, 31, 66). Homocysteine editing to homocysteine thiolactone by the yeast methionyl-tRNA synthetase (39) may also be inhibitory to yeast, as is observed in humans (38, 70). Furthermore, the accumulation of β-aspartate semialdehyde is inhibitory in mutants of the homoserine biosynthetic gene encoding homoserine dehydrogenase, HOM6 (4). Indeed, we also observed some deleterious phenotypes for the hom6Δ mutant such as increased salt and temperature sensitivity compared with the wild type and hom3Δ mutants, although typically to a lesser extent than that observed for thr1Δ and thr4Δ mutants, which could be attributed to β-aspartate semialdehyde accumulation. However, since hom6Δ mutants do not share all of the phenotypes of thr1Δ and thr4Δ mutants, and hom6Δ suppresses thr1Δ and thr4Δ mutant-specific phenotypes, we have ruled out a role for β-aspartate semialdehyde accumulation in the deleterious phenotypes of thr1Δ and thr4Δ mutants.

While phosphohomoserine accumulation may play a role in the toxic phenotypes of thr4Δ mutants, since thr4Δ mutants

FIG. 7. Suppression of the thr1Δ starvation-cidal phenotype by reduced proteasome function. Strains included YJK1766 (thr1Δ), YJK2591 (hom3Δ), YJK2596 (thr1Δ crl3-3), YJK2599 (hom3Δ crl3-3), YJK2620 (thr1Δ crl3-1), YJK2590 (hom3Δ crl3-1), YJK2613 (thr1Δ crl21-1), and YJK2614 (hom3Δ crl21-1). Starvation assays were performed with two isogenic strains for each thr1Δ crl genotype, the results of which were virtually identical, and only one strain is shown.

VIABILITY FOLLOWING STARVATION

DISCUSSION

Myriad phenotypes not typically associated with amino acid auxotrophy, such as sensitivity to pH (21), salts (7), cold (78) DNA-damaging agents (5, 6), and threonine starvation (49) have been described for S. cerevisiae thr1 and thr4 mutants. Here, we report that these mutants are also sensitive to high temperatures, as well as KCl and glycerol when combined with the pmal-114 allele. We dissected the basis of these pheno-
share all of the same phenotypes but phenotypes are generally less deleterious than in thr1Δ mutants, thr4Δ mutants may accumulate homoserine, but to a lesser extent than thr1Δ mutants. When phosphohomoserine levels are high, as seems likely in a thr4Δ mutant, product inhibition of Thr1p (91) would result in homoserine accumulation, or phosphatases may convert phosphohomoserine back to homoserine. Since phosphoserine phosphatase (Ser2p) catalyzes a similar reaction, the dephosphorylation of phosphoserine to serine (62), Ser2p is a candidate phosphohomoserine phosphatase in S. cerevisiae.

What then is the toxic role of homoserine in the cell, and why does it result in such a plethora of phenotypes? We present evidence that homoserine acts as a toxic threonine analog; however, for three of the gene products that metabolize threonine, Chalp, Ivl1p, or Gly1p, we did not observe any evidence of homoserine inhibition or metabolism. It remains possible that homoserine may act as an allosteric inhibitor of an unknown and/or important yeast gene product. Alternatively, homoserine may be converted to homoserine lactone, similar to the toxicity in mammalian cells caused by conversion of the structurally similar homocysteine to homocysteine thiolactone (40); however, we see no evidence of homoserine lactone toxicity in THR1 or thr1Δ strains (data not shown). A final process in which threonine is a substrate is translation. Homoserine is likely not inhibiting threonine incorporation into proteins via inhibition of the threonyl-tRNA synthetase Ths1p, since overexpression of THS1 did not alleviate homoserine toxicity. Similarly, the lack of suppression of homoserine toxicity by expression of the E. coli threonyl-tRNA synthetase, which does not aminoacylate homoserine (30), argues against a role for homoserine incorporation into proteins via only Ths1p-mediated aminoaoylation. However, since translation is required for homoserine toxicity, homoserine may be misacylated by multiple aminocyl tRNA synthetases, as is the case for homocysteine (12, 13, 41–44). Various homoserine-mediated deleterious phenotypes observed for thr1Δ and thr4Δ mutants would be consistent with homoserine incorporation into proteins, creating aberrant proteins that are sensitive to various stresses, analogous to effects mediated by the incorporation into protein of toxic amino acid analogs (93, 96). If homoserine is incorporated into proteins causing misfolding, this would also be consistent with thr1Δ and thr4Δ being synthetic lethal with hsp82Δ, an isoform of the chaperone HSP90 important for protein folding (68), and/or having increased sensitivity to HSP90 inhibitors (57, 97).

Finally, to elucidate the mechanism of homoserine toxicity, we identified suppressors of homoserine toxicity. Interestingly, most of the suppressor genes encoded functions involved in the ubiquitin pathway, which is important for the regulation or destruction of multiple proteins such as signaling, cell cycle control, membrane, or aberrant proteins (35). The involvement of the ubiquitin pathway and proteasome in degrading aberrant proteins provides an intriguing explanation for the role of the ubiquitin pathway in homoserine toxicity. Since dob4Δ and proteasome mutants, which have reduced turnover of ubiquitin-tagged and/or proteasome-bound proteins and thus increased sensitivity to amino acid analogs such as canavanine (3, 32, 59, 80, 90), actually suppress thr1Δ homoserine toxicity, any possible misincorporation of homoserine into proteins may not inactivate proteins per se, but may still induce ubiquitin-mediated turnover of homoserine-containing protein, resulting in undesired proteolysis of one or more essential proteins, such as Pma1-114p, which results in cell death.

ACKNOWLEDGMENTS

We thank the laboratory of Joseph Heitman for plasmid pSF52A, with the permission of Joachim Morschhäuser; John Rohde for the high-copy-number yeast genomic library; Alan Goldstein for plasmid pAG107, and Bradly Nicholson for plasmid pBH05. This study was funded by the National Institutes of Health R21 grant AI072027.

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