Molecular and genetic analysis of the Cryptococcus neoformans MET3 gene and a met3 mutant

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The Cryptococcus neoformans MET3 cDNA (encoding ATP sulfurylase) was cloned by complementation of the corresponding met3 mutation in Saccharomyces cerevisiae. Sequence analysis showed high similarity between the deduced amino acid sequence of the C. neoformans Met3p and other fungal ATP sulfurylases. A C. neoformans met3 mutant was made by targeted insertional mutagenesis, which had the expected auxotrophic phenotype, and reconstituted the met3 mutant to Met+. In vitro, the C. neoformans met3 mutant had a substantial defect in melanin formation, significantly reduced growth rate, and greatly increased thermotolerance. In the murine inhalation infection model, the met3 mutant was avirulent and was deficient in its ability to survive in mice. It is concluded that, in contrast to the yeast form of Histoplasma capsulatum, in C. neoformans the sulfate-assimilation arm of the methionine biosynthetic pathway plays an important role in vitro, even in the presence of abundant exogenous methionine, and is critical for virulence, and indeed for survival, in vivo.

Keywords: Cryptococcus neoformans, methionine auxotroph, ATP sulfurylase, virulence

INTRODUCTION

The methionine/cysteine biosynthetic pathway is highly complex. This complexity, and the important roles of the sulfur-containing amino acids methionine and cysteine in the cell, have resulted in the methionine/cysteine pathway being the focus of a major amount of work in saprophytic fungi (reviewed by Jones & Fink, 1982; Marzluf, 1997; Thomas & Surdin-Kerjan, 1997).

The methionine/cysteine biosynthetic pathway is also of considerable interest in the pathogenic fungi. For example, the saprobiic mycelial form of the human pathogen Histoplasma capsulatum is prototrophic while the pathogenic yeast form requires cysteine (reviewed by Boguslawski & Stetler, 1979; Maresca & Kobayashi, 1989). Similar results are seen in two other dimorphic pathogenic fungi infecting humans, Blastomyces dermatitidis and Paracoccidioides brasilienis (Medoff et al., 1987). In addition, the methionine/cysteine biosynthetic pathway is of interest from the perspective of antifungal drug development. Indeed, the antifungal agent azoxybacilin inhibits growth by repressing transcription of fungal sulfate-assimilation enzymes of the methionine/cysteine biosynthetic pathway (Aoki et al., 1996).

For our initial focus on the methionine/cysteine biosynthetic pathway in the human-pathogenic fungus Cryptococcus neoformans, we decided to examine MET3, which encodes ATP sulfurylase, the first step in the sulfate-assimilation arm of the pathway. One reason for this initial focus on Met3p was a previous report describing hypersensitivity to heat shock of a Saccharomyces cerevisiae met3 mutant (Jakubowski & Goldman, 1993). We report the cloning of the C. neoformans MET3 cDNA by complementation of the corresponding S. cerevisiae met3 mutant as well as the construction and reconstitution of a C. neoformans met3 mutant. We also report the phenotypic analysis of the C. neoformans met3 mutant; specifically, we have determined the effect of the met3 mutation on capsule
and melanin formation, two known virulence factors, as well as on growth rate, thermostolerance and virulence.

**METHODS**

**Strains and media.** The *S. cerevisiae* strains used in this study, which were all isogenic with S288c, were S1 (MATα), S157 (MATαura3A) and S303 (MATαura3Amet3::natMX4). The serotype A *C. neoformans* strains used in this study, which were all isogenic, were H99 (MATα) (Perfect et al., 1993), P99 (MATαura5) (Wang et al., 2001), H99-4 (MATαmet3::URA5 ura5) and H99-6 (MATα MET3 ura3+::cassette URA5). *Escherichia coli* DH10B (Gibco-BRL) was used as a host for cloning and plasmid propagation. Standard yeast media (Rose et al., 1990; Sherman et al., 1974) were used for the culture of both *S. cerevisiae* and *C. neoformans*. LB medium (Sambrook et al., 1989) was used to culture *E. coli*.

**Isolation of the *C. neoformans* MET3 cDNA.** The *C. neoformans* MET3 cDNA was isolated by complementing a met3 mutant of *S. cerevisiae* with a *C. neoformans* cDNA library. To make the *S. cerevisiae* met3 mutant strain, the primer pair ZY002 (5’-CTACGGTCGATCTAGATTGGCCCTACCTTGAAGTGGCCAGCTTACCTTCG-3’) and ZY007 (5’-GCTTGGGTCCTCTCTGCCTGTAACAGTTACGATATCCTGGCAATGCGGACTAGTGGATTCTG-3’) was used to amplify the natMX4 cassette, using the conditions described previously (Goldstein & McCusker, 1999).

The resulting PCR product, containing the drug-expression cassette and 40 bp sequences upstream and downstream of the *S. cerevisiae* MET3 ORF, was used to transform the *S. cerevisiae* ura3 strain S157 to nourseothricin resistance, as described previously (Goldstein & McCusker, 1999). The resulting met3::natMX4 ura3 strain (S303) was isolated by first screening for methionine auxotrophy and then confirming the presence of the met3::natMX4 insertion–deletion mutation by colony PCR with the primer pair JM37 (5’-CTCAGGATCATCTGCGATCTGCG-3’) and ZY063 (5’-GACATGCTGCTATCTCGG-3’).

A cDNA library containing inserts from the *C. neoformans* serotype A strain H99 was a generous gift from Dr Brian Wong (Yale University School of Medicine, New Haven, CT, USA) to the Duke University Mycology Research Unit (Brian Wong and John Perfect, personal communication).

The construction of the cDNA library in the URA3-selectable-marker-containing, galactose-regulated cDNA-expression plasmid pYES2.0 (Invitrogen) has been described previously (Suvarna et al., 2000). Using the lithium acetate transformation protocol (Gietz et al., 1995), plasmid library DNA (150 ng) was transformed into the *S. cerevisiae* met3 ura3 strain S303 followed by selection for Ura+ transformants. Ura+ transformants were replica-plated to synthetic medium lacking methionine and containing galactose as the sole carbon source (SG). Met− colonies from SG plates were streaked onto fresh SG plates for single colonies. A single colony from each Met− transformant was grown in 5 ml uracil drop-out medium (SDC–Ura) at 30 °C overnight to prepare plasmids, as described previously (Hoffman & Winston, 1987). The plasmids were introduced into DH10B cells by electroporation.

**Manipulation of nucleic acids.** Plasmid DNA was isolated using Qiagen Miniprep kits. *C. neoformans* genomic DNA was extracted by grinding cells in liquid nitrogen. Approximately 106 cells from an overnight culture were collected by centrifugation at 3500 r.p.m. for 5 min, washed once with sterile distilled water and, after adding 0.5 ml cracking buffer [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris/HCl (pH 8) and 1 mM Na2EDTA] (Hoffman & Winston, 1987), were transferred to a mortar which contained (and was pre-chilled by) liquid nitrogen. Using a pestle (pre-chilled by liquid nitrogen in the mortar), the cells were ground to fine powder, which was collected in 1.5 ml Eppendorf tubes. After thawing at room temperature and the addition of another 0.5 ml cracking buffer, equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1, Gibco-BRL) were added for extraction. After centrifugation at 14000 r.p.m. for 15 min, the supernatants were transferred to new tubes, treated with an RNase cocktail (Ambion; 2 µl of a 20 µg µl−1 solution) for 20 min at room temperature, and then treated with proteinase K (Roche; 1 µl of a 20 µg µl−1 stock) at 37 °C for 1 h. After extracting with phenol/chloroform once, the DNA was precipitated with 2 vols 100% ethanol and dissolved in TE buffer (pH 8) at a concentration of approximately 1 µg µl−1.

For Southern blot hybridization, approximately 2 µg genomic DNA from each strain was digested with various restriction enzymes and separated on 1% agarose gels. DNA was denatured and transferred to a nylon membrane (Roche) using standard procedures (Sambrook et al., 1989). Probe preparation, hybridization and signal detection were performed according to the manufacturer’s instructions (Roche) except for the substitution of the hybridization solution (0.25 M Na2HPO4, pH 7.2; 1 mM EDTA, 20% SDS, 0.5% blocking reagent) (Engler-Blum et al., 1993).

*C. neoformans* RNA was isolated from H99 cells grown overnight in SD and SD supplemented with 200 µM methionine (SD + Met). After collection of the cells (approx. 109) by centrifugation and washing them in sterile distilled water, the cells were ground in liquid nitrogen in the presence of 200 µl Trizol (mortal and pestle were pre-chilled as described above for DNA isolation from *C. neoformans*). The fine powder was transferred to tubes containing 800 µl Trizol, then 200 µl chloroform was added to the mixture, which was allowed to warm to room temperature for 10 min. After centrifugation at 10000 r.p.m. for 15 min, the aqueous phase was transferred to a clean tube and the RNA was precipitated with 500 µl 2-propanol followed by centrifugation at 13000 r.p.m. for 15 min. The pellet was washed with 500 µl 70% ethanol and the RNA was dissolved in the appropriate volume of diethylpyrocarbonate-treated water. Approximately 10 µg RNA was loaded on a 1.2% 1 M MOPS/formaldehyde gel. Northern blot hybridization was performed according to Sambrook et al. (1989). The ACT1 (1.3 kb) and MET3 (2.3 kb) probes were prepared from gel isolated fragments as template with [α-32P]dCTP and the RediPrime labelling kit according to the instructions provided by the manufacturer (Amersham-Pharmacia Biotech).

For PCR analysis, *S. cerevisiae* and *C. neoformans* genomic DNA was isolated as described previously (Hoffman & Winston, 1987). The PCR programme started with a 4 min incubation at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C and a final 10 min at 72 °C before cooling to 4 °C. Sequencing of the MET3 cDNA and serotype A MET3 gene (pZY25) were performed by the Duke University Cancer Center DNA sequencing facility using synthetic oligonucleotides; a minimum of two over laps were performed to ensure the accuracy of the entire sequence.

**Disruption of the *C. neoformans* MET3 gene.** The MET3 gene was disrupted by insertion of the URA5 marker gene. The primer pair ZY127 (5’-CTCCTCGAGCCCACTCCTA-3’) and ZY128 (5’-TGCTCGCTTTCAACACC-3’), the se...
quences of which were derived from the MET3 cDNA sequence, were used to amplify a 2.1 kb genomic fragment of the MET3 gene from the serotype A strain H99. This PCR product was cloned into the pCR2.1TOPO vector (Invitrogen), confirmed by various restriction enzyme digests and subcloned into the EcoRI site of pUC18, which resulted in pZY25. pZY25 was linearized with EcoRV, dephosphorylated with calf intestinal alkaline phosphatase and ligated to a blunt-ended URA5 expression cassette (excised from pCNCtel1 with BamHI and XhoI, treated with Klenow to create blunt ends), resulting in the met3::URA5-containing disruption plasmid pZY26; 1·3 kb and 0·8 kb of met3 homology flanked the URA5 insertion. Approximately 1 μg pZY26 DNA (linearized with BamHI) was used to biolistically transform (Toffaletti et al., 1993) the ura5 strain F99 (Wang et al., 2001) to Ura-. Ura- transformants were selected on synthetic uracil dropout medium (SD—Ura) containing 1 M sorbitol. Each Ura- transformant was colony-purified by streaking on SDC—Ura medium, and putative disruption mutants were screened by replica-plating onto SD and SD + Met media; Met+ Ura- colonies were further analysed by PCR and Southern blots to confirm the presence of the met3::URA5 construct disrupting the MET3 gene.

Restoration of the C. neoformans MET3 gene into the met3 mutant strain. A 2.1 kb genomic fragment of MET3 was PCR-amplified with the primer pairs ZY127 and ZY128 in the presence of a proofreading enzyme (0·2 U Pfu per reaction). This MET3-containing PCR product was used to biolistically transform the met3::URA5 mutant strain to Met+. Met+ transformants were selected on synthetic medium lacking methionine but containing uracil (SD—Met) in the presence of 1 M sorbitol. Met+ transformants were screened for the loss of the URA5 marker (met3::URA5 ura5 → MET3 ura5) by testing for growth in the presence of uracil and 5-fluoroorotic acid (5FOA). Met+ Ura- 5FOA-resistant transformants were analysed by Southern blotting. The URA5-containing plasmid pCNtel1 (Edman, 1992) was linearized with NotI to delete telomere-containing sequences and self-ligated to generate plasmid pZY22. The URA5-containing plasmid pZY22 was then used to biolistically transform (Toffaletti et al., 1993) the Met+ Ura- 5FOA-resistant cells to Ura-. Ura+ transformants were selected by demanding growth on SD—Ura containing 1 M sorbitol.

Thermotolerance assay. The isogenic C. neoformans wild-type (H99), met3 and MET3 reconstituted (met3+MET3) strains were inoculated into 5 ml YPD medium and grown at 30 °C overnight to mid-exponential phase. Liquid cultures of 1 ml were pelleted, washed twice in water and inoculated into 10 ml SD medium supplemented with 200 μM l-methionine. After shaking at 30 °C for 3 h, each culture (2 ml) was transferred into 15 ml Falcon tubes and incubated at 42 °C for 3 h. Each culture was diluted and spread onto YPD plates before and after the heat shock. For S. cerevisiae wild-type and met3 mutant strains, similar conditions were applied except that 45 °C was used for heat-shock treatment instead of 42 °C.

Capsule production assay. To assay capsule production, strains were grown on fresh YPD plates for 2 days, after which a single colony from each strain was inoculated into Dulbecco’s Modified Eagle’s Medium (D-MEM +22 mM NaHCO3 +25 mM NaMOPS) and grown at 37 °C for 2 days. Capsule production was visualized by standard India ink staining. Packed cell volume measurements, a quantitative assay for capsule production, were performed as described previously (Alspaugh et al., 1997; Granger et al., 1985).

Growth rate. To determine growth rates, a single, freshly streaked colony from each strain was inoculated into 5 ml YPD medium and grown overnight at 30 °C on a roller drum. Cultures were examined microscopically for the presence of budding cells, a characteristic of exponential-phase growth, and cell counts; cells were then diluted for OD measurement. For each strain, cells were inoculated in duplicate into 30 ml YPD in 125 ml flasks at a starting OD of approximately 0·06–0·1. The cultures were shaken at 240 r.p.m. at 37 °C. The OD of the cultures was measured every 2 h for 12 h.

Melanin synthesis assay. Melanin synthesis was assayed on Niger seed agar (Kwon-Chung & Bennet, 1992). Strains were grown on YPD plates for 2–3 days at 30 °C. A colony of the same or very similar size from each strain was resuspended in 100 μl sterile distilled water and 5 μl of each suspension was spotted onto the surface of Niger seed agar supplemented with l-methionine (60 μg ml−1). Plates were incubated at 30 °C and 37 °C for various times.

Test of virulence in the murine inhalation model. The murine cryptococcal inhalation model was used for the virulence test as described by Cox et al. (2000). Briefly, groups of ten 4- to 6-week-old A/Jcr mice (NICI/Charles River Labs) were anaesthetized with pentobarbital and suspended by the incisors on a silk thread. These mice were then infected via nasal inoculation with inocula (105 cells in 50 μl volumes; cultures were pre-grown overnight in YPD at 30 °C) of the MET3 wild-type strain H99, the met3 mutant strain H99-4, and the MET3 reconstituted strain H99-6. The 50 μl inocula were slowly pipetted into the nares of each mouse, after which the mice were left suspended for 10 min to ensure inhalation of the inocula into the lungs. Ten mice were infected with each strain. The mice, which were fed ad libitum, were followed with twice-daily inspections, and mice that appeared moribund or in pain were killed by CO2 inhalation. Survival data were analysed by Kruskal–Wallis test. The mice infected with H99-4 (met3) were killed at 60 days post-infection and their brains were removed and homogenized. The entire volume of each brain homogenate was plated (in aliquots) onto Sabouraud agar containing chloramphenicol, and cultured. Similar intranasal infections, except using 106 cells in 50 μl volumes per mouse, were carried out with H99, H99-4 and H99-6 where three mice per group were killed at day 7 and day 14 post-infection; the lungs of these mice were removed, homogenized and aliquots of the homogenates plated, as described above, to calculate organ load.

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

RESULTS

Isolation of the C. neoformans MET3 gene

The C. neoformans MET3 gene was identified by complementation of a S. cerevisiae met3 mutant. A C. neoformans cDNA library was introduced into the met3Δ::vrzME4Δ ura3 S. cerevisiae strain S303 using the lithium acetate protocol (Gietz et al., 1995); approximately 45000 Ura+ transformants were obtained by demanding complementation of the chromosomal ura3 mutation by the plasmid-borne URA3 gene. After replica-plating the transformants to methionine-deficient medium containing galactose (to induce cDNA
transcription) as the sole carbon source, there were 13 Met\(^+\) colonies. Consistent with the plasmid-borne cDNAs being under the control of the galactose promoter, the Met\(^+\) phenotype of all 13 transformants was galactose-dependent (data not shown). Plasmids isolated from each of these 13 Met\(^+\) transformants were then used to transform S303 to Met\(^+\), which confirmed complementation. Four of these 13 plasmids were then used to transform \(E. coli\) DH10B for plasmid amplification and purification.

Restriction enzyme digestion with EcoRI and NotI of the purified plasmids indicated that the four plasmids contained the same-sized insert of approximately 1.9 kb. Two plasmids that were sequenced from both ends had identical sequences; therefore, only one plasmid was completely sequenced by primer walking. The complete cDNA sequence revealed an ORF of 1746 bp encoding a putative protein of 582 amino acids. The deduced \(C. neoformans\) Met3p sequence showed a high percentage of sequence identity with ATP sulfurylases from \(Aspergillus nidulans\) (GenBank accession AJ292542; 62\% identity), \(Candida albicans\) (GenBank accession AF164103; 53\% identity), \(Schizosaccharomyces pombe\) (GenBank accession D83992; 57\% identity) and \(Saccharomyces cerevisiae\) (GenBank accession X06413; 58\% identity). Based on functional complementation and sequence homology, the cDNA isolated was designated \(CnMET3\) (GenBank accession number AF30556).

The genomic \(MET3\) gene from the serotype A strain H99 was cloned (pZY25) and sequenced (GenBank accession number AF489498). Comparison of the genomic and cDNA sequences revealed the presence of four introns, three of them in the 5′ region (46, 57 and 52 bp) and a fourth in the 3′ region (55 bp). The \(MET3\) cDNA and \(MET3\) genomic sequences were used to search the \(C. neoformans\) genome database of the Stanford Genome Technology Center (http://sequence-www.stanford.edu/group/C.neoformans/index.html), which contains the genomic sequence of the \(C. neoformans\) serotype D strain JEC21. Sequence comparison between the genomic serotype A \(MET3\) gene and serotype D \(MET3\) gene revealed an overall 93\% sequence identity. All four of the introns were located at the same sites in serotypes A and D. However, the first two introns differed in size between the two serotypes; the first intron differed in length by 1 bp (46 bp in serotype A and 47 in serotype D) and the second intron by 6 bp (57 bp in serotype A and 51 bp in serotype D).

**Disruption of the \(C. neoformans MET3\) gene and complementation**

To analyse the function of the \(C. neoformans MET3\) gene, a cloned 2.1 kb PCR product of \(MET3\) amplified from genomic DNA of H99 was disrupted by inserting the \(URA5\) marker into the internal EcoRV site of the gene. The resulting \(met3::URA5\) disruption construct was introduced into the \(ura5\) strain F99 by biolistic transformation (Toffaletti \textit{et al}., 1993). A total of 140 \(Ura^+\) transformants were colony-purified on uracil dropout medium (SDC−Ura) and replica-plated to minimal medium (SD) to screen for methionine auxotrophs. From this screen, one \(Ura^+\) transformant showed methionine-dependent growth. As is the case for the \(S. cerevisiae met3\) mutant, the auxotrophic defect of this \(C. neoformans\) mutant was satisfied by cysteine or sodium sulfite (Na\(_2\)SO\(_3\)), as well as by methionine (data not shown). This \(C. neoformans\) Met\(^−\) \(Ura^+\) transformant was analysed by PCR, which showed that the \(met3::URA5\) construct had integrated homologously (data not shown); in addition, Southern blot analysis showed homologous, single-copy integration of the \(met3::URA5\) construct (Fig. 1). Based on the cDNA complementation of the \(S. cerevisiae met3\) mutant, the auxotrophic phenotypes of the \(C. neoformans\) mutant, and the molecular analysis of the mutant genotype, this \(C. neoformans\) mutant was \(met3\) and was designated H99-4.

To complement the \(met3::URA5\) mutation, a 2.1 kb PCR product of \(MET3\) was amplified from genomic DNA of H99 [using 0·2 units \(Pfu\) (Gibco-BRL) + 4 units \(Taq\)] and used to transform H99-4; 10 Met\(^+\) transformants were isolated. Two of these Met\(^+\) transformants were \(Ura^−\) and 5FOA-resistant. A Southern blot (Fig. 1) showed that a single copy of \(MET3\) had
Cryptococcus neoformans MET3

ATP sulfurylase

SD SD + Met

MET3
(2 kb)

ACT1
(1·3 kb)

Fig. 2. Northern blot analysis of the MET3 transcript. The strain used was wild-type serotype A H99. Growth conditions were SD and SD + 200 µM methionine. The bottom panel shows the actin transcript as a control of the RNA loading in the gel. Numbers at the right show the size of the transcript.

homologously recombined at the met3::URA5 locus in the two Met+ strains; that is met3::URA5 ura5 → MET3 ura5. Since the transformants became ura5 when the met3::URA5 mutation was replaced with MET3, the URA5 marker was reintroduced into the two strains by transforming a modified pCnTel1 plasmid (Edman, 1992) (pCnTel1 digested with NotI and self-ligated to generate pZY22). Three independent Ura+ transformants were colony-purified from each of the two transformations. All six of these met3+ MET3 strains were compared for melanin synthesis and capsule production and no obvious phenotypic differences were observed (data not shown); therefore, one reconstituted strain (designated H99-6) was picked for further detailed analysis (see below).

Northern blot analysis of MET3

To determine if the C. neoformans MET3 gene was transcriptionally regulated by methionine, as is the case in C. albicans (Care et al., 1999), RNA extracted from the wild-type strain H99 growing on SD and SD plus 200 µM methionine was blotted and the blot hybridized (separately) with MET3 and ACT1 probes. The results presented in Fig. 2 show that in C. neoformans, at least at the methionine concentration tested, there is no difference in the level of expression of the MET3 gene between SD and SD + Met.

Delayed melanin biosynthesis by the met3 mutant

H99, H99-4 and H99-6 were compared for their ability to produce melanin, a known virulence factor (Kwon-Chung & Rhodes, 1986; Kwon-Chung et al., 1982; Rhodes et al., 1982), on Niger seed agar supplemented with methionine. Approximately 10⁶ cells from each strain were spotted on the surface of Niger seed agar + methionine plates and incubated at 37 °C. After 30 h incubation, melanin production was readily discernible for the wild-type (H99) and reconstituted (H99-6) strains, but not for the met3 strain (H99-4) (Fig. 3). After 38 h incubation, the met3 strain started to produce melanin as detected by a colour change on the plate (Fig. 3). After 3 days of incubation, there was no obvious difference in the amount of melanin produced by the three strains (Fig. 3); similar behaviour was observed for all three strains grown at 30 °C and when the three strains were grown on separate plates (data not shown). Therefore, the met3 mutant was capable of producing melanin but the process was delayed relative to wild-type.

Normal capsule production of the met3 mutant

H99, H99-4 and H99-6 were compared visually for their ability to produce capsule, a known virulence factor (Chang & Kwon-Chung, 1994). Under inducing conditions (D-MEM + NaHCO₃ + NaMOPS), there were no obvious differences in capsule size between the three strains (data not shown).
Reduced growth rate of the \( \text{met}3 \) mutant

The growth of H99, H99-4 and H99-6 was compared. Exponential-phase cultures of the three strains were inoculated into fresh YPD medium at OD\( \text{600} \) 0.06–0.1, and growth was monitored by determining the OD\( \text{600} \) every 2 h for 12 h. The wild-type strain H99 had a doubling time of 2.06 ± 0.12 h (mean ± s.e., \( n = 3 \)) as compared to the \( \text{met}3 \) strain H99-4, which had a doubling time of 2.87 ± 0.13 h. The slow growth of the \( \text{met}3 \) strain was restored to near wild-type levels in the reconstituted strain H99-6, which had a doubling time of 2.23 ± 0.30 h.

Thermotolerance assay of the \( \text{met}3 \) mutant

Since it had been reported that a \( S. \text{cerevisiae} \) \( \text{met}3 \) mutant was deficient in thermotolerance (Jakubowski & Goldman, 1993), H99, H99-4 and H99-6 were assayed for thermotolerance. Cultures of the three strains were grown in synthetic minimal medium (SD) supplemented with 0.2 mM l-methionine and harvested in mid-exponential phase. Prior to heat shock, each culture was serially diluted to determine the viable c.f.u. To assay thermotolerance, cell suspensions (2 ml) of each culture were incubated at 42 °C for 3 h; after the heat shock, each culture was serially diluted and aliquots were spread onto YPD plates to determine the viable c.f.u. After repeating the experiment four times, it was found that (i) the wild-type strain H99 was the most sensitive to heat shock, with less than 1% of the cells surviving (mean survival ± s.e. \( n = 4 \) 0.8 ± 0.3%), (ii) the reconstituted H99-6 strain was slightly more thermotolerant than the wild-type strain (mean survival 60 ± 10%), and (iii) the \( \text{met}3 \) mutant strain H99-4 was highly thermotolerant, with almost all the cells surviving (mean survival 97.0 ± 3.0%).

Similar thermotolerance experiments were performed with both wild-type (S1) and \( \text{met}3 \) mutant (S303) strains of \( S. \text{cerevisiae} \). After heat shock at 45 °C for 3 h, it was found by spotting assay that, in contrast to a previous report (Jakubowski & Goldman, 1993), virtually 100% of the cells from both the wild-type and the \( \text{met}3 \) mutant cultures survived (data not shown).

Avirulence of the \( \text{met}3 \) mutant

We employed the murine nasal inhalation model (Cox et al., 2000) to test the virulence of the \( \text{met}3 \) mutant. A/Jcr mice were infected, separately, with H99, H99-4 and H99-6. As shown in Fig. 4, the \( \text{met}3 \) strain H99-4 was completely avirulent in A/Jcr mice in comparison with the isogenic wild-type strain H99 and the reconstituted strain H99-6. On average, mice infected with H99 survived 28 days post-infection (mean survival 27.9 ± 2.6 days; \( P < 0.001 \) compared to the \( \text{met}3 \) mutant strain) with 100% mortality occurring by day 33 post-infection. Similarly, mice infected with the reconstituted strain H99-6 survived a mean of 33 days post-infection (mean survival 33.1 ± 3.9 days; \( P < 0.001 \) compared to the \( \text{met}3 \) mutant strain) with 100% mortality occurring on day 38 post-infection. In contrast, all ten mice infected with the \( \text{met}3 \) mutant H99-4 survived to day 60 post-infection. At day 60, the mice infected with the \( \text{met}3 \) mutant were killed and their brains were dissected and homogenized. The complete volumes of all brain homogenates were plated (in aliquots) on Sabouraud agar plus chloramphenicol; no \( C. \text{neoformans} \) \( \text{met}3 \) cells were recovered from the brains of these mice killed at day 60 post-infection.

A/Jcr mice infected, separately, with H99, H99-4 and H99-6 were also killed at day 7 and day 14 post-infection and their lungs were dissected and homogenized. Aliquots of the lung homogenates were plated on Sabouraud agar plus chloramphenicol to determine the number of c.f.u. and the lung burdens per mouse. At day 7 post-infection, the lung burdens (log c.f.u.) were 5.2 ± 0.5 for H99, 4.8 ± 0.5 for H99-6 (\( P = 0.2 \) vs H99) and 2.2 ± 0.6 for H99-4 (\( P = 0.01 \) vs H99). At day 14 post-infection, the lung burdens were 5.3 ± 0.4 for H99, 5.1 ± 0.5 for H99-6 (\( P = 0.1 \) vs H99) and 0.9 ± 0.8 for H99-4 (\( P = 0.02 \) vs H99). Therefore, Met3p was required for both virulence and survival of \( C. \text{neoformans} \) in the murine infection model.

DISCUSSION

This work describes the cloning of the \( C. \text{neoformans} \) \( \text{MET3} \) cDNA, by complementation of a \( S. \text{cerevisiae} \) \( \text{met}3 \) mutant, and the analysis of the corresponding \( C. \text{neoformans} \) \( \text{met}3 \) mutant. The deduced amino acid sequence of the \( C. \text{neoformans} \) \( \text{MET3} \) cDNA shows a high degree of similarity with other fungal ATP sulfurylase sequences. After cloning a genomic fragment, we made an \( \text{in vitro} \) disruption construct of the putative \( C. \text{neoformans} \) \( \text{MET3} \) ORF (\( \text{met}3::\text{URA5} \)). Homologous integration of the \( \text{met}3::\text{URA5} \) construct into a \( \text{ura}5 \) \( C. \text{neoformans} \) strain results in a methionine-auxotrophic strain. The auxotrophic phenotype of this \( \text{met}3::\text{URA5} \) mutant is in turn complemented by reintroduction of the
MET3 gene. Therefore, we have cloned the C. neoformans MET3 gene and made the corresponding met3 mutant.

In spite of transforming with a met3::URA5 disruption construct that has considerable amounts of homology, we observe a much lower frequency of homologous disruptants (1 met3::URA5/140 Ura+) than has been observed at other C. neoformans loci (e.g., Cox et al., 2000, 2001; D’Souza et al., 2001; Yue et al., 1999). This low frequency of MET3 disruption by homologous recombination could be due to the deleterious effects of the met3 mutation. However, we also see a relatively low frequency of reconstitution of met3 (met3::URA5 ura5 → MET3 ura5) by homologous recombination. In combination, the low frequency of disruption of MET3 and reconstitution of the met3::URA5 mutation by homologous recombination suggest that the MET3 region of the C. neoformans genome may be somewhat refractory to homologous recombination.

As with the S. cerevisiae met3 mutant, the auxotrophy of the C. neoformans met3 mutant can be satisfied by methionine or cysteine, which requires that methionine and cysteine be interconvertible. Methionine and cysteine can be interconverted by two distinct transsulfuration pathways. The first pathway, which consists of cystathionine β-synthase and cystathionine γ-lyase and is the only pathway in enteric bacteria, converts cysteine to homocysteine. The second pathway, which consists of cystathionine β-synthase and cystathionine γ-lyase and is the only pathway in mammalian cells, converts homocysteine to cysteine (Thomas & Surdin-Kerjan, 1997). Since the auxotrophy of the C. neoformans met3 mutant can be satisfied by either methionine or cysteine, both transsulfuration pathways must exist in C. neoformans. Therefore, the analysis of the met3 mutant provides additional insight into the metabolic pathways of the human pathogen C. neoformans.

One reason for our focus on C. neoformans Met3p was an earlier report describing the heat-shock-hypersensitive phenotype of a S. cerevisiae met3 mutant (Jakubowski & Goldman, 1993). In our hands, however, a S. cerevisiae met3Δ mutant is indistinguishable from wild-type with respect to heat shock. Nevertheless, we examined the thermostolerance of the C. neoformans met3 mutant. We find that, relative to the parental wild-type strain, the C. neoformans met3 mutant is highly thermostolerant.

The S. cerevisiae met3 mutant grows well in the presence of exogenous methionine while the C. neoformans met3 mutant grows quite slowly. This growth differential suggests that, compared to S. cerevisiae, C. neoformans has a low uptake capacity for methionine. The presence of exogenous methionine had no effect on MET3 expression levels in C. neoformans, consistent with a low uptake capacity for methionine. The slow growth of the C. neoformans met3 mutant in the presence of abundant exogenous methionine is also consistent with a low uptake capacity for methionine and consequent nutrient limitation. Extensive analysis in S. cerevisiae shows that nutrient limitation, including starvation for amino acids and sulfur, induces thermotolerance (e.g., Paris & Pringle, 1983; Plesset et al., 1987; Walton et al., 1979). Therefore, the slow growth rate of the C. neoformans met3 mutant, even in the presence of abundant exogenous methionine, is likely to be the cause of the greatly increased thermotolerance of this auxotroph.

The met3 mutation is pleiotropic with a slower growth rate and an in vitro defect in a known virulence factor, melanin formation. Like the C. neoformans met3 mutant, C. neoformans ade2 and ura5 mutants have in vitro defects in melanin formation although, unlike the met3 mutant, these defects disappear upon supplementation of the medium with adenine and uracil, respectively (J. A. Alspaugh, unpublished). Therefore, probably as a result of their relatively slow growth in vitro, auxotrophic mutants frequently have melanization defects in vitro; that is, the melanization defects of auxotrophic mutants, including met3, are likely to be due to nutrient limitation. Although tissue levels may be different from serum levels, the concentration of methionine in mouse serum (17 µg ml⁻¹; Crispens, 1975) is approximately half that used in defined yeast medium (0.2 mM or ~30 µg ml⁻¹) to measure growth rates. Therefore, we would expect the C. neoformans met3 mutant to be limited for methionine in vivo, and for the in vivo defects in melanin formation and growth rate to be at least as severe as those observed in vitro.

In addition to examining the ecology of infection, the characterization of C. neoformans amino acid auxotrophs genetically evaluates gene products as antifungal drug targets; that is, avirulent auxotrophs identify good potential antifungal drug targets. A further way to evaluate potential antifungal drug targets is to examine the ability of the mutants to survive in vivo; in terms of survival in vivo, the C. neoformans met3 mutant is even more deficient than a C. neoformans ade2 mutant (G. M. Cox, unpublished). By these criteria, Met3p is a good potential antifungal drug target.

In addition to their essential roles in protein synthesis, methionine and cysteine have multiple functions in the cell, including roles in polyamine synthesis and a variety of methylation reactions as well as synthesis of important low-molecular-mass sulfur-containing compounds, including glutathione, coenzyme A and biotin. Therefore, the severe and pleiotropic phenotypes of the C. neoformans met3 mutant are consistent with the inability to synthesize methionine and cysteine. To further test this hypothesis, future work will examine the phenotypes of mutants blocked in different steps in and different arms of the methionine/cysteine biosynthetic pathway, in particular examining and comparing the phenotypes of Met⁺ Cys⁺ mutants, such as met3, with Met⁺ Cys⁻ and Met⁻ Cys⁺ mutants.

The C. neoformans met3 mutant is avirulent and indeed does not survive in vivo. However, the in vivo defect of the C. neoformans met3 auxotrophic mutant stands in contrast to that observed for H. capsulatum, where the
pathogenic yeast phase (but not the saprobic mycelial phase) is auxotrophic due to a deficiency in sulfate reductase (Boguslawski & Stetler, 1979; Maresca & Kobayashi, 1989), a step in sulfate assimilation which occurs after ATP sulfurylase. The different responses of these two species of pathogenic fungi to deficiencies in the sulfate-assimilation arm of the methionine/cysteine biosynthetic pathway, which could be due to differences in their ecological niches or differences in methionine and/or cysteine uptake systems, are intriguing.

The amino acid biosynthetic pathways are a useful window on metabolism, gene structure and gene regulation in the pathogenic fungi that also provide insight into the ecology of infection. In this regard, the complex methionine/cysteine biosynthetic pathway is of particular interest with respect to metabolic and regulatory perspectives (reviewed by Jones & Fink, 1982; Marzluf, 1997; Thomas & Surdin-Kerjan, 1997) as well as the differences between the human-pathogenic fungi H. capsulatum and C. neoformans.

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Cryptococcus neoformans MET3/ATP sulfurylase


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