Cytocidal amino acid starvation of *Saccharomyces cerevisiae* and *Candida albicans* acetolactate synthase (*ilv2Δ*) mutants is influenced by the carbon source and rapamycin

Joanne M. Kingsbury and John H. McCusker

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

The isoleucine and valine biosynthetic enzyme acetolactate synthase (Ilv2p) is an attractive antifungal drug target, since the isoleucine and valine biosynthetic pathway is not present in mammals, *Saccharomyces cerevisiae* *ilv2Δ* mutants do not survive *in vivo*, *Cryptococcus neoformans* *ilv2Δ* mutants are avirulent, and both *S. cerevisiae* and *Cr. neoformans* *ilv2Δ* mutants die upon isoleucine and valine starvation. To further explore the potential of Ilv2p as an antifungal drug target, we disrupted *Candida albicans ILV2*, and demonstrated that *Ca. albicans* *ilv2Δ* mutants were significantly attenuated in virulence, and were also profoundly starvation-cidal, with a greater than 100-fold reduction in viability after only 4 h of isoleucine and valine starvation. As fungicidal starvation would be advantageous for drug design, we explored the basis of the starvation-cidal phenotype in both *S. cerevisiae* and *Ca. albicans ilv2Δ* mutants. Since the mutation of *ILV1*, required for the first step of isoleucine biosynthesis, did not suppress the *ilv2Δ* starvation-cidal defects in either species, the cidal phenotype was not due to α-ketobutyrate accumulation. We found that starvation for isoleucine alone was more deleterious in *Ca. albicans* than in *S. cerevisiae*, and starvation for valine was more deleterious than for isoleucine in both species. Interestingly, while the target of rapamycin (TOR) pathway inhibitor rapamycin further reduced *S. cerevisiae* *ilv2Δ* starvation viability, it increased *Ca. albicans ilv1Δ* and *ilv2Δ* viability. Furthermore, the recovery from starvation was dependent on the carbon source present during recovery for *S. cerevisiae ilv2Δ* mutants, reminiscent of isoleucine and valine starvation inducing a viable but non-culturable-like state in this species, while *Ca. albicans ilv1Δ* and *ilv2Δ* viability was influenced by the carbon source present during starvation, supporting a role for glucose wasting in the *Ca. albicans* cidal phenotype.

INTRODUCTION

Due to the high level of conserved pathways between fungi and mammals, effective antifungal therapy relies upon drugs from only three main drug classes, the polyenes, azoles and echinocandins, which target only two cellular components, the cell membrane and cell wall (Tkacz & DiDomenico, 2001). In addition to the cell wall, another fundamental difference between fungi and mammals is the ability of fungi to synthesize all amino acids, while mammals must acquire many amino acids, such as isoleucine and valine, in their diet. Certain *in vivo* environments occupied by fungal pathogens are likely to be limiting in amino acids, as serum amino acid concentrations are low (Crispens, 1975; Cynober, 2002), genes for the biosynthesis and transport of various amino acids and the general control regulator Gcn4p are induced upon exposure of fungi to neutrophils, macrophages or blood (Fradin *et al.*, 2003, 2005; Lorenz *et al.*, 2004), and various amino acid auxotrophs are unable to survive *in vivo* and/or are avirulent (Goldstein & McCusker, 2001; Kingsbury *et al.*, 2004a, b, 2006; Kingsbury & McCusker, 2008; Liebmann *et al.*, 2004; Nazi *et al.*, 2007; Pascon *et al.*, 2004; Yang *et al.*, 2002). Therefore, various amino acid biosynthetic enzymes offer an attractive alternative class of antifungal targets.

Since many fungal infections occur in immunocompromised patients, and fungistatic drugs require a healthy...
immune system to clear the infection so that it does not recrudesce upon drug removal, targeting biosynthetic enzymes for which the resulting auxotrophy is fungicidal rather than fungistic is advantageous. The degree of viability loss during starvation is nutrient-dependent; for example, while starvation for methionine, phosphate or sulfate is generally cytostatic (Barclay & Little, 1977; Boer et al., 2008; Kingsbury et al., 2004a; Unger & Hartwell, 1976), starvation for leucine or uracil is minimally cytoidal [50 % reduction after 2 days (Boer et al., 2008)], while inositol starvation is highly cytoidal [≥99 % reduction after 1 day (Culbertson & Henry, 1975; Henry et al., 1975)]. Viability during starvation has been shown to correlate with how rapidly the cell cycle arrests upon starvation, with methionine-, phosphate- and sulfate-starved cells undergoing a prompt and uniform arrest in the G1/G0 (unbudded) stage (Saldanha et al., 2004; Unger & Hartwell, 1976), while leucine-starved cells do not undergo uniform arrest (Saldanha et al., 2004), and waste the excess glucose present (Brauer et al., 2008). The degree of viability reduction is also dependent on the carbon source available during starvation and the genetic background (Boer et al., 2008). Finally, the starvation-cidal phenotype may be contingent on which biosynthetic enzyme of the particular pathway is inhibited or mutated; for example, Cryptococcus neoformans (and Saccharomyces cerevisiae) met3 mutants are cytostatic for methionine starvation, while Cr. neoformans met6 mutants, which accumulate the toxic intermediate homocysteine, are cytoidal (Pascon et al., 2004).

Enzymes of the isoleucine and valine biosynthetic pathway such as acetohydroxy acid synthase (Ilv2p) are of particular interest from the perspective of antifungal drug targets, since S. cerevisiae and Cr. neoformans ilv2Δ mutants rapidly lose viability during isoleucine and valine starvation [90 % reduction after 1 day (Kingsbury et al., 2004a)], and do not survive in vivo and/or are avirulent (Kingsbury et al., 2004a, 2006). The first step of isoleucine biosynthesis (reviewed by Chipman et al., 1998; Fig. 1) involves the deamination of threonine to α-ketobutyrate by threonine deaminase (Ilv1p). The second step, catalysed by Ilv2p, combines α-ketobutyrate and pyruvate to yield α-acetolactate. Ilv2p also converts two pyruvate molecules to α-aceto-α-hydroxybutyrate, an intermediate in valine and leucine biosynthesis. The absence of acetolactate synthase activity in bacteria results in elevated levels of α-ketobutyrate, which has been hypothesized to inhibit growth by interfering with utilization of glucose (LaRossa & Van Dyk, 1987; LaRossa et al., 1987; Van Dyk et al., 1987). Therefore, α-ketobutyrate accumulation may explain the deleterious phenotypes of fungal ilv2Δ mutants, such as the starvation-cidal phenotype.

Since Cr. neoformans and S. cerevisiae ilv2Δ mutants are avirulent and/or unable to survive in vivo (Kingsbury et al., 2004a, 2006), and die upon starvation for isoleucine and valine, we further investigated the potential of acetolactate synthase as a drug target in the highly clinically relevant pathogen Candida albicans. Importantly, we found that Ca. albicans ilv2Δ mutants were significantly attenuated in virulence, and undergo a dramatic decline in viability upon isoleucine and/or valine starvation. By looking at the starvation phenotypes of other isoleucine–valine pathway mutants, we tested the two following hypotheses to explain the basis of the starvation-cidal effect in both S. cerevisiae and Ca. albicans: first, that death is due to toxic α-ketobutyrate accumulation; and second, that death is a consequence of isoleucine, leucine and/or valine starvation.

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**Fig. 1.** Isoleucine, valine and leucine biosynthetic pathways.
We exclude the α-ketobutyrate hypothesis, and demonstrate that the cidal effect is due to starvation for isoleucine and/or valine, with valine starvation being more deleterious. We examined the effects of rapamycin on starvation, which we show to be deleterious for \( S.\ cerevisiae \) survival, yet beneficial for \( C.\ albicans \) survival. We further determine that the extent of loss of viability is governed by the carbon source present during starvation recovery for \( S.\ cerevisiae \), and the carbon source present during starvation for \( C.\ albicans \).

**METHODS**

**Strains, media and growth conditions.** \( C.\ albicans \) strains used in this study were isogenic with strain SC5314 (Gillum et al., 1984), and \( S.\ cerevisiae \) strains were isogenic with S288c or YJM145 (McCusker et al., 1994) (Table 1). Standard yeast culture media including yeast extract peptone dextrose (YPD), yeast extract peptone ethanol glycerol (YPEG) and synthetic dextrose (SD) were prepared as described previously (Ito-Harashima et al., 2002; Sherman et al., 1974). SD (proline) contained 1% (w/v) proline instead of ammonium sulfate. Yeast extract peptone maltose [YP(maltose)] was prepared as for YPD, expect that maltose (2%, w/v) replaced glucose. Synthetic ethanol glycerol (SEG) liquid media contained succinic acid (1%, w/v), glycerol (2%, v/v) (mixed, adjusted to pH 5.5 with potassium hydroxide), yeast nitrogen base with succinic acid (1%, w/v), glycerol (2%, v/v) (mixed, adjusted to pH 5.5 with potassium hydroxide), yeast nitrogen base with ammonium sulfate (0.67%, w/v) and ethanol (2.5%, v/v), and was filter-sterilized. Where specified, media were supplemented with isoleucine (0.23 mM), valine (1.28 mM) or leucine (0.46 mM), nourseothricin (Nat; 100 \( \mu \)g ml\(^{-1}\) for \( S.\ cerevisiae \) selection, 200 \( \mu \)g ml\(^{-1}\) for \( C.\ albicans \) selection; Hans Knoll Institute für Naturstoff-Forschung), hygromycin B (300 \( \mu \)g ml\(^{-1}\), Calbiochem) or geneticin (200 \( \mu \)g ml\(^{-1}\), Life Technologies).

**Strain construction.** \( S.\ cerevisiae \) genes were replaced with the natMX4, kanMX4, hphMX4 or loxP-kanMX4-loxP cassettes by PCR-mediated gene deletion, as described previously (Goldstein & McCusker, 1999; Guldener et al., 1996; Wach et al., 1994). To construct strains containing multiple deletions, separate strains containing deletions with different drug markers were crossed, crosses were sporulated and dissected, and segregants with the appropriate genotype were selected for further analysis. Gene deletions were confirmed by PCR and by acquisition or loss of a phenotype.

\( C.\ albicans \) genes were replaced with the SAT1 flipper cassette from plasmid pSF52A (Reuss et al., 2004) using a similar PCR-mediated disruption strategy, in which the SAT1 flipper cassette 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 PCR product by electroporation (Reuss et al., 2004), and Nat-resistant transformants were purified and verified by PCR analysis. To induce FLP-mediated excision of the SAT1 cassette, transformants were grown for 2 h in YP(maltose), leaving an FLP recombination target (FRT) site. Natsensitive +/Δ strains then underwent a second round of transformation to disrupt the second allele. To reintroduce the wild-type gene, homoygous disruption strains were transformed with the wild-type gene of interest, and amplified using primers homologous to

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*All \( C.\ albicans \) strains were isogenic with SC5314 and all \( S.\ cerevisiae \) strains were isogenic with S288c, except YJK564, which was isogenic with YJM145 (McCusker et al., 1994).
sequences upstream and downstream of the deleted region. Transformants in which the wild-type allele had replaced a disrupted allele were chosen; thus, the complementing gene was expressed from its original chromosomal location. All disruptions and mutant complementations were verified by PCR, phenotype where available, and Southern hybridization analysis (Supplementary Fig. S1).

All primers used are listed in Supplementary Table S1.

**Manipulation of DNA.** *S. cerevisiae* and *Ca. albicans* genomic DNA was extracted as described previously (Hoffman & Winston, 1987). For Southern hybridization analyses, *Ca. albicans* DNA (2 µg) was digested with various restriction enzymes, separated by gel electrophoresis in a 1 % (w/v) agarose gel, denatured and transferred to a nylon membrane (Roche), as described previously (Sambrook et al., 1989). Hybridization probes were PCR-amplified using the primer pairs JO784 + JO787 (ILV1), JO515 + JO518 (ILV2), and JO662 + JO663 (MET2). Probes were purified following agarose gel electrophoresis using the QIAquick Gel Extraction kit (Qiagen) and labelled with [32P]dCTP (Perkin-Elmer) using the Rediprime II Random Prime Labeling system (Amersham Biosciences), as described by the manufacturer. Prehybridizations and hybridizations were carried out in ULTRAhyb buffer (Ambion), membranes were washed according to manufacturer’s instructions, and hybridized bands were visualized using a Typhoon 9200 Variable Mode imager (Molecular Dynamics).

**Mouse infections.** The virulence of *Ca. albicans* strains was tested by injecting 6-week-old male CD-1 mice with 1 × 10⁶ cells suspended in sterile PBS, via the lateral tail vein. Typically, 10 mice were infected per *Ca. albicans* strain tested. Mice were fed ad libitum for the course of the experiment. Mice were observed and weighed twice daily, and animals that appeared moribund (judged by >15 % loss of body weight, lethargy, or being unable to access food) were euthanized. Mice that remained healthy after 28 days were euthanized, and their kidneys, liver and spleen were recovered and homogenized in 3 ml PBS + streptomycin + ampicillin. The entire spleen and 250 µl of each liver and kidney homogenate were plated on YPD + Nat to ascertain whether the infection had been cleared. Mouse survival data were analysed using the Kaplan–Meier test. Mouse experiments met with institutional guidelines and were approved by the Institutional Animal Care and Use Committee.

**Starvation assays.** To assess the ability of auxotrophic strains to survive amino acid starvation, overnight cultures grown in YPD or YPEG were pelleted, washed twice in sterile distilled water, and added to 5 ml SD or SEG to a concentration of approximately 10⁷ cells ml⁻¹. Cultures were incubated at 30 °C with aeration, and at various time points, aliquots were removed, serially diluted and plated to YPD or YPEG to determine viable c.f.u. Freshly poured (≤2 weeks) YPD plates were used to circumvent as much as possible the considerable variation observed for ilv2Δ mutant starvation recovery on different batches of YPD. For approximate numbers, 5 ml volumes of 10-fold dilutions were plated, while 100 µl volumes were plated for more exact estimations; experiments were typically repeated in triplicate. *S. cerevisiae* colonies were counted after 3 days of incubation at 30 °C on YPD plates or 4 days of incubation on YPEG, and *Ca. albicans* colonies were counted after 2 days of incubation on YPD or 3 days of incubation on YPEG.

**α-Keto butyrate inhibition assay.** To determine the level of α-keto butyrate inhibition, strains were grown overnight in YPD, washed twice in water, and resuspended to a concentration of approximately 1–2 × 10⁶ c.f.u. ml⁻¹ in YPD. Volumes of 90 µl of cells were added to 10 µl of a twofold dilution series of α-keto butyrate in flat-bottomed microtitre plate (Corning) wells. Assays were performed in triplicate and incubated at 30 °C for 1–2 days.

**Growth assays.** To determine the effect of isoleucine and valine auxotrophy on growth, wild-type, ilv1Δ and ilv2Δ strains were grown overnight in YPD, washed twice in water, and resuspended in YPD or SD + isoleucine + valine media to OD₆₀₀ 0.05. One hundred micro-litre volumes of strains were incubated in 96-well microtitre plates (Corning) at 30 °C, and OD₆₀₀ readings were taken half-hourly using an automated Tecan Sunrise absorbance reader. Experiments were performed in triplicate.

**RESULTS**

**Ca. albicans ilv1Δ and ilv2Δ mutants have attenuated virulence**

Since *C. neoformans* ilv2Δ mutants are virulent (Kingsbury et al., 2004a) and *S. cerevisiae* ilv2Δ mutants do not survive in vivo (Kingsbury et al., 2006), we constructed and tested the virulence in mice of *Ca. albicans* ilv1Δ, ilv2Δ and ilv1Δ ilv2Δ mutants. As shown in Fig. 2, compared with the wild-type (2.6 ± 0.8 days, P=0.0254) and ILV1-complemented strain (CJK142; 1.6 ± 1.3 days, P=0.0008), mice infected with the ilv1Δ mutant (CJK140) survived longer on average (6.6 ± 5.5 days). Therefore, isoleucine auxotrophy has a modest effect on *Ca. albicans* virulence.

Relative to ilv1Δ mutants, the ilv2Δ mutant (CJK27) was far more attenuated in virulence, with 73 % of mice still surviving after 28 days; P values were <0.0029 and <0.0012 compared with the wild-type and ILV2-complemented strains, respectively. Examination of the kidneys, liver and spleen from the eight mice still surviving after 28 days revealed the presence of viable ilv2Δ mutants in the kidneys of six mice; thus, two mice had cleared the infection (<22 c.f.u. g⁻¹ of tissue). Therefore, isoleucine auxotrophy combined with valine auxotrophy has a more detrimental effect on *Ca. albicans* virulence than isoleucine auxotrophy alone.

The ilv1Δ ilv2Δ mutant, CJK132, was similarly shown to be significantly attenuated in virulence (P<0.0004 compared with the wild-type), with 80 % of the mice still surviving after 28 days, of which, three of eight had also cleared the infection. Since Ilv1p is required for the biosynthesis of the intermediate α-ketobutyrate, and the ilv1Δ disruption did not suppress the attenuated virulence of the ilv2Δ mutant, α-ketobutyrate accumulation plays no role in the greatly attenuated virulence of the ilv2Δ mutant.

**S. cerevisiae and Ca. albicans ilv2Δ mutants die rapidly upon isoleucine and valine starvation**

Since *S. cerevisiae* and *C. neoformans* ilv2Δ mutants are avirulent and die upon isoleucine and valine starvation (Kingsbury et al., 2004a), survival during amino acid starvation is likely to be important in vivo. Therefore, the ability of *S. cerevisiae* and *Ca. albicans* ilv2Δ mutants to survive isoleucine and valine starvation was compared. Averaged over three separate experiments, the *S. cerevisiae*
The \textit{S. cerevisiae} and \textit{Ca. albicans} \textit{ilv2Δ} starvation-cidal phenotype is not due to \(\alpha\)-ketobutyrate accumulation

The \textit{ilv2Δ} mutant fungicidal phenotype upon isoleucine and valine starvation may be due to toxicity caused by \(\alpha\)-ketobutyrate accumulation. If \(\alpha\)-ketobutyrate accumulation is toxic, \textit{ilv2Δ} mutants would likely be hypersensitive to exogenously added \(\alpha\)-ketobutyrate. To test whether \(\alpha\)-ketobutyrate is actually transported by cells, we first assessed the ability of exogenous \(\alpha\)-ketobutyrate to satisfy the auxotrophy of \textit{S. cerevisiae} and \textit{Ca. albicans} \textit{ilv1Δ} mutants (YJK2484 and CJK140), when added to a filter disk (50 \(\mu\)l of 100 mg ml\(^{-1}\)) on SD or SD (proline) plates to which the strains were applied. While the \(\alpha\)-ketobutyrate only minimally satisfied the \textit{S. cerevisiae} \textit{ilv1Δ} mutant auxotrophy, \(\alpha\)-ketobutyrate supported robust growth of the \textit{Ca. albicans} \textit{ilv1Δ} mutant on both media types (data not shown). The ability of \(\alpha\)-ketobutyrate to inhibit growth was then determined for \textit{S. cerevisiae} and \textit{Ca. albicans} wild-type (S094 and SC5314), \textit{ilv2Δ} (YJK2486 and CJK27), and \textit{ilv1Δ ilv2Δ} (YJK2487 and CJK132) strains in YPD. Even at the highest concentration of \(\alpha\)-ketobutyrate (10 mg ml\(^{-1}\)), no growth inhibition was observed for any strain, which argues against the hypothesis that \(\alpha\)-ketobutyrate is toxic in fungi.

To further test the \(\alpha\)-ketobutyrate accumulation hypothesis, we compared the amino acid starvation phenotypes of \textit{S. cerevisiae} and \textit{Ca. albicans} \textit{ilv1Δ ilv2Δ} mutants and \textit{ilv2Δ} mutants. Viability following starvation of both the \textit{S. cerevisiae} \textit{ilv1Δ ilv2Δ} mutant (YJK2487) and the \textit{Ca. albicans} \textit{ilv1Δ ilv2Δ} mutant (CJK132) was indistinguishable from that of the \textit{S. cerevisiae} and \textit{Ca. albicans} \textit{ilv2Δ} mutants, respectively (Figs 3a and 4a). Since disruption of \textit{ILV1} did not suppress the starvation-cidal phenotype of the \textit{ilv2Δ} mutants in either \textit{S. cerevisiae} or \textit{Ca. albicans}, \(\alpha\)-ketobutyrate accumulation does not play a role in this phenotype.

The \textit{ilv2Δ} mutant starvation-cidal phenotype is due to valine starvation in \textit{S. cerevisiae}, and multiple amino acid auxotrophies in \textit{Ca. albicans}

The \textit{ilv2Δ} mutants are auxotrophic for isoleucine, valine and leucine (Fig. 1). We therefore assessed various individual and combined contributions of starvation for isoleucine, valine or leucine to the starvation-cidal phenotype.

We first determined the effect of isoleucine starvation. If the cidal phenotype is due to isoleucine starvation alone, supplementation of media with isoleucine during starvation, which results in valine and leucine starvation alone, may enhance \textit{ilv2Δ} viability. However, neither \textit{S. cerevisiae} nor \textit{Ca. albicans} \textit{ilv2Δ} mutant viability was improved by isoleucine addition (Figs 3b and 4b). Since we observed considerably reduced growth of both \textit{S. cerevisiae} and \textit{Ca. albicans} \textit{ilv1Δ ilv2Δ} mutants in SD medium supplemented with isoleucine (and valine) (Supplementary Fig. S2), the lack of effect of isoleucine supplementation could be attributed to poor isoleucine uptake by \textit{ilv2Δ} mutants. Alternatively, the
results may indicate that, in both species, valine and/or leucine starvation can account for all of the starvation-cidal phenotypes.

To further test whether isoleucine starvation is cidal, the viability following amino acid starvation was tested for *S. cerevisiae* and *Ca. albicans ilv1* Δ mutants (YJK2484 and CJK40, respectively), which are auxotrophic only for isoleucine. The *S. cerevisiae ilv1* Δ mutant viability remained unchanged following 24 h starvation (c.f.u. were, on average, 111% of initial levels; Fig. 3a). Therefore, isoleucine starvation alone is not cidal for *S. cerevisiae* and hence does not explain the *ilv2* Δ cidal phenotype. In contrast, the *Ca. albicans ilv1* Δ mutant reduced in viability over time, although to a lesser extent than the *ilv2* Δ mutant, with an average 32-fold reduction in viability from input levels after 4 h, 200-fold reduction after 8 h, and 400-fold reduction after 24 h (Fig. 4a). Therefore, starvation for isoleucine alone is cytocidal for *Ca. albicans* and partly explains the *Ca. albicans ilv2* Δ starvation-cidal phenotype.

We next investigated the individual effect of leucine starvation on the *ilv2* Δ cidal phenotype. Since *S. cerevisiae* *leu2* mutants die upon leucine starvation (50% reduction after 2 days) in a different background containing at least one additional auxotrophy (Boer et al., 2008), the viability of *S. cerevisiae leu1* Δ (strain S3782, accumulates α-isopropylmalate), *leu2* Δ (strain S3807, accumulates β-isopropylmalate) and *leu4* Δ *leu9* Δ (strain S3783, no
intermediate accumulation) leucine auxotrophs was determined following leucine starvation. Following incubation in SD for 24 h, the leu1Δ, leu2Δ and leu4Δ leu9Δ mutant viability was 103, 84 and 90% of input levels, respectively (Fig. 3a). Therefore, in this strain background and experimental protocol, S. cerevisiae leucine starvation results in little to no reduction in viability over a short (24 h) period. Furthermore, leucine supplementation of starvation media, which results in isoleucine and valine starvation alone, did not alleviate S. cerevisiae ilv2Δ survival, and had little to no effect on Ca. albicans ilv2Δ survival (Figs 3b and 4b). Therefore, in both species, leucine starvation alone also does not explain the ilv2Δ starvation-cidal phenotypes. Rather, valine starvation alone (both species) or together with isoleucine starvation (Ca. albicans only) can account for most, if not all, of the starvation-cidal phenotypes.

Finally, we assessed the effect of valine starvation. Since the leucine auxotrophy can also be satisfied by valine due to the transamination of valine to the valine and leucine intermediate α-ketoisovalerate, valine supplementation results in isoleucine starvation alone (Fig. 1). Although the lack of an effect of isoleucine or leucine starvation alone, as described above, implied that the ilv2Δ starvation-cidal phenotype was due to valine starvation, valine supplementation did not alleviate the S. cerevisiae ilv2Δ survival defect during starvation (Fig. 3b). The lack of an effect of valine supplementation may be due to poor uptake of valine by the ilv2Δ strain in these conditions, such that cells continue to experience a certain state of valine starvation. Consistent with this hypothesis, S. cerevisiae ilv2Δ mutants grew more slowly than the wild-type and ilv1Δ mutants in both YPD and SD media supplemented with isoleucine and valine (Supplementary Fig. S2). In contrast to S. cerevisiae ilv2Δ mutants, valine supplementation significantly increased survival following starvation for the Ca. albicans ilv2Δ mutant at each time point, with survival kinetics more closely resembling those of the Ca. albicans ilv1Δ mutant (Fig. 4b). Results therefore indicate a significant role for valine starvation in the S. cerevisiae and Ca. albicans ilv2Δ starvation-cidal phenotype.
Since both isoleucine and valine starvation appeared to be more cytocidal for \textit{Ca. albicans} than \textit{S. cerevisiae}, to determine whether \textit{Ca. albicans} is more sensitive to amino acid starvation in general than \textit{S. cerevisiae}, the viability of \textit{Ca. albicans} met2Δ (CJK103) and \textit{S. cerevisiae} met2Δ (YJK564) methionine auxotrophs was determined following starvation for 24 h in medium lacking methionine (SD). After 24 h starvation, the \textit{S. cerevisiae} and \textit{Ca. albicans} met2Δ strains were at 116 and 115\% of input levels, respectively (Fig. 3a). Therefore, rather than being a general consequence of amino acid starvation in \textit{Ca. albicans}, the significantly reduced viability following starvation of \textit{Ca. albicans} ilv1Δ and ilv2Δ mutants is a unique feature of these mutants.

**Recovery from, and sensitivity to, isoleucine and valine starvation is highly influenced by carbon source and rapamycin**

Extreme differences were observed in the colony size of \textit{S. cerevisiae} ilv2Δ mutants recovering from isoleucine and valine starvation, which were reminiscent of petite formation (Fig. 5). Since \textit{Ca. albicans} is generally considered petite-negative (Bulder, 1964), the hypothetical induction of petite formation by isoleucine and valine starvation might explain why \textit{S. cerevisiae} ilv2Δ mutants survive this stress at orders of magnitude higher than \textit{Ca. albicans} ilv2Δ mutants. To test whether starvation induced petite formation in \textit{S. cerevisiae} ilv2Δ mutants, recovery from starvation was compared on YPD and YPEG. Surprisingly, instead of resulting in a decreased recovery on YPEG plates compared with YPD plates, consistent with petite formation, we observed considerably increased recovery, with c.f.u. approximately the same as input levels on YPEG plates (Fig. 3b). Therefore, rather than dying, \textit{S. cerevisiae} ilv2Δ mutants enter into a stage upon isoleucine and valine starvation that is technically viable, but non-recoverable on YPD.

In contrast to \textit{S. cerevisiae} ilv2Δ results, little difference (less than twofold) was observed for recovery of \textit{Ca. albicans} ilv2Δ mutants on YPEG compared with YPD following starvation, although a minor improvement (two- to threefold) in recovery was observed for cultures that had been supplemented with valine during starvation (Fig. 4c). However, similar to \textit{S. cerevisiae} ilv2Δ results, we found increased recovery on YPEG compared with YPD following the amino acid starvation of \textit{Ca. albicans} ilv1Δ, particularly at earlier time points, with approximately 10- and 30-fold improvements in recovery at 4 and 8 h, respectively (Fig. 4c). Therefore, a proportion of \textit{Ca. albicans} ilv1Δ mutants also appear to enter into a state that is viable, but non-recoverable on YPD.

Viability loss upon starvation for certain nutrients has been attributed to an inability to enter into a rapid and safe cell-cycle arrest upon starvation (Boer et al., 2008). Since this entry is regulated by the carbon source (Gray et al., 2004; Schneper et al., 2004), the carbon source present during nutrient starvation can influence viability (inhibition of arrest in the presence of glucose, arrest in the presence of ethanol and glycerol) (Boer et al., 2008). To investigate whether the carbon source present influences the isoleucine and valine starvation-cidal phenotype, we compared the survival of mutants in SD and SEG starvation media, with or without isoleucine, valine or leucine supplementation. The c.f.u. of \textit{S. cerevisiae} ilv2Δ and ilv1Δ ilv2Δ mutants recovered on YPD following starvation in SEG were no higher than when starved in SD, while numbers recovered on YPEG were again at input levels (Fig. 3b). Therefore, the carbon source present during starvation did not influence \textit{S. cerevisiae} ilv2Δ mutant survival.

Again contrasting with \textit{S. cerevisiae} results, we found that survival of the \textit{Ca. albicans} ilv2Δ mutant was significantly enhanced at earlier time points when starved in SEG compared with SD with the respective amino acid supplementations; for example, the average increases in survival in SEG, SEG+isoleucine, SEG+valine and SEG+leucine incubation at 4 h, were 90-, 37-, 31- and 84-fold, respectively. However, levels surviving more closely approximated those observed for starvation in SD supplemented with the respective amino acids after 24 h incubation (Fig. 4d includes SEG results, other results not shown). Furthermore, starvation in SEG was completely cytostatic for the ilv1Δ mutant after 24 h (Fig. 4d). Therefore, results are consistent with a role for the lack of an orderly cell cycle arrest, particularly in the isoleucine starvation-cidal phenotype in \textit{Ca. albicans}.

Since the TOR (target of rapamycin) pathway inhibits entry into the quiescent state and the cessation of glucose fermentation in nutrient-rich conditions (Gray et al., 2004; Zaman et al., 2008), we investigated whether starvation in the presence of rapamycin influenced the viability of the \textit{S. cerevisiae} ilv2Δ and \textit{Ca. albicans} ilv1Δ and ilv2Δ mutants. Rapamycin was added to SD at a concentration of 100 nM, since this was the concentration used in starvation assays

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**Fig. 5.** Effect of starvation on \textit{S. cerevisiae} ilv2Δ colony size. Cells were starved in SD medium and streaked onto YPD plates at the times indicated.
by Boer et al. (2008); this is the MIC$_{80}$ for wild-type $S$. cerevisiae (S094) and Ca. albicans (SC5314), and is not fungicidal at this concentration (data not shown). Starvation in SD + rapamycin (100 nM) significantly reduced the recovery of $S$. cerevisiae ilv2Δ mutants, with an average 54-fold lower recovery on YPD compared with the no-drug control, and an approximately 2000-fold overall decrease after 24 h (Fig. 3c). In contrast, starvation in SD + rapamycin suppressed the starvation-cidal phenotype of Ca. albicans ilv1Δ mutants, with an average sixfold and 76-fold higher recovery following 8 and 24 h starvation, respectively, compared with the no-drug control (Fig. 4d). Rapamycin also improved Ca. albicans ilv2Δ survival, with average recovery rates fivefold (after 8 h starvation) and 10-fold (after 24 h starvation) higher than that of the no-drug control. These results provide further support for the lack of an orderly arrest of the cell cycle contributing to the starvation-cidal phenotype in Ca. albicans ilv1Δ and ilv2Δ mutants.

DISCUSSION

Our previous demonstration that $S$. cerevisiae and Cr. neoformans ilv2 mutants are starvation-cidal and are unable to survive in vivo and/or are avirulent (Kingsbury et al., 2004a, 2006) provided promise for the exploitation of fungal acetolactate synthase as a novel antifungal drug target. In this study, we further strengthen the drug target utility of this enzyme by determining that Ca. albicans target. In this study, we further strengthen the drug target fungal acetolactate synthase as a novel antifungal drug (2004a, 2006) provided promise for the exploitation of

We considered the hypothesis that the starvation-cidal phenotypes of fungal ilv2Δ mutants were due to accumulation of the biosynthetic intermediate $\alpha$-ketobutyrate, since $\alpha$-ketobutyrate or its transamination product, $\alpha$-aminobutyric acid, accumulates upon inhibition of acetolactate synthase in plants and bacteria (LaRossa et al., 1987; Rhodes et al., 1987; Shaner & Singh, 1993). Although results from other researchers have shown a lack of correlation between $\alpha$-ketobutyrate accumulation and growth inhibition (Epelbaum et al., 1996; Landstein et al., 1990; Shaner & Singh, 1993), the high potency associated with the inhibition of acetolactate synthase has often been attributed to $\alpha$-ketobutyrate toxicity (Daniel et al., 1983, 1984; LaRossa & Van Dyk, 1987; LaRossa et al., 1987; Rhodes et al., 1987; Van Dyk et al., 1987). However, since neither $S$. cerevisiae nor Ca. albicans ilv2Δ mutants were sensitive to high exogenous levels of $\alpha$-ketobutyrate, and ilv1Δ ilv2Δ mutants that cannot accumulate $\alpha$-ketobutyrate were as starvation-cidal as ilv2Δ mutants, we ruled out the $\alpha$-ketobutyrate accumulation hypothesis (Epelbaum et al., 1996) as the explanation for the $S$. cerevisiae and Ca. albicans ilv2Δ starvation-cidal phenotypes.

Interestingly, isoleucine-auxotrophic Ca. albicans ilv1Δ mutants were also starvation-cidal, while $S$. cerevisiae ilv1Δ mutants were starvation-static over the same time period; thus, isoleucine starvation is more deleterious in Ca. albicans than in $S$. cerevisiae. The more severe starvation-cidal phenotype in ilv2Δ mutants compared with ilv1Δ mutants in both species and leucine auxotrophs in $S$. cerevisiae, together with the 10-fold increased survival upon supplementation of Ca. albicans ilv2Δ mutants with valine during starvation, but not isoleucine or leucine, suggest that valine starvation is more deleterious than isoleucine or leucine starvation in both species.

The carbon source present during recovery from starvation had a major effect on recovery from starvation of $S$. cerevisiae ilv2Δ and Ca. albicans ilv1Δ mutants, with greatly enhanced recovery when ethanol and glycerol were the carbon sources compared with glucose. The carbon source-dependent recovery is reminiscent of the viable but non-culturable phenomenon explored extensively in bacteria, whereby following exposure to various stresses such as starvation, cells that are metabolically active fail to grow under classical culture conditions, but may be able to be resuscitated upon administration of a certain trigger (Kell et al., 1998; Oliver, 2005). An analogous state has also been recorded for Saccharomyces, Candida and other yeast species following alcoholic fermentation and SO$_2$ addition during wine production (Divol & Lonvaud-Funel, 2005; Mills et al., 2002).

In contrast to $S$. cerevisiae ilv2Δ mutants, viability following starvation of Ca. albicans ilv1Δ and ilv2Δ mutants was strongly influenced by the carbon source present during starvation, with enhanced survival when mutants were incubated in ethanol and glycerol compared with glucose.
Further contrasting with \textit{S. cerevisiae ilv2}\(\Delta\) mutants, in which rapamycin reduced viability upon starvation, we observed a suppression of cell death upon starvation of both \textit{Ca. albicans ilv1}\(\Delta\) and \textit{ilv2}\(\Delta\) mutants when starved in the presence of rapamycin, an inhibitor of the TOR pathway that controls entry into stationary phase and cessation of glucose utilization when nutrients are plentiful (Gray et al., 2004; Zaman et al., 2008). Taken together, these \textit{Ca. albicans} results are similar to the findings of Boer et al. (2008), who proposed that since glucose represses pathways that activate entry into a resting state (Gray et al., 2004; Schnepner et al., 2004), the cells are failing to undergo a rapid and prompt cell arrest when glucose is the carbon source, and are wasting glucose, analogous to the Warburg effect described in tumours (Warburg, 1956).

Since \textit{S. cerevisiae} met2\(\Delta\) and \textit{Ca. albicans} met2\(\Delta\) mutants were equally starvation-static upon methionine starvation, amino acid starvation is not generally more starvation-cidal in \textit{Ca. albicans} than \textit{S. cerevisiae}. Therefore, two questions remain: first, precisely why \textit{ilv2}\(\Delta\) mutants are starvation-cidal; and second, why \textit{Ca. albicans ilv2}\(\Delta\) mutants die, or fail to recover, from isoleucine and valine starvation at such a rapid rate and to a significantly higher degree than \textit{Cr. neoformans} and \textit{S. cerevisiae ilv2}\(\Delta\) mutants. Possible mechanisms may involve differences in the levels of misincorporation of other amino acids into proteins in the absence of valine and/or isoleucine and leucine, or differences in the timing or degree of cellular arrest upon starvation. The effect of rapamycin on the \textit{ilv2}\(\Delta\) starvation phenotype indicates an intimate association of the TOR pathway with this phenotype in both species, and the species-specific differences in the rapamycin response and starvation severity may be consequences of differences in the wiring of the TOR pathway between species. Further research is required to better understand both the species-specific differences in the rapamycin response and starvation severity, as well as why in both species, starvation for one amino acid, such as methionine, is static, while starvation for others, such as isoleucine and valine, is cidal.

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