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Cryptococcus neoformans Ilv2p confers resistance to sulfometuron methyl and is required for survival at 37 °C and *in vivo*

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Acetolactate synthase catalyses the first common step in isoleucine and valine biosynthesis and is the target of several classes of inhibitors. The *Cryptococcus neoformans ILV2* gene, encoding acetolactate synthase, was identified by complementation of a *Saccharomyces cerevisiae ilv2* mutant. *C. neoformans* is highly resistant to the commercially available acetolactate synthase inhibitor, sulfometuron methyl (SM). Expression of *C. neoformans ILV2* in *S. cerevisiae* conferred SM resistance, indicating that the SM resistance of *C. neoformans* is due, at least in part, to *C. neoformans* Ilv2p. The *C. neoformans ILV2* gene was disrupted. The *ilv2* mutants were auxotrophic for isoleucine and valine and the auxotrophy was satisfied by these amino acids only when proline, and not ammonium, was the nitrogen source, indicating nitrogen regulation of amino acid transport. *ilv2* mutants rapidly lost viability at 37 °C and when starved for isoleucine and valine. Consistent with these phenotypes, an *ilv2* mutant was avirulent and unable to survive in mice. Because *C. neoformans* Ilv2p is required for virulence and survival *in vivo*, inhibitors of branched-chain amino acid biosynthesis may make valuable antifungal agents.

INTRODUCTION

Opportunistic fungal infections, such as fungal meningitis caused by the human pathogen *Cryptococcus neoformans* (Casadevall & Perfect, 1998; Mitchell & Perfect, 1995), are an increasing problem because of the expanding immunodeficient population of AIDS, organ transplant and cancer chemotherapy patients. This problem is compounded by the limited availability of effective antifungal drug treatments, to which fungi often become resistant, and which can cause severe side effects.

Amino acid biosynthetic pathways are interesting from the perspective of potential antifungal drug targets as (1) various fungal auxotrophs are reduced in virulence (see, for example, Goldstein & McCusker, 2001; Manning *et al.*, 1984; Namiki *et al.*, 2001; Yang *et al.*, 2002); (2) these pathways are conserved throughout fungi; (3) many of these pathways do not exist in humans; and (4) various amino acid biosynthetic inhibitors have already been identified for use as herbicides. The branched-chain amino acids (isoleucine, valine and leucine) are examples of amino acids that can be synthesized by fungi, but which are essential in mammals. The first common step in the biosynthesis of these amino acids is catalysed in plants, bacteria and fungi by acetolactate (also acetohydroxyacid) synthase (EC 2.2.1.6). The reaction involves the condensation either of two pyruvate molecules to form 2-acetolactate (a precursor for valine, leucine and pantothenate biosynthesis), or of pyruvate with 2-ketobutyrate to yield 2-acetohydroxybutyrate (a precursor for isoleucine biosynthesis) (reviewed by Chipman *et al.*, 1998). Acetolactate synthase has been studied extensively as it has been shown to be the target of several structurally different classes of inhibitors widely used as herbicides, particularly the sulfonylureas, imidazolinones and sulfonanilides (reviewed by Stetter, 1994). Moreover, as acetolactate synthases are highly conserved, many of these herbicides also inhibit this enzyme in bacteria and fungi (see, for example, Falco & Dumas, 1985; Grandoni *et al.*, 1998; LaRossa & Schloss, 1984).

Because many well-studied inhibitors of acetolactate synthase exist, we reasoned that this enzyme would be an ideal antifungal drug target if mutation of the gene that encodes it (designated *ILV2* in *Saccharomyces cerevisiae*; Falco *et al.*, 1985) abolishes virulence in *C. neoformans*. We found that a *C. neoformans ilv2* mutant was indeed avirulent and unable to survive in a murine inhalation model of infection. The avirulence of the *C. neoformans ilv2* mutant may be a consequence of an inability to survive at 37 °C and in isoleucine and valine starvation conditions, and/or

Abbreviations: Nat, nourseothricin; SM, sulfometuron methyl.

GenBank accession numbers: AF394891(*ILV2* cDNA); AY450850 (*ILV2* genomic sequence).

an inability to transport sufficient amino acids and/or pantothenate to suppress the isoleucine, valine, leucine and pantothenate auxotrophy of this strain. As *C. neoformans ilv2* mutants were avirulent, we examined the efficacy of sulfometuron methyl (SM) as an anti-cryptococcal agent. *C. neoformans* was highly resistant to SM, and we demonstrated that the resistance was due, at least in part, to *C. neoformans* Ilv2p. Although the intrinsic resistance of *C. neoformans* Ilv2p rules out the use of SM as an anti-cryptococcal agent, acetolactate synthase is an excellent potential target for antifungal agents.

METHODS

Strains, media and growth conditions. All *S. cerevisiae* and *C. neoformans* strains used in this study are listed in Table 1. *S. cerevisiae* strains were isogenic with S288c and *C. neoformans* strains were isogenic with serotype A strain H99 (Perfect *et al.*, 1993), unless specified otherwise. One Shot TOP10 Chemically Competent *Escherichia coli* (Invitrogen) was used for cloning purposes, and *E. coli* DH10β (Gibco-BRL) was used for other plasmid propagation. Standard yeast and bacterial culture media were prepared as described by Sambrook *et al.* (1989) and Sherman *et al.* (1974). Where specified, media were supplemented with isoleucine (0·23 mM), valine (1·28 mM), H-valine-isoleucine-OH (Val-Ile; 0·22 mM), H-isoleucine-valine-OH (Ile-Val; 0·22 mM), nourseothricin (Nat; 100 μg ml^{-1}), proline (5 g l^{-1}) or galactose (2 %, w/v). Unless specified otherwise, *S. cerevisiae* and *C. neoformans* cultures were incubated at 30 °C, and *E. coli* cultures at 37 °C.

Manipulation of nucleic acids. Plasmid DNA was isolated from *E. coli* using the QIAprep Spin Miniprep kit (Qiagen). Isolation of plasmid DNA from *S. cerevisiae* and genomic DNA from *C. neoformans* and *S. cerevisiae* for PCR analysis was performed as described by Hoffman & Winston (1987). *C. neoformans* genomic DNA for Southern analysis was isolated from *C. neoformans* strains with the Wizard Genomic DNA Purification Kit (Promega) using a modification of the 'Isolation of Genomic DNA from Yeast' protocol supplied by the manufacturers. Five-millilitre cultures of cells, grown for approximately 16 h in YPD, were pelleted by centrifugation. Cell pellets were resuspended in 600 μl 10 mM EDTA. Following the addition of 0·1 ml glass beads (425–600 μm), tubes were vortexed for 1 min. Tubes were allowed to sit long enough for the glass beads to settle, the cell supernatants were transferred to clean tubes, and cells were centrifuged briefly. Cells were resuspended in 290 μl 10 mM EDTA, and 10 μl 'Gluconex' Lysing Enzymes (Sigma; 0·1 mg ml^{-1}) was added. Tubes were incubated at 37 °C for 2 h, and allowed to cool to room temperature before a brief centrifugation. Pellets were resuspended in 300 μl Nuclei Lysis Solution, and subsequent protein precipitation and DNA purification steps were performed as described by the manufacturers.

The DNA (2 μg) for Southern analysis was digested with various restriction enzymes, separated by electrophoresis using a 0·7 % (w/v) agarose gel, denatured and transferred to nylon membranes (Roche) as described by Sambrook *et al.* (1989). Probes for Southern analysis were prepared from PCR products and labelled with the DIG non-radioactive labelling kit (Roche) according to the manufacturer's instructions. Blots were prehybridized in ULTRAhyb hybridization buffer (Ambion) for 1 h. Blots were hybridized overnight in the same buffer, following addition of the denatured probe. Washing and detection of hybridized DNA bands were performed according to the manufacturer's instructions (Roche).

Table 1. Strain list

Strain	Genotype	Source
S. cerevisiae		
S157	*MAT*α *ura3*Δ	Yang *et al.* (2002)
S311	*MAT*α *ura3*Δ *ilv2*Δ::natMX4	This study
S1415	*MAT***a** *lys5*Δ	McCusker laboratory collection
S3501	*MAT*α *ura3*Δ *ilv2*Δ::cn*ILV2* cDNA	This study
S3502	*MAT*α *ura3*Δ *ilv2*Δ::cn*ILV2* cDNA	This study
S3553	*MAT*α/*MAT***a** *lys5*Δ/*LYS5 ura3*Δ/*URA3 ILV2*/*ILV2*	This study
S3554	*MAT*α/*MAT***a** *lys5*Δ/*LYS5 ura3*Δ/*URA3 ILV2*/*ilv2*Δ::cn*ILV2* cDNA	This study
S3555	*MAT*α/*MAT***a** *lys5*Δ/*LYS5 ura3*Δ/*URA3 ILV2*/*ilv2*Δ::cn*ILV2* cDNA	This study
C. neoformans		
H99	*MAT*α serotype A	Perfect *et al.* (1993)
H99-4	*MAT*α serotype A *met3*::*URA5 ura5*	Yang *et al.* (2002)
H99-20	*MAT*α serotype A *ilv2*::*NAT1*	This study
H99-21	*MAT*α serotype A *ilv2*::*NAT1*	This study
H99-22	*MAT*α serotype A *ILV2* (*ILV2*-reconstituted strain)	This study
H99-23	*MAT*α serotype A *ILV2* (*ILV2*-reconstituted strain)	This study
CDC1	Serotype A	J. Xu, McMaster University, personal communication
CDC2	Serotype A	J. Xu, personal communication
B4495	Serotype B	J. Xu, personal communication
B4496	Serotype B	J. Xu, personal communication
ATCC34883	*MAT*α serotype C	Brandt *et al.* (1993); Schmeding *et al.* (1981)
ATCC34880	*MAT*α serotype C	Brandt *et al.* (1993); Schmeding *et al.* (1981)
JEC20	*MAT*α serotype D	Heitman *et al.* (1999); Kwon-Chung *et al.* (1992)

PCR amplification reactions were performed in a PTC-200 Peltier Thermal Cycler (MJ Research). *Taq* polymerase (Invitrogen BRL) was used in most PCR reactions except those in which DNA was amplified for sequencing, construction of the *ilv2*::*NAT1* targeting allele and reconstitution of the *C. neoformans ilv2*::*NAT1* allele, and for replacing the *S. cerevisiae ilv2*Δ::natMX4 allele with the *C. neoformans ILV2* cDNA. For these reactions, MEGA-Frag DNA polymerase (Denville Scientific) was used for its 3′–5′ proofreading activity. When *Taq* polymerase was used, amplification conditions typically consisted of 94 °C for 3 min, an annealing temperature of 55 °C for 30 s and an extension step of 72 °C for 1 min per 1 kb of DNA to be amplified. These steps were then repeated 29 times except that the initial 94 °C denaturation incubation was reduced to 30 s in subsequent cycles. The final cycle included a 10 min extension step at 72 °C. When MEGA-Frag DNA polymerase was used, cycle conditions were identical except that extension steps were carried out at 68 °C. All primers used are listed in Table 2.

Isolation of *C. neoformans ILV2*. The *C. neoformans ILV2* cDNA was isolated by complementation of a *S. cerevisiae ilv2*Δ mutant with a *C. neoformans* cDNA library. To construct a *S. cerevisiae ilv2*Δ strain, a PCR product, containing the natMX4 cassette flanked by 40 bp sequences upstream and downstream of the *S. cerevisiae ILV2* ORF, was amplified using the primer pair ZY019 and ZY034, and using the conditions described previously (Goldstein & McCusker, 1999). This product was used to transform the *S. cerevisiae ura3* strain S157 to nourseothricin (Nat) resistance, as described previously (Goldstein & McCusker, 1999). Nat-resistant transformants were screened for isoleucine and valine auxotrophy and by PCR using the primer pair JM37 and ZY054.

Table 2. Primer list

Primer	Sequence (5′ to 3′)	Comments
JM37	CCTCGACATCATCTGCCC	Confirmation of *S. cerevisiae ILV2* replacement by natMX4
ZY019	CTAAACCCTTTGAGCTAAGAGGAGATAAATACAACAGAATCAGCTGAAGCTTCGTACGC	Disruption primer for *S. cerevisiae ILV2*
ZY034	CAGTGCTTACCGCCTGTACGCTTATGACGTAATTCAGGCATAGGCCACTAGTGGATCTG	Disruption primer for *S. cerevisiae ILV2*
ZY054	CATGACTACCGATTTGG	Confirmation of *S. cerevisiae ILV2* replacement by natMX4; confirmation of natMX4 replacement by *C. neoformans ILV2* cDNA in *S. cerevisiae*
ZY079	AGCTCGTCTTCTCAGACG	Creation of *in vitro ilv2*::*NAT1* allele for *C. neoformans ILV2* disruption; amplification of *C. neoformans ILV2* for use as probe in Southern analysis
ZY080	AGACAGAAATGGCAGACC	Creation of *in vitro ilv2*::*NAT1* allele for *C. neoformans ILV2* disruption; amplification of *C. neoformans ILV2* for use as probe in Southern analysis
ZY089	TCATTTATCGGTCTCTCC	Sequencing of *C. neoformans ILV2*
ZY091	TGCACCTGAGTCCTTTGG	Sequencing of *C. neoformans ILV2*; confirmation of *C. neoformans ILV2* disruption
ZY097	AAGGGATGGTCGAGCAATG	Sequencing of *C. neoformans ILV2*
ZY109	TCACTTCGAAATCCAACCC	Sequencing of *C. neoformans ILV2*
ZY110	AACGAAGCTTTCAAGATTGC	Sequencing of *C. neoformans ILV2*; confirmation of *C. neoformans ILV2* disruption; confirmation of natMX4 replacement by *C. neoformans ILV2* cDNA in *S. cerevisiae*
JO89	GGACTCACATAAGCATGCAG	Confirmation of *C. neoformans ILV2* disruption
JO90	ACTAGCTTCCTGGTTTCAGA	Confirmation of *C. neoformans ILV2* disruption
JO262	TTAACTCTCATCGACAGTCA	Sequencing of *C. neoformans ILV2*; amplification of genomic *C. neoformans ILV2* for reconstitution of *ilv2*::*NAT1* mutant
JO265	AGGCATATTTGTTGGTCCAG	Amplification of genomic *C. neoformans ILV2* for reconstitution of *ilv2*::*NAT1* mutant
JO284	CTGCTTTGGATTTTCCTCAC	Sequencing of *C. neoformans ILV2*
JO334	GAGCTAAGAGGAGATAAATACAACAGAATCAATTTTCAAATGCTTACACGCCAAGCTCG	Amplification of *C. neoformans ILV2* cDNA ORF for replacement of *S. cerevisiae ILV2*
JO335	TTTTTACTGAAAATGCTTTTGAAATAAATGTTTTTGAAATTTACTCAGAGCCGTTCCGAA	Amplification of *C. neoformans ILV2* cDNA ORF for replacement of *S. cerevisiae ILV2*
TMG001	ATTCCTGTGACCACCACTGCTGCGAGGATGTGAGCTGGA	Creation of *in vitro ilv2*::*NAT1* allele for *C. neoformans ILV2* disruption
TMG002	TGCCGATCATGTGTAAACAGAAGAGATGTAGAAACTAGC	Creation of *in vitro ilv2*::*NAT1* allele for *C. neoformans ILV2* disruption
TMG003	TCCAGCTCACATCCTCGCAGCAGTGGTGGTCACAGGAAT	Creation of *in vitro ilv2*::*NAT1* allele for *C. neoformans ILV2* disruption
TMG004	AGAAGAGATGTAGAAACTAGCGTTTACACATGATCGGCA	Creation of *in vitro ilv2*::*NAT1* allele for *C. neoformans ILV2* disruption

A *C. neoformans* cDNA library, containing inserts from the H99 strain placed under control of the *S. cerevisiae GAL* promoter in the *URA3*-containing vector pYES2.0 (InVitrogen) (Suvarna *et al.*, 2000), was a generous gift from Brian Wong (Yale University School of Medicine, New Haven, CT, USA) to the Duke University Mycology Research Unit (Brian Wong & John Perfect, personal communication). Library plasmid DNA was used to transform *S. cerevisiae* S311 (*ura3 ilv2*Δ::natMX4) to uracil (Ura) prototrophy using the lithium-acetate-mediated transformation protocol (Gietz *et al.*, 1995). Ura^+ transformants were screened for isoleucine and valine prototrophy on isoleucine- and valine-deficient medium containing galactose as the sole carbon source. Plasmid DNA was recovered from transformants and introduced into *E. coli* DH10*β* by electroporation (Smith *et al.*, 1990). Plasmid DNA, isolated from *E. coli* DH10*β*, was sequenced by the Duke University Cancer Center Sequencing Facility.

The chromosomal *C. neoformans ILV2* gene sequence was isolated by PCR amplification using primers designed from *ILV2* cDNA sequence (JO262) and sequence obtained from the *C. neoformans* serotype D BLAST database that showed a high degree of similarity to *ILV2* cDNA sequence (JO265). The PCR product was run on a 1 % (w/v) agarose gel, extracted using the QIAquik Gel Extraction Kit (Qiagen) and cloned into pCR2.1-TOPO according to the manufacturer's instructions (Invitrogen). Inserts from two plasmids, arising from independent PCR amplification reactions (pJO219 and pJO220), were sequenced to ensure sequence integrity. The *ILV2* ORF was predicted by comparing the chromosomal and cDNA sequences, and by comparing the predicted amino acid sequence with that of other fungal acetolactate synthases.

Disruption of the *C. neoformans ILV2* gene. *C. neoformans ILV2* was disrupted by insertion of the *NAT1* gene (McDade & Cox, 2001), resulting in the replacement of bases 1217 to 1234 of the *ILV2* coding region. First, an *ilv2*::*NAT1* targeting allele was constructed using a modified PCR overlap technique (Davidson *et al.*, 2002). In the first round of amplification, a 1·18 kb fragment containing the 5′ end of *ILV2* and a 1·36 kb fragment consisting of the 3′ end of *ILV2* were amplified from *C. neoformans* H99 chromosomal DNA using primer pairs ZY079 and TMG003, and ZY080 and TMG004, respectively. In addition, a 1·70 kb product containing *NAT1*, under the control of the *C. neoformans* actin gene promoter and *TRP1* terminator, was amplified from template pGMC200 (McDade & Cox, 2001) using primers TMG001 and TMG002. The amplified products were run on an agarose gel and extracted. Based on the complementarity of primers TMG001 and TMG003, and TMG002 and TMG004, the three amplified products were then used as templates for an overlap reaction with primers ZY079 and ZY080, to yield the 4·17 kb *ilv2*::*NAT1* disruption allele. The PCR product was gel-extracted and cloned into pCR2.1-TOPO (pTG25).

The *ilv2*::*NAT1* allele was amplified from pTG25 using primers ZY079 and ZY080 and inserted into *C. neoformans* H99 by biolistic transformation using a Bio-Rad model PDS-1000/He biolistic particle delivery system (Toffaletti *et al.*, 1993). Transformation was carried out on YPD plates supplemented with 1 M sorbitol as an osmotic stabilizer. Following incubation at 30 °C for 4 h, cells were washed off plates and plated on YPD + Nat medium. Purified Nat-resistant transformants were streaked onto synthetic medium lacking amino acids (SD medium) to screen for acquisition of isoleucine and valine auxotrophy. Disruption of *ILV2* was confirmed by PCR analysis (using primer pairs JO89 and ZY091, JO90 and ZY110, and ZY110 and ZY091), and by Southern analysis (Fig. 1).

Replacement of the *C. neoformans ilv2*::*NAT1* allele with *ILV2*. A 2·68 kb genomic fragment of *ILV2* that extended beyond the *ILV2* sequence present in the *ilv2*::*NAT1* targeting allele was PCR-amplified from *C. neoformans* H99 genomic DNA using primers JO262 and JO265. The *ILV2*-containing PCR product was used to transform the *ilv2*::*NAT1* strains H99-20 and H99-21 to isoleucine (Ile) and valine (Val) prototrophy by biolistic transformation. Ile^+ Val^+ transformants were selected on SD medium containing 1 M sorbitol. Purified transformants were screened for the loss of the *NAT1* marker by testing for growth on YPD + Nat medium. Replacement of the *ilv2*::*NAT1* allele by *ILV2* was confirmed by PCR and Southern hybridization analyses using the same conditions as described for confirmation of the *C. neoformans ilv2*::*NAT1* disruption.

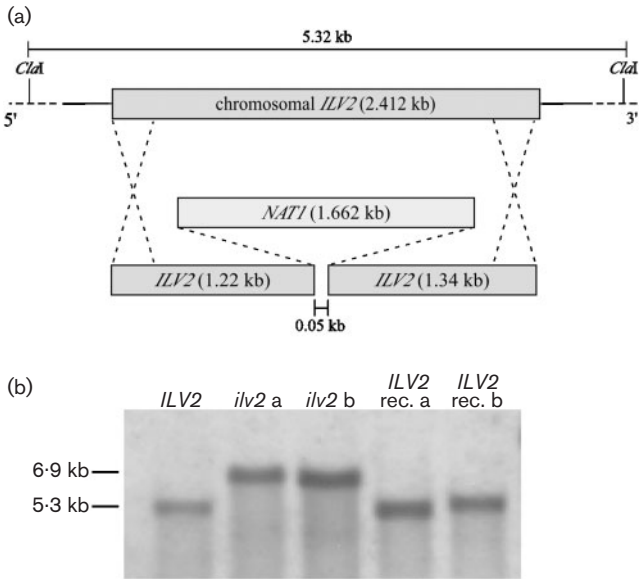

Fig. 1. (a) *ILV2* gene disruption construction. An *ilv2*::*NAT1* targeting allele was constructed by PCR-overlap technology, resulting in the replacement of bases at positions 1·153 to 1·194 kb of *ILV2* sequence by the 1·66 kb *NAT1* gene, flanked by a 1·16 kb fragment containing the 5′ end of *ILV2* and a 1·34 kb fragment consisting of the 3′ end of *ILV2*. The targeting allele was introduced into *C. neoformans* H99 to disrupt *ILV2* by homologous recombination. (b) Southern blot analysis of H99 (*ILV2*), *ilv2*::*NAT1* strains H99-20 (*ilv2* a) and H99-21 (*ilv2* b), and *ILV2*-reconsitituted strains H99-22 (*ILV2* rec. a) and H99-23 (*ILV2* rec. b). Genomic DNA was digested with *Cla*I, which does not cut within *ILV2* or *NAT1*. The blot was hybridized under high-stringency conditions with the genomic *ILV2* PCR product, amplified using primers ZY079 and ZY080.

Growth rate comparisons. Growth rates of the *ilv2* mutants (H99-20 and H99-21), the wild-type (H99) and the *ILV2*-reconstituted strain (H99-22) were compared in liquid YPD medium, incubated at 30 and 37 °C. Cell concentrations were determined from exponential-phase cultures using a haemocytometer, and strains were inoculated into 50 ml YPD medium in 125 ml flasks to a final concentration of 1×10^4 cells ml^{-1}. Cultures were shaken at 250 r.p.m. at 30 °C and 37 °C, and aliquots were removed after 0, 3, 6, 9, 12 and 24 h, serially diluted and plated for enumeration. Each experiment was performed in duplicate.

Melanin production assay. Melanin production was assayed on Niger seed agar (Kwon-Chung & Bennett, 1992) for the wild-type (H99), *ilv2* mutants (H99-20 and H99-21) and an *ILV2*-reconstituted strain (H99-22). Strains were grown on YPD plates for 2 days at

30 °C. Approximately equivalent-sized colonies of each strain were scraped off plates and resuspended in 100 µl sterile water. Cell numbers were counted using a haemocytometer, and cell suspensions were adjusted to contain 2×10^7 cells ml^{-1}. Volumes of 5 µl were spotted onto Niger seed agar supplemented with Ile-Val and Val-Ile dipeptides, as well as isoleucine and valine. Melanin development was observed following incubation of plates at 30 °C.

Capsule production assay. Capsule production by the wild-type (H99), *ilv2* mutants (H99-20 and H99-21) and *ILV2*-reconstituted strain (H99-22) was assayed in Dulbecco's Modified Eagle's Medium (DMEM) + 22 mM $NaHCO_3$ + 25 mM NaMOPS, supplemented with Ile-Val and Val-Ile dipeptides. The medium was inoculated to a concentration of approximately 1×10^6 cells ml^{-1}, with colonies from YPD plates that had been grown for 2 days at 30 °C. Cultures were incubated for 2 days at 30 °C with reduced aeration. To visualize capsule, suspensions of cells in India ink were observed and photographed under differential interference microscopy with a 100× oil-immersion objective, using a Zeiss Axioskop 2 Plus microscope, equipped with an AxioCam MRM digital camera. The mean capsule thickness was determined for each strain by measuring the distance from the cell wall to the outside edge of the capsule for 29 cells from each strain using AxioVision software.

Test of virulence using the murine inhalation model. The murine cryptococcal nasal inhalation model (Cox *et al.*, 2000) was used to compare the virulence of a *C. neoformans ilv2* mutant (H99-20) with the *ILV2* wild-type strain (H99) and the *ILV2* reconstituted strain (H99-22). Groups of ten 4- to 6-week-old female A/Jcr mice (NCI/Charles River Labs) for each *C. neoformans* strain tested were anaesthetized with pentobarbital via intraperitoneal injection and suspended by the incisors on a silk thread. Inocula for infections were prepared from YPD-grown cells that had been washed three times in sterile phosphate-buffered saline (PBS) and resuspended in PBS at a concentration of 2×10^6 cells ml^{-1}, using a haemocytometer to determine cell numbers. The mice were then infected by slowly pipetting 50 µl inoculum into a single nare, and then were left suspended for 10 min to ensure inhalation of the inocula into the lungs. The mice were fed *ad libitum* and were observed twice-daily. Mice that appeared moribund or in pain were euthanized by CO_2 inhalation. Survival data were analysed using the *t*-test. The mice infected with H99-20 (*ilv2*::*NAT1*) were killed at 70 days post-infection and their brains and lungs were removed and homogenized in 1 ml PBS. The entire volume of each homogenate was plated (in aliquots) onto YPD medium containing chloramphenicol, and cultured.

The design of all murine experiments met with institutional guidelines and was approved by the institutional animal care and use committee.

Replacement of *S. cerevisiae ILV2* by *C. neoformans ILV2* cDNA. A *S. cerevisiae* strain was constructed in which the *ilv2*Δ::natMX4 allele had been replaced by the *C. neoformans ILV2* cDNA ORF. First, the *C. neoformans ILV2* cDNA ORF was amplified using primers JO334 and JO335 and plasmid pZY27 as the template. The 20 bases at the 3′ end of primers JO334 and JO335 were homologous to *C. neoformans ILV2*; the 5′ ends were homologous to the 39 bases immediately upstream of the *S. cerevisiae ILV2* start codon and the 40 bases immediately downstream of the *ILV2* termination codon, respectively. Based on the homology of the 5′ and 3′ ends of the PCR product to *S. cerevisiae* sequence, the purified product was introduced into *S. cerevisiae* S311 (*ilv2*Δ::natMX4) to replace the *ilv2*Δ::natMX4 allele. Transformants were selected by acquisition of isoleucine and valine prototrophy. Replacement of the *ilv2*Δ::natMX4 allele by the *C. neoformans ILV2* cDNA ORF was confirmed by reversion to Nat sensitivity and by PCR analysis. The *ilv2*Δ::natMX4 allele was no longer detected using PCR primers JM37 and ZY054, and the presence of the *C. neoformans ILV2* allele at the *S. cerevisiae ILV2* site was confirmed using PCR primers ZY110 and ZY054. Two positive transformants (*S. cerevisiae* S3501 and S3502) were selected for further experiments. The *C. neoformans ILV2* cDNA-derived ORF from these *S. cerevisiae* transformants was PCR-amplified, cloned into pCR2.1-TOPO and sequenced to confirm the integrity of the *C. neoformans ILV2* cDNA sequence.

MIC assays comparing SM sensitivity. For MIC assays, 80 µl aliquots of a series of twofold dilutions of SM, dissolved in 2·5 % (v/v) DMSO, were dispensed into flat-bottomed microdilution plate wells (Corning). The inocula were prepared from strains that had been grown overnight in SD + Ura medium. A 20 µl volume of cells, at a concentration of approximately 5×10^{-3} to 1×10^{-4} c.f.u. ml^{-1} in 5×-concentrated SD + Ura, with or without isoleucine and valine amino acids and dipeptides, was added to the SM dilutions. The final concentrations of SM ranged from 0·2 to 200 µg ml^{-1}, and a control included no SM. The plates were incubated at 30 °C for 48 h. After incubation, the turbidity in each well was measured at 620 nm with a microdilution plate reader (Sunrise, Tecan). Readings were corrected for the background (wells with 100 µl of 2 %, v/v, DMSO and no cells added), and expressed as percentages of the turbidity recorded for the no-drug controls. MICs were defined as the SM concentration in which growth was at least 80 % less than the no-drug control (MIC_{80}). Experiments were performed in duplicate.

RESULTS

Isolation of the *C. neoformans ILV2* gene

Using a *URA3*-marked *C. neoformans* cDNA library, containing cDNAs under the expression of a *S. cerevisiae GAL* promoter (Suvarna *et al.*, 2000), the *C. neoformans ILV2* cDNA was isolated by complementation of the isoleucine and valine auxotrophy of *S. cerevisiae* strain S311 (*ura3 ilv2*Δ::natMX4). From approximately 30 000 Ura^+ transformants screened, a single isolate was identified that had acquired isoleucine and valine prototrophy on galactose-containing, but not glucose-containing, medium. Consistent with plasmid dependence of the phenotype, the transformant was unable to grow when plated on minimal galactose medium that lacked isoleucine and valine but contained uracil and 5-fluoroorotic acid to counterselect the plasmid-borne *URA3* marker. Finally, isolation of the plasmid (designated pZY27) and its subsequent reintroduction into a naïve *ilv2* strain conferred galactose-dependent prototrophy.

The plasmid DNA insert that conferred complementation of the auxotrophic phenotype was sequenced and determined to be 2377 bp. The deduced *C. neoformans* Ilv2p has 719 amino acid residues, which share over 50 % sequence identity with acetolactate synthases from other fungi: 59 % identity with *Schizosaccharomyces pombe* Ilv2p, 56 % identity with *Magnaporthe grisea* Ilv2p and 56 % identity with *S. cerevisiae* Ilv2p. The *ILV2* gene is present in a single copy in the genome based on Southern blot analysis (data not shown).

The genomic *ILV2* sequence was determined and a comparison between cDNA and genomic *ILV2* DNA sequence revealed the presence of five introns, of 48, 53, 53, 54 and

50 bp. A search for sequence similar to *C. neoformans* serotype A *ILV2*, using the Stanford Genome Technology Center *C. neoformans* Genome Project serotype D database, revealed sequence that was 95 % identical at the nucleotide level over the predicted coding region, with 98 % amino acid identity (contig cneo030328.b3501.C0627). All five predicted introns were in the same position as in the serotype A sequence; however, introns 3 and 4 were each 2 bp larger for the serotype D sequence.

Disruption of the *C. neoformans ILV2* gene results in auxotrophy

To investigate the functions of *C. neoformans* Ilv2p, we disrupted the *C. neoformans ILV2* gene in strain H99. An *ilv2*::*NAT1* disruption allele, constructed *in vitro* by PCR overlap technology, was introduced into H99 by biolistic transformation and Nat-resistant transformants were selected. Nine isoleucine and valine auxotrophic isolates were identified from a total of 81 purified Nat-resistant transformants. PCR analyses using primers that recognized sequences within the *NAT1* cassette (JO89 and JO90), paired with primers homologous to *ILV2* DNA (ZY110 and ZY091), showed that, unlike the wild-type, all putative *ilv2* transformants contained the *NAT1* allele flanked by *ILV2* sequence. In addition, amplification of the *ILV2* region in the putative *ilv2* mutants, using primers ZY110 and ZY091, revealed a single band that was approximately 1·6 kb larger than that observed for the wild-type, consistent with the homologous integration of a single copy of the *ilv2*::*NAT1* allele (data not shown). Finally, Southern analyses performed on two independently isolated transformants that were positive by PCR analyses (H99-20 and H99-21) confirmed that the *ILV2* gene was disrupted by a single copy of the *NAT1* gene (Fig. 1).

The *C. neoformans ilv2* mutant auxotrophy is satisfied by isoleucine and valine amino acids and dipeptides

Satisfaction of the auxotrophic phenotype of the *C. neoformans ilv2* mutants by the addition of isoleucine and valine to growth media would provide further evidence that the gene disrupted encodes acetolactate synthase. Growth of the wild-type (H99) and *ilv2* mutants (H99-20 and H99-21) was compared by streaking on minimal medium (SD) supplemented with isoleucine and valine. Interestingly, no growth was observed for the *ilv2* mutants (Fig. 2), even after 5 days of incubation at 30 °C.

The inability of *ilv2* mutants to grow on isoleucine- and valine-supplemented SD medium may be attributable to negative regulation of isoleucine and/or valine permeases by preferred nitrogen sources such as ammonium ions. To test this hypothesis, the growth of the *ilv2* mutants was compared on SD or Yeast Nitrogen Base medium containing the non-repressing nitrogen source proline (YNB + proline), supplemented with isoleucine and valine. After 3 days of incubation at 30 °C, growth was observed for *ilv2* strains on YNB + proline when isoleucine and valine were added together, but not individually (Fig. 2). These results are consistent with the transport of valine and/or isoleucine being subject to a nitrogen-repressible control in *C. neoformans*.

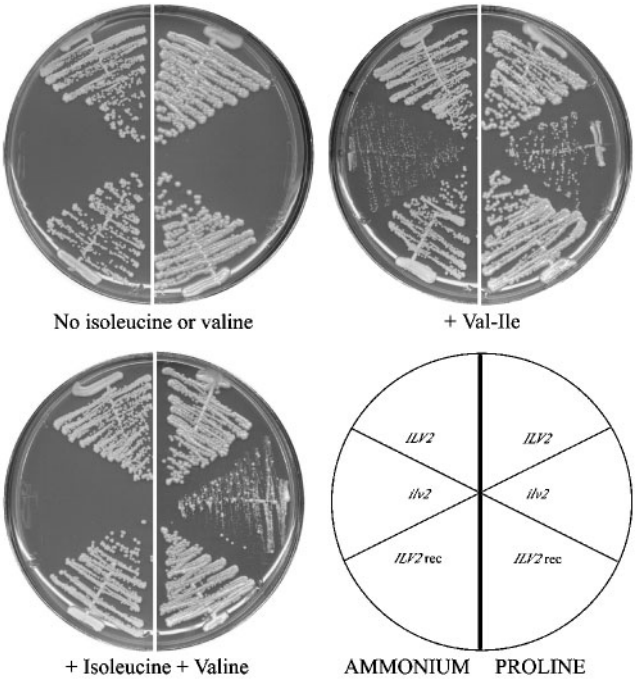

Fig. 2. Supplementation of the *C. neoformans ilv2* mutant auxotrophy using isoleucine, valine and Val-Ile dipeptide. Strains H99 (*ILV2*), H99-20 (*ilv2*) and H99-22 (*ILV2* rec) were streaked onto SD (left) or YNB + proline (right) media without additional supplements, or supplemented with either the amino acids isoleucine (0·23 mM) and valine (1·28 mM), or the dipeptide Val-Ile (0·22 mM), as indicated. Plates were incubated for 3 days at 30 °C.

The *ilv2* mutants grew considerably better when plated on YPD than on minimal medium supplemented with isoleucine and valine, even when proline was the nitrogen source. YPD contains peptides in addition to amino acids and thus *C. neoformans* may transport peptides better than amino acids. Growth of *ilv2* mutants was therefore compared on SD or YNB + proline medium that was supplemented with Ile-Val or Val-Ile dipeptides. The *ilv2* mutants were able to grow significantly, although considerably less well than wild-type, when Val-Ile (Fig. 2) or Ile-Val was added to SD plates. Moreover, as judged by colony size, the mutants were able to grow better when Val-Ile (Fig. 2) or Ile-Val (data not shown) was added to YNB + proline plates than to SD plates. These findings indicate that isoleucine and valine can better restore prototrophic growth of *C. neoformans ilv2* mutants when supplied as Ile-Val or Val-Ile dipeptides than as amino acids. Moreover, like isoleucine and valine transport, the uptake of these dipeptides also appears to be regulated by

the nitrogen source. The ability of the amino acids isoleucine and valine and the Ile-Val and Val-Ile dipeptides to satisfy the auxotrophy of the *ilv2* disruption further supports the designation of *C. neoformans* Ilv2p as an acetolactate synthase.

Reintroduction of *C. neoformans ILV2* restores prototrophy

To confirm that the phenotypes of *C. neoformans ilv2* mutants were due to the disruption of *ILV2*, the wild-type copy of *ILV2* was reintroduced into *ilv2* mutants. Transformants were selected by reversion to isoleucine and valine prototrophy and were subsequently screened for sensitivity to Nat: 16 out of 18 Ile^+ Val^+ transformants were Nat-sensitive, indicating that *ILV2* had replaced the *ilv2*::*NAT1* allele in these transformants. PCR analyses of the Nat-sensitive transformants, using the same primers as used to confirm disruption of *ILV2* (JO89 and ZY110, JO90 and ZY091, and ZY110 and ZY091), no longer detected the presence of *NAT1* flanked by *ILV2* sequence, and all products were of identical size to products from wild-type DNA (data not shown). Also, the sizes of DNA fragments generated by restriction digestion that hybridized to the *ILV2* probe in Southern analyses were indistinguishable between DNA from wild-type and *ILV2*-reconstituted strains (Fig. 1b). Therefore, genetic and phenotypic analyses confirmed that reversion to prototrophy was a result of the replacement of the disrupted *ilv2* allele by *ILV2*. Two strains that were phenotypically and genotypically positive for *ILV2* replacement, H99-22 (H99-20 reconstituted with *ILV2*) and H99-23 (H99-21 reconstituted with *ILV2*), were used in subsequent experiments.

ilv2 mutants are temperature sensitive

Since the ability to survive and proliferate at 37 °C is important for the virulence of *C. neoformans* (Odom *et al.*, 1997), we investigated whether *ilv2* mutants displayed temperature-sensitive growth. *ilv2* mutants (H99-20 and H99-21), wild-type (H99) and *ILV2*-reconstituted strains (H99-22 and H99-23) were streaked onto YPD plates and incubated at 30 °C or 37 °C. Unlike the significant amount of growth observed for the wild-type and *ILV2*-reconstituted strains, no growth of the *ilv2* mutants was observed when incubated at 37 °C for 3 days (Fig. 3a). The lack of growth on YPD medium at 37 °C was a consequence of the 37 °C incubation rather than a YPD medium-specific defect as *ilv2* mutant colonies were only slightly smaller than the wild-type and *ILV2*-reconstituted colonies when grown on

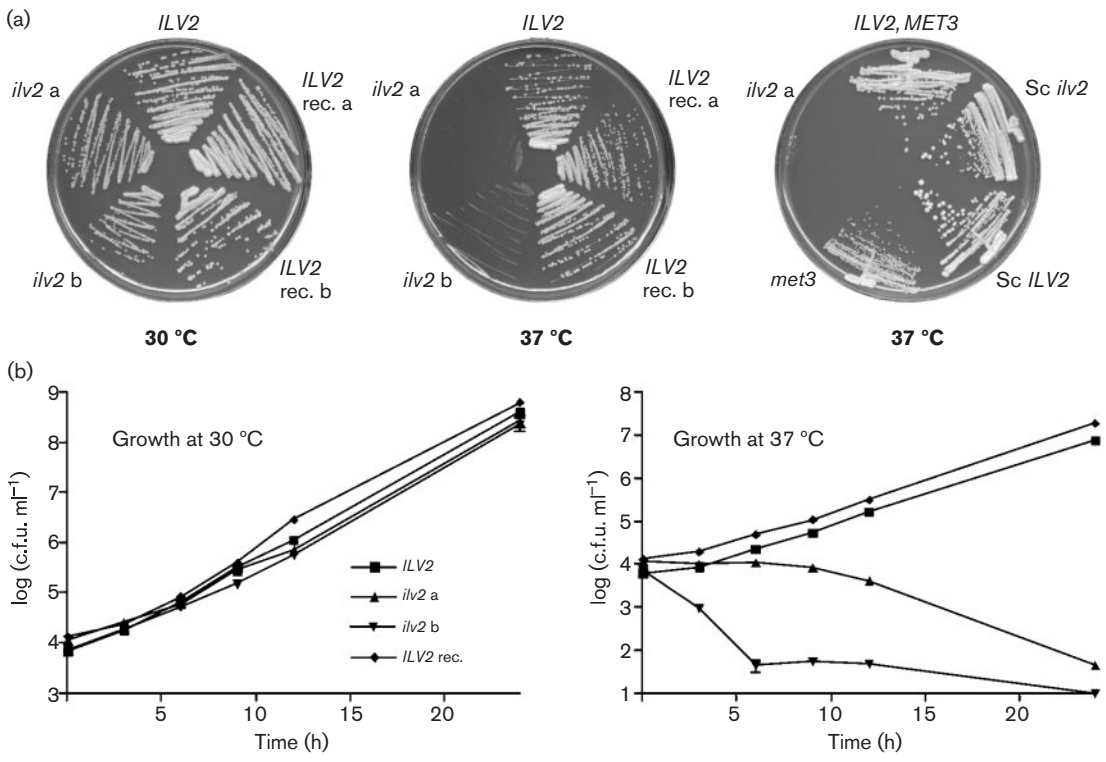

Fig. 3. Temperature sensitivity of *C. neoformans ilv2* mutants. (a) Strains H99 (*ILV2*), H99-20 (*ilv2* a), H99-21 (*ilv2* b), H99-22 (*ILV2* rec. a), H99-23 (*ILV2* rec. b) and H99-4 (*met3*), and *S. cerevisiae* S157 (Sc *ILV2*) and S311 (Sc *ilv2*) were streaked onto YPD medium and incubated at 30 °C or 37 °C, as indicated, for 2 days (30 °C) or 3 days (37 °C). (b) Growth rates were determined in liquid YPD medium at 30 °C and 37 °C. Experiments were performed in duplicate.

YPD medium at 30 °C (Fig. 3a). The temperature-sensitive defect was also observed on YNB + proline medium that was supplemented with Ile-Val and Val-Ile dipeptides.

To investigate whether incubation at 37 °C had a static or cidal effect on *ilv2* mutant growth, we determined the c.f.u. of *ilv2* mutants, the wild-type and an *ILV2*-reconstituted strain, following incubation in liquid YPD at 37 °C for 0, 3, 6, 9, 12 and 24 h. As can be seen in Fig. 3(b), incubation at 37 °C was cidal to two independent *ilv2* mutants: after 24 h, the *ilv2* strain c.f.u. values were approximately 0·32 % and $<0{\cdot}14$ % of initial values. In contrast, the wild-type and *ILV2*-reconstituted strain proliferated at this temperature (Fig. 3b), with mean generation times of approximately 2·54 and 2·63 h, respectively. Growth rates were also compared at 30 °C. Unlike on YPD plates, where *ilv2* mutants appeared to have a slight growth disadvantage compared with the wild-type, in liquid YPD medium, the wild-type, *ilv2* mutants and the *ILV2*-reconstituted strain all had very similar growth rates (Fig. 3b). Mean generation times were 1·63, 2·00, 1·92 and 1·55 h for the wild-type, two *ilv2* mutants and the *ILV2*-reconstituted strain, respectively.

The cidal effect of incubation at 37 °C is not a general phenomenon of fungal *ilv2* mutants as a *S. cerevisiae ilv2* mutant (S311) grew at a similar rate as the wild-type at this temperature, as judged by colony size (Fig. 3a). In addition, the cidal effect is not a common feature of all *C. neoformans* amino acid auxotrophs as a *met3* methionine auxotroph (H99-4) grew at this temperature (Fig. 3a).

The cidal effect may be a consequence of possible starvation for isoleucine and/or valine by the *C. neoformans ilv2* mutants at 37 °C. To investigate whether starvation for isoleucine and valine has a cidal effect at 30 °C, we determined the c.f.u. of the two *ilv2* mutants following incubation in liquid SD medium. Experiments were performed essentially the same as the growth rate experiments, except that the starting inoculum was approximately 1×10^5 c.f.u. ml^{-1}, and subsequent c.f.u. were determined at 24 and 48 h post-inoculation. Incubation of these strains in SD medium at 30 °C resulted in a reduction of c.f.u. over time. At 24 h post-inoculation, c.f.u. for the *ilv2* mutants were on average 8·53 % and 26·9 % of initial levels, and at 48 h post-inoculation, levels had reduced further to 0·52 % and 4·0 % of the initial values. This cidal effect of isoleucine and/or valine starvation may be a specific feature of *ilv2* mutants rather than a general consequence of cryptococcal nitrogen or amino acid starvation. Consistent with this, we found that a *S. cerevisiae ilv2* mutant also showed the same phenotype. Following isoleucine and valine starvation for 24 h, c.f.u. for the *S. cerevisiae ilv2* mutant were on average 11·9 % of initial levels, with numbers further dropping to 0·49 % of initial levels after 48 h. Conversely, the c.f.u. of nitrogen-starved (the wild-type incubated in YNB with no nitrogen source added) or methionine-starved (the *met3* mutant incubated in SD) *C. neoformans* cultures both increased slightly over 48 h incubation. At this time, the c.f.u. for the *met3* mutant were on average 2·0-fold higher than initial c.f.u. and the nitrogen-starved wild-type c.f.u. increased by 7·46-fold. Therefore, like the incubation of *C. neoformans ilv2* mutants at 37 °C, isoleucine and/or valine-specific starvation also results in the loss of cell viability.

Melanin and capsule production by *ilv2* mutants

We investigated whether the *ilv2*::*NAT1* mutation influences the ability of the strain to synthesize melanin, another important virulence determinant for *C. neoformans* (Salas *et al.*, 1996; Torres-Guererro & Edman, 1994; Williamson, 1997). Pigment production was followed for wild-type, *ilv2* mutants and *ILV2*-reconstituted strains, which had been spotted on Niger seed agar supplemented with Ile-Val or Val-Ile dipeptides, or with isoleucine and valine. Following incubation at 30 °C for 38 h, melanin production was evident for all strains and no differences in colour development were discernible between the *ilv2* mutants, wild-type and *ILV2*-reconstituted strains. Therefore, Ilv2p is not important for the synthesis of melanin.

Since capsule production is important for cryptococcal virulence (Bulmer *et al.*, 1967; Chang & Kwon-Chung, 1994; Fromtling *et al.*, 1982; Kwon-Chung & Rhodes, 1986), we examined capsule formation by *ilv2* mutants. After 2 days incubation at 30 °C in capsule-induction medium supplemented with Ile-Val and Val-Ile dipeptides, visual examination of India-ink-stained cells revealed that capsule production by the *ilv2* mutants was significantly decreased compared with the wild-type. Mean capsule thicknesses for the *ilv2* mutants was $0{\cdot}52\pm0{\cdot}26$ μm and $0{\cdot}53\pm0{\cdot}34$ μm, compared with $4{\cdot}33\pm1{\cdot}25$ μm for the wild-type. The poor capsule production by *ilv2* mutants was a consequence of *ILV2* disruption as the capsule production by the *ILV2*-reconstituted strain was indistinguishable from that of the wild-type, with a mean capsule thickness of $4{\cdot}40\pm1{\cdot}03$ μm.

While the c.f.u. of the wild-type and *ILV2*-reconstituted strains increased 270·8- and 1076·2-fold, respectively, during incubation in capsule-inducing medium for 2 days, the c.f.u. of the *ilv2* mutants fell to 28·6 % and 20·9 % of initial levels. Therefore, due to *ilv2* mutant lethality in standard capsule production medium, even when supplemented with isoleucine and valine amino acids and dipeptides, we were unable to determine the basis for the observed effect of the *ilv2* mutation on capsule production.

The *ilv2* mutant is avirulent and does not survive *in vivo*

The virulence of an *ilv2* mutant was compared with the virulence of wild-type and *ILV2*-reconstituted strains using the murine nasal inhalation model of cryptococcal infection (Cox *et al.*, 2000). Mice infected with the wild-type survived for a mean of 19 days ($18{\cdot}6\pm0{\cdot}49$ days, $t_{18}=232{\cdot}5$, $P<0{\cdot}001$ compared with the *ilv2* mutant), and all mice were dead by 21 days post-infection (Fig. 4). In

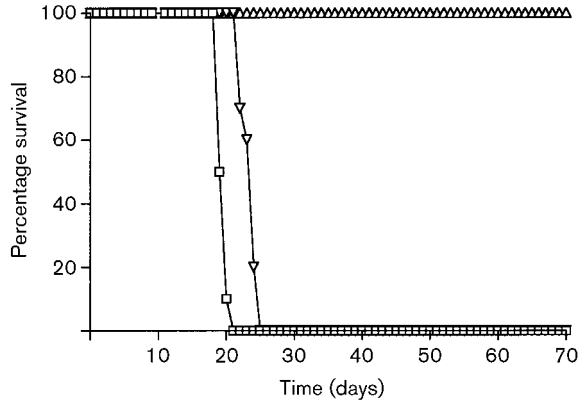

Fig. 4. Virulence of *C. neoformans ILV2*, *ilv2* and *ILV2*-reconstituted strains. Groups of 10 female A/Jcr mice were infected with H99 (*ILV2*; □), H99-20 (*ilv2*; △) and H99-22 (*ILV2*-rec.; ▽) by nasal inhalation, and observed twice-daily for mortality. *P* values were $<0{\cdot}001$ for the comparisons between the *ilv2* mutant and the wild-type and the *ILV2*-reconstituted strain.

contrast, all mice infected with the *ilv2* mutant remained healthy to 70 days post-infection. Following this time, the mice were sacrificed and their brains and lungs were recovered, homogenized, and plated (in aliquots) on YPD medium containing chloramphenicol to determine if any *ilv2* mutants remained. No *ilv2* mutants were recovered from any of the organs. Furthermore, the avirulence and inability to survive *in vivo* was a result of *ILV2* disruption, as mice that were infected with the *ILV2*-reconstituted strain survived for a similar length of time as the wild-type. The mean survival time for these mice was 23 days ($22{\cdot}5 \pm 1{\cdot}39$ days, $t_{18}=127{\cdot}5$, $P<0{\cdot}001$ compared with the *ilv2* mutant), and all mice were dead by 25 days post-infection. Therefore, consistent with the 37 °C and isoleucine and/or valine starvation lethal phenotypes, the *C. neoformans ilv2* mutant was avirulent and did not survive *in vivo*.

Ilv2p sensitivity to SM

As the *C. neoformans ilv2* mutant was avirulent and unable to survive *in vivo*, acetolactate synthase inhibitors which inhibit *S. cerevisiae* (Duggleby *et al.*, 2003; Falco & Dumas, 1985) may make excellent antifungal drugs. Therefore, we investigated whether the sulfonylurea SM inhibited *C. neoformans* growth. We determined the SM MICs for the *ILV2* wild-type strains *C. neoformans* H99 and *S. cerevisiae* S157 in SD + Ura medium, with or without isoleucine and valine amino acids and dipeptides added. The SM MIC_{80} for *S. cerevisiae* S157 was $6{\cdot}25$ μg ml^{-1}, and the inhibition was alleviated when isoleucine and valine amino acids and dipeptides were present. In contrast, no inhibition of *C. neoformans* H99 growth was observed, even at the highest level of SM tested (200 μg ml^{-1}). Due to the low solubility of SM, higher SM concentrations were not tested. *C. neoformans* SM resistance was strain-independent, as two other serotype A strains (CDC1 and CDC2) were also SM-resistant in MIC assays. *C. neoformans* SM resistance was also serotype-independent, as serotype B strains (B4495 and B4496), serotype C strains (ATCC34883 and ATCC34880) and a serotype D strain (JEC20) were all SM-resistant in MIC assays.

A higher level of resistance to SM by *C. neoformans* compared with *S. cerevisiae* may be due to *C. neoformans* Ilv2p being intrinsically more resistant than *S. cerevisiae* Ilv2p. To determine if differences in *C. neoformans* and *S. cerevisiae* Ilv2p sequences were responsible for *C. neoformans* SM resistance, we compared the SM sensitivity of *S. cerevisiae* strains containing either the *S. cerevisiae ILV2* ORF (S157) or the *C. neoformans ILV2* cDNA ORF (*S. cerevisiae* S3501 and S3502). The latter strains were independent Ile^+ Val^+ Nat-sensitive transformants resulting from the replacement of the *S. cerevisiae* S311 *ilv2*Δ::natMX4 allele by the *C. neoformans ILV2* cDNA allele; thus the *C. neoformans ILV2* cDNA allele was expressed from the same promoter as *S. cerevisiae ILV2*. The sequence of the *C. neoformans ILV2* cDNA-derived ORF from these *S. cerevisiae* transformants was identical to that of the original cDNA; therefore, there were no PCR-generated mutations. In contrast to *S. cerevisiae* S157, but like *C. neoformans* H99, *S. cerevisiae* strains containing the *C. neoformans ILV2* cDNA ORF (S3501 and S3502) were SM-resistant, with MIC_{80} values of >200 μg ml^{-1}.

To determine whether the SM resistance conferred by the *C. neoformans ILV2* allele was dominant over the SM sensitivity of a *S. cerevisiae ILV2* strain, we crossed the *S. cerevisiae* S157, S3501 and S3502 strains (all *MAT*α *ura3*Δ) with *S. cerevisiae* S1415 (*MAT***a** *lys5*), and compared the SM resistance of the resulting Lys^+ Ura^+ diploid strains (*S. cerevisiae* S3553, S3554 and S3555). Like the dominance of SM resistance previously reported for various *S. cerevisiae ILV2* mutants (Falco & Dumas, 1985), we observed that the SM resistance conferred by the *C. neoformans ILV2* allele was dominant over the SM sensitivity conferred by the *S. cerevisiae ILV2* allele. The MIC_{80} values were $6{\cdot}25$ μg ml^{-1} for *S. cerevisiae* S3553 (*ILV2*/*ILV2*) and >200 μg ml^{-1} for *S. cerevisiae* S3554 and S3555 (both *ILV2*/*ilv2*Δ::cn*ILV2* cDNA). Therefore, the difference in SM sensitivity between *C. neoformans* and *S. cerevisiae* is, at least in part, because *C. neoformans* and *S. cerevisiae* Ilv2p differ in their sensitivity to SM.

DISCUSSION

To better understand factors that are important for fungal pathogenesis and survival *in vivo*, and to identify possible antifungal drug targets, we employ a genetic approach to study the role of fungal-specific amino acid biosynthetic genes in the clinically relevant human pathogen *C. neoformans*. In this paper, we have described the identification of the *C. neoformans ILV2* gene, encoding acetolactate

synthase, and the construction and analysis of the phenotypes of *C. neoformans ilv2* mutants. The auxotrophic phenotype of *C. neoformans ilv2* mutants could be satisfied by the addition of Ile-Val or Val-Ile dipeptides to growth media, or by the reintroduction of *ILV2*. Thus, the gene isolated and disrupted in *C. neoformans* was confirmed as encoding acetolactate synthase.

In contrast to the auxotrophic phenotype of *S. cerevisiae ilv2* mutants, the *C. neoformans ilv2* mutant auxotrophy could not be satisfied by the addition of isoleucine and valine to minimal (SD) medium. However, we observed growth in the presence of these amino acids when proline was the nitrogen source, consistent with the transport of isoleucine and/or valine being negatively repressed in the presence of preferred nitrogen sources, and derepressed when poorer nitrogen sources are available. Therefore, the lack of growth of the *C. neoformans ilv2* mutants when supplemented with isoleucine and valine with ammonium as the nitrogen source suggests that *C. neoformans* may possess far fewer amino acid permeases than *S. cerevisiae*, or a greater proportion which are subject to ammonium repression.

The dipeptides Ile-Val and Val-Ile satisfied the auxotrophy of the *C. neoformans ilv2* mutants in the presence of either ammonium or proline as a nitrogen source. As we found that isoleucine and valine were unable to support the auxotrophy when ammonium was the nitrogen source, satisfaction of the auxotrophic requirement by dipeptides in these conditions could not have occurred by transport of amino acids that resulted from external dipeptide hydrolysis. Thus, our results provide evidence that at least one dipeptide transport system exists in *C. neoformans*; structurally similar peptide transporters are ubiquitous throughout fungi. Also, we observed increased growth of *ilv2* mutants when minimal, dipeptide-supplemented medium contained proline as the nitrogen source instead of ammonium. These results indicate that, like transport of isoleucine and/or valine by this strain and dipeptide transport by *S. cerevisiae* (Becker & Naider, 1977; Nisbet & Payne, 1979) and *C. albicans* (Logan *et al.*, 1979; Payne *et al.*, 1991), *C. neoformans* dipeptide transport may also be regulated by nitrogen source.

Two factors known to be important for virulence of *C. neoformans* include the production of a polysaccharide capsule (Bulmer *et al.*, 1967; Chang & Kwon-Chung, 1994; Fromtling *et al.*, 1982; Kwon-Chung & Rhodes, 1986) and melanin production (Salas *et al.*, 1996; Torres-Guererro & Edman, 1994; Williamson, 1997). While melanin development for the *ilv2* mutants was similar to that observed for the wild-type, we observed significantly decreased capsule production by the *ilv2* mutants compared with the wild-type. However, since viable cell numbers of the mutant decreased over time in capsule induction medium, compared with the proliferation of the wild-type, the reduced capsule production may be a consequence of low metabolic activity and cell death by the *ilv2* mutants in the capsule induction medium.

A third factor important for cryptococcal virulence and survival *in vivo* is the ability to survive and proliferate at the physiologically relevant temperature of 37 °C (Odom *et al.*, 1997). Interestingly, we found that the *ilv2* mutants were highly temperature-sensitive in both minimal and rich media at 37 °C. Furthermore, incubation at this temperature was cidal to *ilv2* mutants. This is not a general feature of all fungal *ilv2* mutants as *S. cerevisiae ilv2* mutants grow at 37 °C under the same conditions. The temperature sensitivity may be related to starvation by *C. neoformans ilv2* mutants for isoleucine, valine, leucine and/or pantothenate at 37 °C. Starvation could be a consequence of an enhanced requirement for these compounds at 37 °C, or a reduction in their transport at higher temperatures, caused by negative regulation of permease production or function, or enhanced permease turnover. Starvation for amino acids at 37 °C is certainly not a feature of all *C. neoformans* amino acid auxotrophs, however, as *met3* mutants survive and grow at this temperature. This model assumes that starvation for isoleucine and/or valine is cidal to cells. We established that unlike general starvation for nitrogen or other single amino acids (demonstrated for methionine), starvation for the same length of time for isoleucine and/or valine results in a significant reduction in *C. neoformans* viability, similar to the 37 °C cidal effect. Finally, disruption of *ILV2* may result in the accumulation of an intermediate that may be toxic at higher temperatures. Indeed, in *Salmonella typhimurium*, inhibition of acetolactate synthase has been reported to result in the accumulation of 2-ketobutyrate. Some evidence indicates that these elevated 2-ketobutyrate levels may inhibit bacterial growth by interfering with utilization of glucose as a carbon and energy source (LaRossa & Van Dyk, 1987; LaRossa *et al.*, 1987; Van Dyk *et al.*, 1987).

Consistent with the inability to survive and proliferate at 37 °C, we found an *ilv2* mutant to be avirulent and unable to survive *in vivo* using a murine inhalation model of infection. We cannot distinguish between the *in vivo* survival and virulence effects being due to the temperature-sensitive phenotype, accumulation of 2-ketobutyrate, or a possible inability to acquire sufficient isoleucine, valine, leucine and/or pantothenate *in vivo*. As starvation for isoleucine and/or valine results in the loss of *C. neoformans* viability and may be responsible for the capsule and temperature-sensitive defects of *ilv2* mutants, any possible *in vivo* starvation for these amino acids may be detrimental on several different levels.

The avirulence and lack of survival *in vivo* of the *C. neoformans ilv2* mutant validates acetolactate synthase as an excellent potential anti-cryptococcal drug target. As many different acetolactate synthase inhibitors have already been identified and used successfully as herbicides, these inhibitors make attractive lead compounds to evaluate as useful antifungal agents. Indeed, *S. cerevisiae* has been

shown to be sensitive to several of these, particularly compounds from the sulfonylurea family of inhibitors such as SM (Duggleby *et al.*, 2003; Falco & Dumas, 1985). However, we found that *C. neoformans* is considerably more resistant to inhibition by SM than *S. cerevisiae*. This may be because this compound is poorly transported by *C. neoformans*, or SM may bind *C. neoformans* Ilv2p less well due to differences between the *C. neoformans* and *S. cerevisiae* Ilv2p sequences. At least 10 amino acid substitutions have been identified which confer resistance of *S. cerevisiae* Ilv2p to sulfonylureas (Duggleby *et al.*, 2003; Falco *et al.*, 1989; Pang *et al.*, 2003; Xie & Jimenez, 1996). When we compared the *S. cerevisiae* and *C. neoformans* serotype A and D Ilv2p sequences, we found that one of these residues differs: *C. neoformans* Ilv2p contained an alanine instead of a proline at position 226, equivalent to *S. cerevisiae* position 192. Consistent with this, we observed that expression of the *C. neoformans ILV2* cDNA ORF in *S. cerevisiae* confers a substantial increase in SM resistance. Therefore, although we cannot address SM permeability or efflux, our results indicate that *C. neoformans* Ilv2p is intrinsically resistant to SM.

Our genetic approach for identifying good antifungal drug targets in the amino acid biosynthetic pathways has advantages over an alternative approach consisting of testing existing inhibitors for their ability to affect the course of experimental infections. The latter approach is hindered by the availability of relatively few inhibitors for the enzymes in the amino acid biosynthetic pathways; therefore, most enzymes could not be evaluated as targets. In addition, even for those enzymes with currently available inhibitors, these inhibitors have not been designed, or optimized, for low toxicity, solubility, oral availability, half-life within the host, or for their ability to enter the fungal cell. For example, *C. neoformans* is highly resistant to SM, a compound that was designed for use as a herbicide. Therefore, although the resistance of *C. neoformans* rules out the clinical use of SM, Ilv2p remains a very attractive anti-cryptococcal target for other inhibitors. As isoleucine and/or valine may be limiting in various *in vivo* environments, and acetolactate synthases are conserved throughout fungi, this enzyme may also have a more general utility as an antifungal drug target.

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