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Cytocidal amino acid starvation of *Saccharomyces cerevisiae* and *Candida albicans* acetolactate synthase (*ilv2*Δ) mutants is influenced by the carbon source and rapamycin

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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 ilv2*Δ mutants do not survive *in vivo*, *Cryptococcus neoformans ilv2* mutants are avirulent, and both *S. cerevisiae* and *Cr. neoformans ilv2* 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 ilv2*Δ 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 ilv2*Δ mutants. Since the mutation of *ILV1*, required for the first step of isoleucine biosynthesis, did not suppress the *ilv2*Δ 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 ilv2*Δ starvation viability, it increased *Ca. albicans ilv1*Δ and *ilv2*Δ viability. Furthermore, the recovery from starvation was dependent on the carbon source present during recovery for *S. cerevisiae ilv2*Δ mutants, reminiscent of isoleucine and valine starvation inducing a viable but non-culturable-like state in this species, while *Ca. albicans ilv1*Δ and *ilv2*Δ viability was influenced by the carbon source present during starvation, supporting a role for glucose wasting in the *Ca. albicans* cidal phenotype.

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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

Abbreviations: FRT, FLP recombination target; Nat, nourseothricin; TOR pathway, target of rapamycin pathway.

Two supplementary figures, showing the construction of *Candida albicans* deletion mutants, Southern hybridization analysis confirming gene replacements and growth of *S. cerevisiae* and *Ca. albicans* wild-type, *ilv1*Δ and *ilv2*Δ strains in YPD and SD + isoleucine + valine media, and a supplementary table, listing primers used in this study, are available with the online version of this paper.

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 fungistatic 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 cytocidal [50 % reduction after 2 days (Boer *et al.*, 2008)], while inositol starvation is highly cytocidal [$\geqslant$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 G_1/G_0 (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 cytocidal (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 ilv2*Δ 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 *ilv2*Δ mutants, such as the starvation-cidal phenotype.

Since *Cr. neoformans* and *S. cerevisiae ilv2*Δ 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 ilv2*Δ 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.

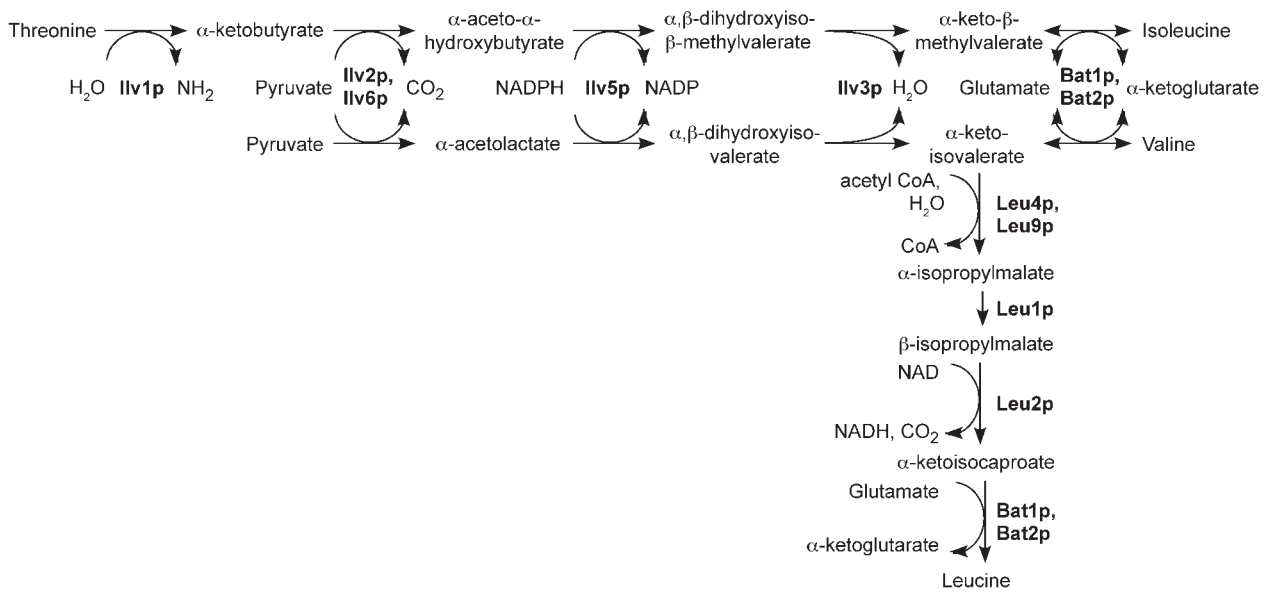

Fig. 1. Isoleucine, valine and leucine biosynthetic pathways.

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 *Ca. 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 *Ca. albicans*.

METHODS

Strains, media and growth conditions. *Ca. 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 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 μg ml^{-1} for *S. cerevisiae* selection, 200 μg ml^{-1} for *Ca. albicans* selection; Hans Knöll Institute für Naturstoff-Forschung), hygromycin B (300 μg ml^{-1}, Calbiochem) or geneticin (200 μg 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.

Ca. albicans genes were replaced with the SAT1 flipper cassette from plasmid pSFS2A (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. Nat-sensitive +/Δ strains then underwent a second round of transformation to disrupt the second allele. To reintroduce the wild-type gene, homozygous disruption strains were transformed with the wild-type gene of interest, and amplified using primers homologous to

Table 1. Strains used in this study

Strain	Genotype*	Reference or source
Ca. albicans		
SC5314	Wild-type	Gillum *et al.* (1984)
CJK14	*ILV2/ilv2*Δ::SAT1	This study
CJK23	*ILV2/ilv2*Δ::FRT	This study
CJK27	*ilv2*Δ::SAT1/*ilv2*Δ::FRT	This study
CJK80	*MET2/met2*Δ::SAT1	This study
CJK98	*MET2/met2*Δ::FRT	This study
CJK103	*met2*Δ::SAT1/*met2*Δ::FRT	This study
CJK131	*ILV1/ilv1*Δ::SAT1	This study
CJK132	*ilv1*Δ::SAT1/*ilv1*Δ::FRT *ilv2*Δ::FRT/*ilv2*Δ::FRT	This study
CJK133	*ILV2/ilv2*Δ::SAT1 (*ILV2*-complemented strain)	This study
CJK138	*ILV1/ilv1*Δ::FRT	This study
CJK140	*ilv1*Δ::SAT1/*ilv1*Δ::FRT	This study
CJK142	*ILV1/ilv1*Δ::SAT1 (*ILV1*-complemented strain)	This study
S. cerevisiae		
S094	*MAT***a**	McCusker laboratory strain collection
S3782	*MATα leu1*Δ::hphMX4	This study
S3783	*MATα leu4*Δ::loxP-kanMX4-loxP *leu9*Δ::natMX4	This study
S3807	*MAT***a** *leu2*Δ::hphMX4	This study
YJK564	*MAT***a**/*MATα met2*Δ::natMX4/*met2*Δ::natMX4	This study
YJK2484	*MAT***a** *ilv1*Δ::kanMX4	This study
YJK2486	*MAT***a** *ilv2*Δ::natMX4	This study
YJK2487	*MAT***a** *ilv1*Δ::kanMX4 *ilv2*Δ::natMX4	This study

*All *Ca. 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 [α-^{32}P]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 1×10^6 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 10^7 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 *ilv2*Δ mutant starvation recovery on different batches of YPD. For approximate numbers, 5 μl 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-Ketobutyrate inhibition assay. To determine the level of α-ketobutyrate inhibition, strains were grown overnight in YPD, washed twice in water, and resuspended to a concentration of approximately $1–2 \times 10^3$ 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-ketobutyrate 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, *ilv1*Δ and *ilv2*Δ strains were grown overnight in YPD, washed twice in water, and resuspended in YPD or SD + isoleucine + valine media to OD_{600} 0.05. One hundred microlitre volumes of strains were incubated in 96-well microtitre plates (Corning) at 30 °C, and OD_{600} readings were taken half-hourly using an automated Tecan Sunrise absorbance reader. Experiments were performed in triplicate.

RESULTS

*Ca. albicans ilv1*Δ and *ilv2*Δ mutants have attenuated virulence

Since *Cr. neoformans ilv2*Δ mutants are avirulent (Kingsbury *et al.*, 2004a) and *S. cerevisiae ilv2*Δ mutants do not survive *in vivo* (Kingsbury *et al.*, 2006), we constructed and tested the virulence in mice of *Ca. albicans ilv1*Δ, *ilv2*Δ and *ilv1*Δ *ilv2*Δ 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 *ilv1*Δ mutant (CJK140) survived longer on average (6.6 ± 5.5 days). Therefore, isoleucine auxotrophy has a modest effect on *Ca. albicans* virulence.

Relative to *ilv1*Δ mutants, the *ilv2*Δ 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 *ilv2*Δ 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 *ilv1*Δ *ilv2*Δ 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 *ilv1*Δ disruption did not suppress the attenuated virulence of the *ilv2*Δ mutant, α-ketobutyrate accumulation plays no role in the greatly attenuated virulence of the *ilv2*Δ mutant.

S. cerevisiae and *Ca. albicans ilv2*Δ mutants die rapidly upon isoleucine and valine starvation

Since *S. cerevisiae* and *Cr. neoformans ilv2* 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 ilv2*Δ mutants to survive isoleucine and valine starvation was compared. Averaged over three separate experiments, the *S. cerevisiae*

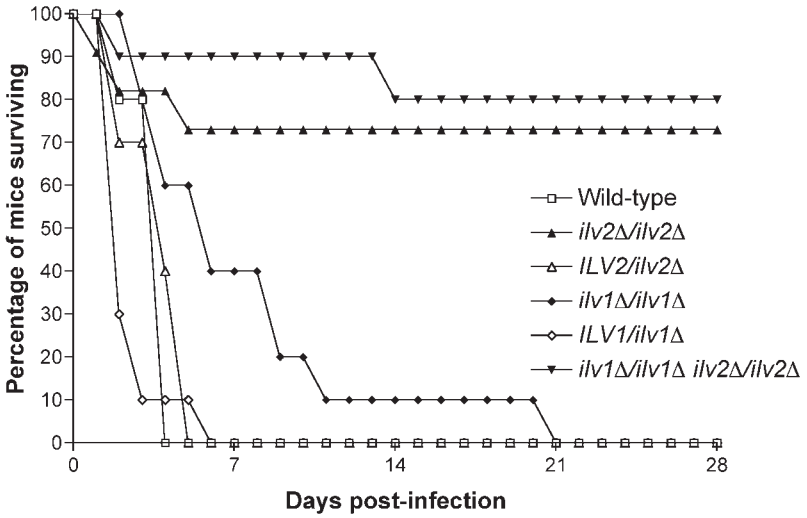

Fig. 2. Virulence phenotypes of *Ca. albicans* isoleucine and valine biosynthetic mutants. Strains included SC5314 (wild-type), CJK27 (*ilv2*Δ/*ilv2*Δ), CJK133 (*ILV2*/*ilv2*Δ, *ILV2*-complemented strain), CJK140 (*ilv1*Δ/*ilv1*Δ), CJK142 (*ILV1*/*ilv1*Δ, *ILV1*-complemented strain) and CJK132 (*ilv1*Δ/*ilv1*Δ *ilv2*Δ/*ilv2*Δ).

*ilv2*Δ mutant (YJK2486) was recovered at approximately 13-fold reduced levels after 24 h of amino acid starvation (Fig. 3a), similar to previous findings (Kingsbury *et al.*, 2004a). The *Ca. albicans ilv2*Δ mutant (CJK27) underwent a substantially more dramatic decline in viability upon incubation in SD medium, with an average 167-fold reduction after only 4 h, and further decreasing to a 658-fold reduction after 8 h, and a 2320-fold reduction after 24 h (Fig. 4a). Microscopic examination revealed that cells were predominantly single or budded; thus, cell clumping was excluded as an explanation for reduced c.f.u. Therefore, *Ca. albicans ilv2*Δ mutants are starvation-cidal like *S. cerevisiae* and *Cr. neoformans ilv2*Δ mutants, but to a significantly greater degree.

The *S. cerevisiae* and *Ca. albicans ilv2*Δ starvation-cidal phenotype is not due to α-ketobutyrate accumulation

The *ilv2*Δ mutant fungicidal phenotype upon isoleucine and valine starvation may be due to toxicity caused by α-ketobutyrate accumulation. If α-ketobutyrate accumulation is toxic, *ilv2*Δ mutants would likely be hypersensitive to exogenously added α-ketobutyrate. To test whether α-ketobutyrate is actually transported by cells, we first assessed the ability of exogenous α-ketobutyrate to satisfy the auxotrophy of *S. cerevisiae* and *Ca. albicans ilv1*Δ mutants (YJK2484 and CJK140), when added to a filter disk (50 µl of 100 mg ml^{-1}) on SD or SD (proline) plates to which the strains were applied. While the α-ketobutyrate only minimally satisfied the *S. cerevisiae ilv1*Δ mutant auxotrophy, α-ketobutyrate supported robust growth of the *Ca. albicans ilv1*Δ mutant on both media types (data not shown). The ability of α-ketobutyrate to inhibit growth was then determined for *S. cerevisiae* and *Ca. albicans* wild-type (S094 and SC5314), *ilv2*Δ (YJK2486 and CJK27), and *ilv1*Δ *ilv2*Δ (YJK2487 and CJK132) strains in YPD. Even at the highest concentration of α-ketobutyrate (10 mg ml^{-1}), no growth inhibition was observed for any strain, which argues against the hypothesis that α-ketobutyrate is toxic in fungi.

To further test the α-ketobutyrate accumulation hypothesis, we compared the amino acid starvation phenotypes of *S. cerevisiae* and *Ca. albicans ilv1*Δ *ilv2*Δ mutants and *ilv2*Δ mutants. Viability following starvation of both the *S. cerevisiae ilv1*Δ *ilv2*Δ mutant (YJK2487) and the *Ca. albicans ilv1*Δ *ilv2*Δ mutant (CJK132) was indistinguishable from that of the *S. cerevisiae* and *Ca. albicans ilv2*Δ mutants, respectively (Figs 3a and 4a). Since disruption of *ILV1* did not suppress the starvation-cidal phenotype of the *ilv2*Δ mutants in either *S. cerevisiae* or *Ca. albicans*, α-ketobutyrate accumulation does not play a role in this phenotype.

The *ilv2*Δ mutant starvation-cidal phenotype is due to valine starvation in *S. cerevisiae*, and multiple amino acid auxotrophies in *Ca. albicans*

The *ilv2*Δ 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 *ilv2*Δ viability. However, neither *S. cerevisiae* nor *Ca. albicans ilv2*Δ mutant viability was improved by isoleucine addition (Figs 3b and 4b). Since we observed considerably reduced growth of both *S. cerevisiae* and *Ca. albicans ilv1*Δ 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 *ilv2*Δ mutants. Alternatively, the

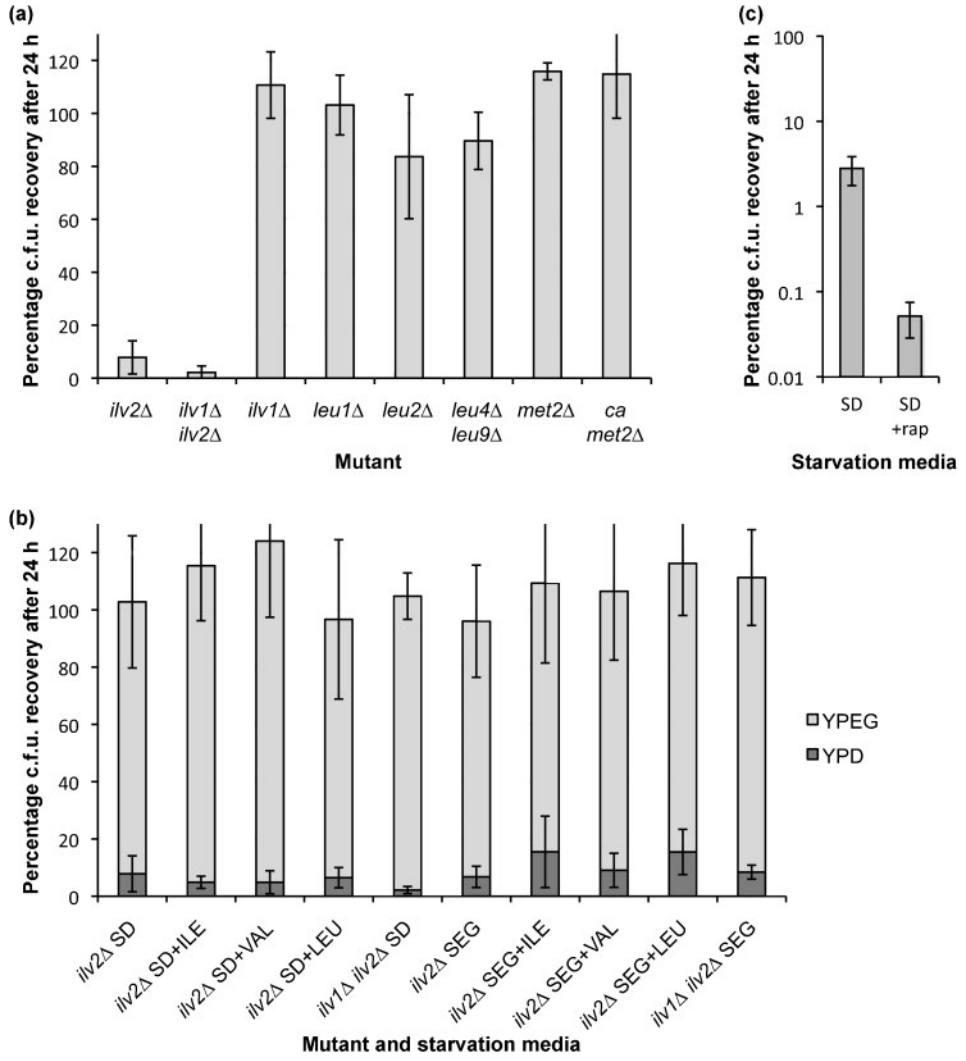

Fig. 3. (a) Recovery on YPD following starvation of *S. cerevisiae* (and *Ca. albicans met2*Δ) auxotrophs in SD. (b) Recovery on YPD and YPEG following starvation in SD or SEG ± amino acid supplementation, as indicated. (c) Recovery of *S. cerevisiae ilv2*Δ mutant on YPD following starvation in SD or SD + rapamycin (SD + rap). Strains included *S. cerevisiae* YJK2486 (*ilv2*Δ), YJK2487 (*ilv1*Δ *ilv2*Δ), YJK2484 (*ilv1*Δ), S3782 (*leu1*Δ), S3807 (*leu2*Δ), S3783 (*leu4*Δ *leu9*Δ), YJK564 (*met2*Δ) and *Ca. albicans* CJK103 (*ca met2*Δ).

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

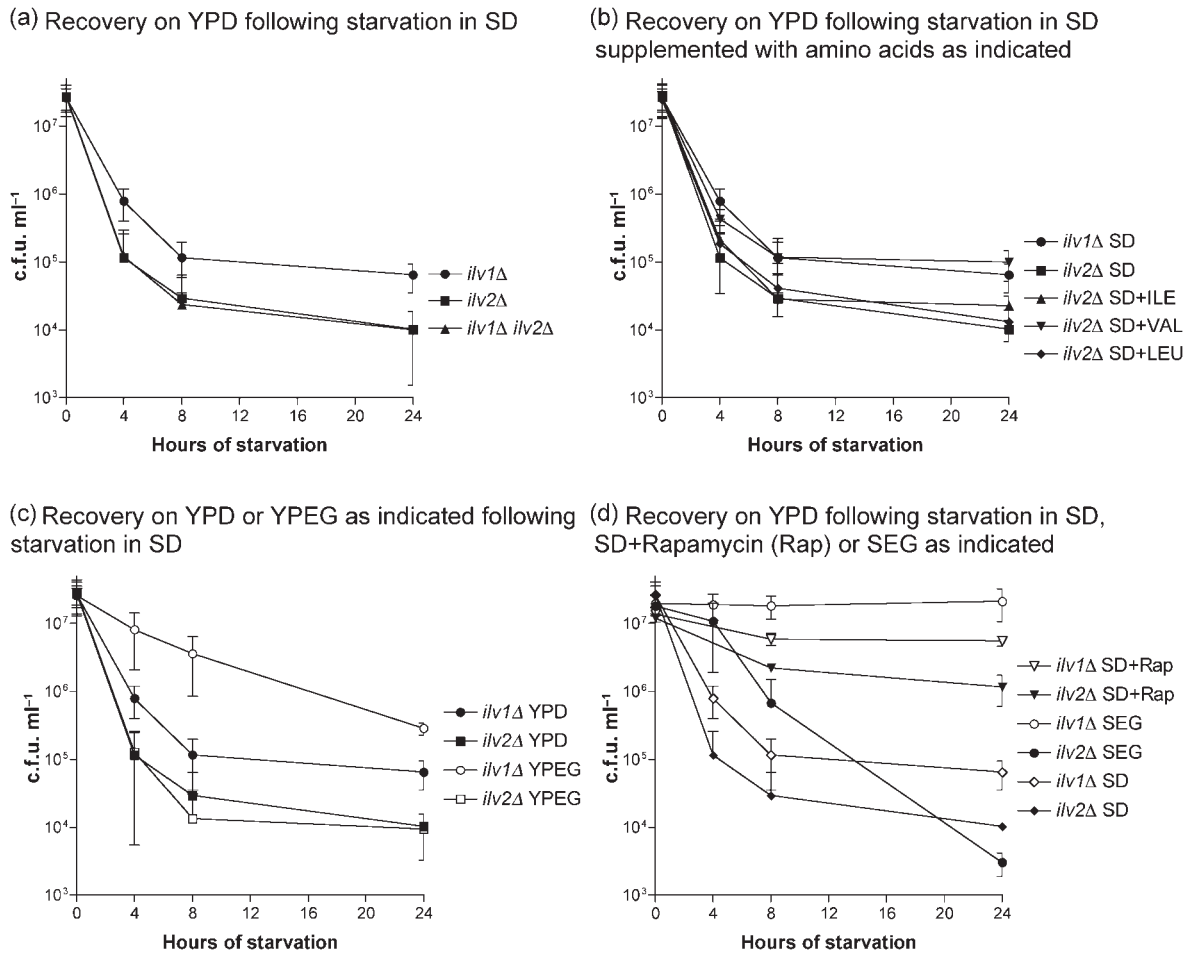

Fig. 4. Influence of genotype, amino acid addition, carbon source present during recovery and starvation, and rapamycin on the viability of *Ca. albicans* mutants following starvation. Strains included CJK27 (*ilv2*Δ), CJK132 (*ilv1*Δ *ilv2*Δ) and CJK140 (*ilv1*Δ).

intermediate accumulation) leucine auxotrophs was determined following leucine starvation. Following incubation in SD for 24 h, the *leu1*Δ, *leu2*Δ and *leu4*Δ *leu9*Δ 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 ilv2*Δ survival, and had little to no effect on *Ca. albicans ilv2*Δ survival (Figs 3b and 4b). Therefore, in both species, leucine starvation alone also does not explain the *ilv2*Δ 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 *ilv2*Δ starvation-cidal phenotype was due to valine starvation, valine supplementation did not alleviate the *S. cerevisiae ilv2*Δ survival defect during starvation (Fig. 3b). The lack of an effect of valine supplementation may be due to poor uptake of valine by the *ilv2*Δ strain in these conditions, such that cells continue to experience a certain state of valine starvation. Consistent with this hypothesis, *S. cerevisiae ilv2*Δ mutants grew more slowly than the wild-type and *ilv1*Δ mutants in both YPD and SD media supplemented with isoleucine and valine (Supplementary Fig. S2). In contrast to *S. cerevisiae ilv2*Δ mutants, valine supplementation significantly increased survival following starvation for the *Ca. albicans ilv2*Δ mutant at each time point, with survival kinetics more closely resembling those of the *Ca. albicans ilv1*Δ mutant (Fig. 4b). Results therefore indicate a significant role for valine starvation in the *S. cerevisiae* and *Ca. albicans ilv2*Δ starvation-cidal phenotype.

Since both isoleucine and valine starvation appeared to be more cytocidal for *Ca. albicans* than *S. cerevisiae*, to determine whether *Ca. albicans* is more sensitive to amino acid starvation in general than *S. cerevisiae*, the viability of *Ca. albicans met2*Δ (CJK103) and *S. cerevisiae met2*Δ (YJK564) methionine auxotrophs was determined following starvation for 24 h in medium lacking methionine (SD). After 24 h starvation, the *S. cerevisiae* and *Ca. albicans met2*Δ strains were at 116 and 115 % of input levels, respectively (Fig. 3a). Therefore, rather than being a general consequence of amino acid starvation in *Ca. albicans*, the significantly reduced viability following starvation of *Ca. albicans ilv1*Δ and *ilv2*Δ 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 *S. cerevisiae ilv2*Δ mutants recovering from isoleucine and valine starvation, which were reminiscent of petite formation (Fig. 5). Since *Ca. albicans* is generally considered petite-negative (Bulder, 1964), the hypothetical induction of petite formation by isoleucine and valine starvation might explain why *S. cerevisiae ilv2*Δ mutants survive this stress at orders of magnitude higher than *Ca. albicans ilv2*Δ mutants. To test whether starvation induced petite formation in *S. cerevisiae ilv2*Δ 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, *S. cerevisiae ilv2*Δ mutants enter into a stage upon isoleucine and valine starvation that is technically viable, but non-recoverable on YPD.

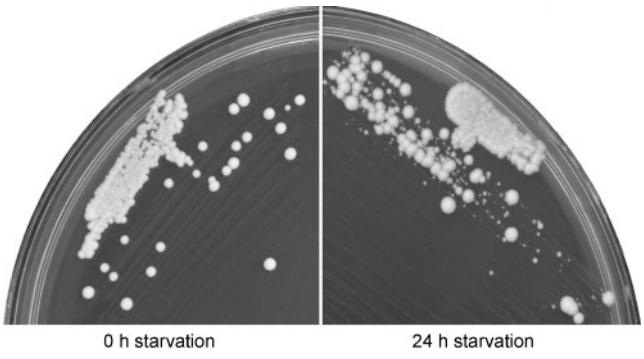

Fig. 5. Effect of starvation on *S. cerevisiae ilv2*Δ colony size. Cells were starved in SD medium and streaked onto YPD plates at the times indicated.

In contrast to *S. cerevisiae ilv2*Δ results, little difference (less than twofold) was observed for recovery of *Ca. albicans ilv2*Δ 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 *S. cerevisiae ilv2*Δ results, we found increased recovery on YPEG compared with YPD following the amino acid starvation of *Ca. albicans ilv1*Δ, 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 *Ca. albicans ilv1*Δ 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 *S. cerevisiae ilv2*Δ and *ilv1*Δ *ilv2*Δ 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 *S. cerevisiae ilv2*Δ mutant survival.

Again contrasting with *S. cerevisiae* results, we found that survival of the *Ca. albicans ilv2*Δ 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 *ilv1*Δ 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 *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 *S. cerevisiae ilv2*Δ and *Ca. albicans ilv1*Δ and *ilv2*Δ mutants. Rapamycin was added to SD at a concentration of 100 nM, since this was the concentration used in starvation assays

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 ilv2*Δ 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 ilv1*Δ 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 ilv2*Δ 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 ilv1*Δ and *ilv2*Δ 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 ilv2*Δ mutants die rapidly and at profoundly high levels upon isoleucine and valine starvation, and are highly attenuated in virulence.

The highly amino acid starvation-cidal phenotype of *Ca. albicans ilv2*Δ mutants indicates that inhibition of Ilv2p in amino acid-limited environments such as *in vivo* should be fungicidal, rather than fungistatic. Numerous herbicides belonging to three drug classes have already been identified that inhibit acetolactate synthase (Whitcomb, 1999). While inhibitors of the *Ca. albicans* leucine biosynthetic pathway alone would have little utility *in vivo*, as leucine auxotrophs are virulent (Kirsch & Whitney, 1991; Noble & Johnson, 2005), since the toxic effects in *ilv2*Δ mutants are likely to be due to isoleucine and valine starvation, inhibitors of other isoleucine and valine biosynthetic enzymes would likely also be effective. Furthermore, given that isoleucine starvation is also fungicidal and that the *ilv1*Δ mutant is somewhat attenuated in virulence in *Ca. albicans*, any inhibitor targeting an isoleucine biosynthetic enzyme should have a clinically beneficial interaction with any of the various isoleucyl tRNA synthetase inhibitors currently available with antifungal activity, such as the antibacterial agent mupirocin (Nicholas *et al.*, 1999) or the anticandidal drug BAY 10-8888 (PLD-118) (Ziegelbauer, 1998; Ziegelbauer *et al.*, 1998). Finally, since the growth rate of *ilv2*Δ mutants was reduced compared with the wild-type even in the presence of abundant levels of isoleucine and valine, Ilv2p inhibition may not require complete absence of isoleucine and valine from the environment to have a therapeutic benefit.

We considered the hypothesis that the starvation-cidal phenotypes of fungal *ilv2*Δ mutants were due to accumulation of the biosynthetic intermediate α-ketobutyrate, since α-ketobutyrate or its transamination product, α-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 α-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 α-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 ilv2*Δ mutants were sensitive to high exogenous levels of α-ketobutyrate, and *ilv1*Δ *ilv2*Δ mutants that cannot accumulate α-ketobutyrate were as starvation-cidal as *ilv2*Δ mutants, we ruled out the α-ketobutyrate accumulation hypothesis (Epelbaum *et al.*, 1996) as the explanation for the *S. cerevisiae* and *Ca. albicans ilv2*Δ starvation-cidal phenotypes.

Interestingly, isoleucine-auxotrophic *Ca. albicans ilv1*Δ mutants were also starvation-cidal, while *S. cerevisiae ilv1*Δ 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 *ilv2*Δ mutants compared with *ilv1*Δ mutants in both species and leucine auxotrophs in *S. cerevisiae*, together with the 10-fold increased survival upon supplementation of *Ca. albicans ilv2*Δ 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 ilv2*Δ and *Ca. albicans ilv1*Δ 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 ilv2*Δ mutants, viability following starvation of *Ca. albicans ilv1*Δ and *ilv2*Δ 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 *S. cerevisiae ilv2*Δ mutants, in which rapamycin reduced viability upon starvation, we observed a suppression of cell death upon starvation of both *Ca. albicans ilv1*Δ and *ilv2*Δ 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 *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; Schneper *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 *S. cerevisiae met2*Δ and *Ca. albicans met2*Δ mutants were equally starvation-static upon methionine starvation, amino acid starvation is not generally more starvation-cidal in *Ca. albicans* than *S. cerevisiae*. Therefore, two questions remain: first, precisely why *ilv2*Δ mutants are starvation-cidal; and second, why *Ca. albicans ilv2*Δ mutants die, or fail to recover, from isoleucine and valine starvation at such a rapid rate and to a significantly higher degree than *Cr. neoformans* and *S. cerevisiae ilv2*Δ 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 *ilv2*Δ 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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