**Cryptococcus neoformans** methionine synthase: expression analysis and requirement for virulence

Renata C. Pascon, Tonya M. Ganous, Joanne M. Kingsbury, Gary M. Cox and John H. McCusker

Department of Molecular Genetics and Microbiology, Department of Medicine, Infectious Disease Division, Duke University Medical Center, Durham, NC 27710, USA

This paper describes (i) the expression profile of the methionine synthase gene (**MET6**) in the human pathogenic fungus *Cryptococcus neoformans* and (ii) the phenotypes of a *C. neoformans* **met6** mutant. In contrast to the **MET3** gene, which showed no significant change in expression in any environmental condition tested, the **MET6** gene showed a substantial induction in response to methionine and a dramatic transcriptional induction in response to homocysteine. Like a **met3** mutant, the **met6** mutant was a methionine auxotroph. However, relative to a **met3** mutant, the **met6** mutant grew very slowly and was less heat-shock resistant. In contrast to a **met3** mutant, the **met6** mutant lost viability when starved of methionine, and it was deficient in capsule formation. Like a **met3** mutant, the **met6** mutant was avirulent. In contrast to a **met3** mutant, the **met6** mutant was hypersensitive to fluconazole and to the calcineurin inhibitors FK506 and cyclosporin A. A synergistic fungicidal effect was also found between each of these drugs and **met6**. The phenotypic differences between the **met3** and **met6** mutants may be due to the accumulation in **met6** mutants of homocysteine, a toxic metabolic intermediate that inhibits sterol biosynthesis.

**INTRODUCTION**

The methionine–cysteine biosynthetic pathway has been extensively studied in saprophytic fungi because of interest in gene regulation, the length and complexity of the pathway, and because of the central role of the pathway in metabolism (e.g. Brzywcyz et al., 2002; Marzluf, 1997; Thomas & Sardiner-Kerjan, 1997). The methionine–cysteine biosynthetic pathway is also of considerable interest in the pathogenic fungi. For example, the pathogenic fungi *Blastomyces dermatitidis*, *Histoplasma capsulatum* and *Paracoccidioides brasiliensis* are cysteine prototrophic in the mycelial (non-pathogenic) form, but cysteine auxotrophic in the yeast (pathogenic) form (Boguslawski & Stetler, 1979; Maresca & Kobayashi, 1989; Medoff et al., 1987). Finally, the methionine–cysteine biosynthetic pathway is of interest as a target for novel antifungal drugs, since elements of this pathway are essential for survival in the host or during infection. For example, in *Cryptococcus neoformans*, a causal agent of fungal meningitis, **MET3**, encoding ATP sulfurylase, is required for virulence and survival in vivo (Yang et al., 2002).

These results prompted us to examine other steps in the methionine–cysteine biosynthetic pathway as antifungal drug targets. We chose to examine *C. neoformans* methionine synthase (**MET6**), encoded by the **MET6** gene, for two reasons. First, the fungal Met6p is cobalamin-independent, differing from the human methionine synthase, which is cobalamin-dependent (Banerjee & Matthews, 1990; González et al., 1992). Second, lack of methionine synthase activity not only causes methionine auxotrophy, but it is also expected to lead to the accumulation of homocysteine, reported to be a toxic intermediate that interferes with ergosterol biosynthesis (Hatanaka et al., 1974; McCammon & Parks, 1981; Parks & Casey, 1995). Many antifungal drugs, such as fluconazole, also target the sterol biosynthetic pathway (Sanglard, 2002; Sheehan et al., 1999; White et al., 1998). In addition, there is evidence that drugs targeting ergosterol biosynthesis are fungicidal in combination with calcineurin inhibitors such as FK506 and cyclosporin A (CSA) in *C. neoformans* and *Candida* spp. (Cruz et al., 2000, 2002; Del Poeta et al., 2000; Maesaki et al., 1998; Marchetti et al., 2000a, b, 2003; Onyewu et al., 2003; Sanglard et al., 2003). Therefore, there is the possibility of synergistic interactions between a **met6** mutation and ergosterol and calcineurin inhibitors.

In this work, we describe the identification of the **MET6** gene of *C. neoformans*. We determined the transcriptional profile of the **MET6** gene in response to homocysteine and methionine. We constructed a **met6** mutant in serotype **A** *C. neoformans* and found that the **met6** mutant grew very slowly and lost viability upon methionine starvation. Like a *C. neoformans* **met3** mutant, the **met6** mutant was avirulent in experimental infections. However, unlike the **met3** gene, for two reasons. First, the fungal Met6p is cobalamin-independent, differing from the human methionine synthase, which is cobalamin-dependent (Banerjee & Matthews, 1990; González et al., 1992). Second, lack of methionine synthase activity not only causes methionine auxotrophy, but it is also expected to lead to the accumulation of homocysteine, reported to be a toxic intermediate that interferes with ergosterol biosynthesis (Hatanaka et al., 1974; McCammon & Parks, 1981; Parks & Casey, 1995). Many antifungal drugs, such as fluconazole, also target the sterol biosynthetic pathway (Sanglard, 2002; Sheehan et al., 1999; White et al., 1998). In addition, there is evidence that drugs targeting ergosterol biosynthesis are fungicidal in combination with calcineurin inhibitors such as FK506 and cyclosporin A (CSA) in *C. neoformans* and *Candida* spp. (Cruz et al., 2000, 2002; Del Poeta et al., 2000; Maesaki et al., 1998; Marchetti et al., 2000a, b, 2003; Onyewu et al., 2003; Sanglard et al., 2003). Therefore, there is the possibility of synergistic interactions between a **met6** mutation and ergosterol and calcineurin inhibitors.
mutant, and consistent with the homocysteine accumulation hypothesis, the \textit{met6} mutant was hypersensitive to the antifungal drugs fluconazole, FK506 and CsA, and slightly more resistant to amphotericin B.

**METHODS**

**Strains, media and growth conditions.** All serotype A \textit{C. neoformans} strains constructed and characterized in this study are described in Table 1. \textit{Escherichia coli} DH10B was used as a host for cloning and plasmid propagation. Standard yeast media (Rose et al., 1990), including yeast extract, peptone, dextrose (glucose) (YEPD), synthetic dextrose minimal (SD) and synthetic dextrose complete (SDC) media, were used for the culture of \textit{C. neoformans}. \textit{Escherichia coli} Bertani medium was used to culture \textit{E. coli} strains constructed and characterized in this study are

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Strain & Genotype & Source \\
\hline
H99 & \textit{MATx} (serotype A) & Perfect et al. (1993) \\
TG076 & \textit{MATx met6A::NAT1} (serotype A) & This work \\
TG077 & \textit{MATx met6A+MET6} (serotype A) & This work \\
H99-4 & \textit{MATx met3::URA5 ura5} (serotype A) & Yang et al. (2002) \\
H99-6 & \textit{MATx MET3 ura5+ ectopic URA5} & Yang et al. (2002) \\
\hline
\end{tabular}
\caption{\textit{C. neoformans} strains used in this work}
\end{table}

Nucleic acid manipulations. Plasmid DNA was recovered from \textit{E. coli} using the QIAprep Spin Miniprep kit (Qiagen), following the manufacturer's instructions. Genomic DNA from \textit{C. neoformans} was isolated as described by Hoffman & Winston (1987) with modifications (Yang et al., 2002).

For Southern analysis, \textit{C. neoformans} genomic DNA was digested with various restriction enzymes, subjected to gel electrophoresis on 0.7% (w/v) agarose gels, denatured, and transferred to nylon membranes. Probe preparation was performed according to instructions in the Ready-To-Go DNA kit (Amersham Pharmacia Biotech). Hybridization and signal detection were performed according to instructions in the Bio-Rad Micro Bio-Spin P-30 Tris Chromatography Column Kit.

All PCR primers are described in Table 2. PCR amplification reactions were performed using a PTC-200 Peltier Thermal Cycler (MJ Research). For high-fidelity DNA amplification, MegaFrag DNA polymerase (Invitrogen) was used for its 3'-5' proofreading capabilities. All other DNA amplifications were performed with Taq polymerase (Invitrogen). The PCR programme consisted of one cycle at 94°C for 5 min, 25 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min, and one final extension at 72°C for 10 min was typically used. Variations on the annealing temperature were used as necessary depending on the primer pair.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
Primer & Sequence (5' to 3') \\
\hline
TMG005 & TGAGAATTGGATGAAAAACGAGT \\
TMG006 & TCCAGCTCACAATCCGTGCACGGG- \\
& GTTGAAATATTTGGCCGAGCA \\
TMG007 & GCCGTCAGGAAAGGAGCTGACC \\
TMG009 & TGCCTGCGAAAGATTGGAAACCGG- \\
& CTGCGAGATGTGACGTGAGGCA \\
TMG011 & CACCTGCGGTATTATATAAACA \\
TMG012 & GGCAATACAGATGGATCTGCA \\
TMG013 & AAGTTGTGATGTCACATCCCGG- \\
& AAGTAGATGAAACTAG \\
TMG014 & CATGTTTCTCACATCTCCGCCG- \\
& GTAGTTGATCTACACGAACTT \\
JO280 & CTGTCAGTCTAGTGGATGCTCC \\
JO281 & TCTGAAACCGGGAGACTGAT \\
JO223 & ATGGTGCTCAGGAGTTGGAAT \\
JO225 & AGCTGAAAGGCTTGGACAAAG \\
\hline
\end{tabular}
\caption{Primer list}
\end{table}

Deletion of \textit{C. neoformans} \textit{MET6}. First, an insertion–deletion construct was generated by fusion PCR (Davidson et al., 2002) in which a region containing part of the \textit{MET6} coding region was replaced with the \textit{NAT1} cassette (McDade & Cox, 2001). The initial round of PCR reactions amplified a 1 kb fragment upstream of the \textit{MET6} partial coding region using primers TMG005 and TMG006, and a 1 kb fragment downstream of the \textit{MET6} partial coding region using primers TMG007 and TMG014. These PCR fragments were generated using \textit{C. neoformans} H99 chromosomal DNA as template. Also, a 1-6 kb product containing the \textit{NAT1} marker cassette was amplified from template pGM200 using primers TMG009 and TMG013. The products were gel purified and used as the templates for the final fusion reaction, using primers TMG005 and TMG007, to yield the 3-6 kb \textit{met6A::NAT1} insertion–deletion construct which was cloned into pCR2.1-TOPO (pCRP19).

Second, the \textit{met6A::NAT1} insertion–deletion construct was amplified from pCRP19 using primers TMG005 and TMG007, and introduced into \textit{C. neoformans} H99 by biolistic transformation (Toffaletti et al., 1993) using a Bio-Rad model PDS-1000/He biolistic particle delivery system. Transformants were incubated on YEPD agar plates supplemented with 1 M sorbitol at 30°C overnight; cells were then scraped off and replated onto YEPD+nourseothricin (Nat, 100 μg ml⁻¹) medium. Nat-resistant transformants were purified and screened for inability to grow on synthetic medium lacking methionine. Replacement of the \textit{MET6} gene by the targeting allele was confirmed by PCR and Southern hybridization analysis. The resulting \textit{met6A::NAT1} mutant (TG076) was selected for use in further experiments.

Reconstitution of the \textit{C. neoformans} \textit{met6} mutation. A 3-8 kb region containing the \textit{C. neoformans} serotype A \textit{MET6} gene was amplified using MegaFrag DNA polymerase and primers TMG011 and TMG012. The \textit{MET6}-containing PCR product was introduced into TG076 (\textit{met6A::NAT1}) by biolistic transformation. \textit{Met}⁺ transformants were selected on SD medium containing 1 M sorbitol and lacking methionine. Purified \textit{Met}⁺ transformants were tested for the loss of the \textit{Nat} marker by assaying growth on YEPD+Nat. Replacement of the \textit{met6A::NAT1} allele by the wild-type allele was confirmed by PCR and Southern blot analysis; one of the reconstituted \textit{Met}⁺ strains thereby generated, TG077, was used for further analysis.
Growth rate. A single colony of each strain was inoculated into 5 ml YEPD and grown overnight at 30 °C in a roller drum. The overnight cultures were diluted and cells were counted in a Neubauer counting chamber. A total of 1 × 10⁶ cells ml⁻¹ of each strain was inoculated in 50 ml YEPD in a 250 ml flask. The cultures were incubated at 30 °C with rotation of 250 r.p.m. for up to 24 h. Aliquots of each strain, which were collected every 2 h for the first 10 h, with a final aliquot collected at 24 h, were diluted and plated onto YEPD agar plates to calculate the number of c.f.u. Growth rate experiments were performed in triplicate.

Spot dilutions were also used to assay growth in medium containing different sulfur and nitrogen sources. For these assays, the overnight cultures were washed twice in sterile distilled water, the cells were counted, and the culture was diluted to 1 × 10⁶ cells ml⁻¹ and serially diluted; 5 μl of each serial dilution (10⁻¹ to 10⁻⁵) was spotted onto plates containing 200 μM methionine, 5 mM homocysteine or 200 μM cysteine, with 38 mM NH₄(SO₄)₂ or 43 mM proline as the nitrogen source. The plates were incubated at 30 °C for 72 h.

Northern analysis. First, a single colony of the prototrophic strain H99 was inoculated into 5 ml SD medium and grown overnight at 30 °C. Cells were collected by centrifugation, washed twice in sterile distilled water, and counted in a Neubauer chamber. H99 cells were then inoculated to an initial concentration of 1 × 10⁶ cells ml⁻¹ into 50 ml volumes of the following: (i) SD, (ii) SD plus 200 μM methionine, (iii) SD plus 2 mM homocysteine, and (iv) SD plus 5 mM homocysteine. These cultures were grown for 6 h at 30 °C, rotating at 250 r.p.m. After 6 h, cells were harvested by centrifugation, washed once in diethyl-pyrocarbonate-treated water and the RNA was extracted by using Trizol (Invitrogen) as described previously (Yang et al., 2002). A total of 5 μg RNA was loaded per lane, transferred to nitrocellulose filters and hybridized, as described by Sambrook et al. (1989). The probes for detecting transcripts of MET6, MET3 and GPD (the glyceraldehyde-3-phosphate dehydrogenase gene) were generated by radiolabelling gel-purified, PCR-amplified DNA with [(α-³²P)dCTP and the RediPrimeII labelling kit, according to the manufacturer’s instructions (Amersham Pharmacia Biotech). The PCR-generated DNAs were amplified with the gene-specific primers described in Table 2 (TMG005 and TMG007 for MET6, ZY127 and ZY128 for MET3, and J0223 and J0225 for GPD), and H99 genomic DNA as the template. The signal was detected by exposure of the blot to a phosphoscreen, which was scanned using a Typhoon 9200 Variable Mode Imager (Molecular Dynamics), and band intensity was quantified using ImageQuaNT 5.2 software (Molecular Dynamics).

Melanin production assay. Melanin synthesis was determined as described by Kwon-Chung & Bennett (1992). Briefly, 5 ml YEPD was inoculated with a single colony of each strain that was then grown at 30 °C. The overnight cultures were washed twice in sterile water, diluted, and the cells were counted in a Neubauer chamber. A total of 10 μl of a cell suspension containing 1 × 10⁶ cells ml⁻¹ from each strain was spotted onto Niger seed agar plates (Kwon-Chung & Bennett, 1992) supplemented with 200 μM methionine, 5 mM sodium MOPS and 200 μM methionine, or 10 % (v/v) heat-inactivated fetal bovine serum (Sigma) in phosphate-buffered saline (PBS). The strains were incubated in conical tubes, tightly closed to decrease aeration, at 30 °C for 48 h for incubation in supplemented DMEM medium, or at 37 °C for 24 h for incubation in 10 % (v/v) serum. Capsule formation was assayed by mixing 10 μl cell suspension with 10 μl standard Indian ink and spotting the mixture onto a slide. The slide was covered with a coverslip and examined microscopically. To determine the growth or viability of strains following incubation in capsule-inducing conditions, cells were diluted and plated on YEPD medium before and after capsule induction and the numbers of c.f.u. were compared.

Thermotolerance assay. Thermotolerance was assayed as described previously (Yang et al., 2002). Briefly, 5 ml YEPD inoculated with a single colony of each strain was grown overnight at 30 °C. The overnight cultures were washed twice with sterile distilled water, diluted, and the cells were counted in a Neubauer chamber. A total of 1 × 10⁶ cells from each strain was inoculated into 10 ml SD supplemented with 200 μM methionine and incubated for 3 h at 30 °C. After this period, an aliquot was collected from each strain, diluted, and plated onto YEPD agar plates to calculate the number of c.f.u. In parallel, 2 ml of each of the cultures was transferred to 15 ml conical tubes, which were then placed in a 42 °C water bath for 3 h. After this period the heat-treated cells were diluted and plated onto YEPD to determine the number of c.f.u. The thermotolerance experiment was done in triplicate.

Starvation assay. First, 5 ml YEPD inoculated with a single colony of each strain was grown overnight at 30 °C. These overnight cultures were washed twice with sterile distilled water, diluted, and the cells were counted in a Neubauer chamber. A total of 1 × 10⁶ cells ml⁻¹ from each strain was inoculated into SD medium and incubated at 30 °C, with shaking at 250 r.p.m. Aliquots were removed at time zero and every 24 h up to 96 h, and appropriate dilutions were plated onto YEPD to determine the number of c.f.u. The starvation assay experiment was performed in duplicate.

Test of virulence in the murine inhalation model. The murine cryptococcal inhalation model (Cox et al., 2000) was used to assay virulence. Briefly, groups of ten 4- to 6-week-old A/Jcr mice (NCI/Charles River Laboratories) were anaesthetized with pentobarbital and suspended on a silk thread. These mice were then infected via nasal inhalation with the wild-type strain H99, the met6 mutant strain TG076, or the MET6 reconstituted strain TG077. The inoculum (10⁶ cells in a 50 μl volume, prepared from cultures which were pre-grown overnight in YEPD at 30 °C) was slowly administered by pipette into the nares of each mouse, after which the mouse was left suspended for 10 min to ensure inhalation of the inoculum into the lungs. Ten mice were infected with each strain. Mice were fed ad libitum. Mice were examined twice a day, and those that appeared moribund or in pain were sacrificed by CO₂. Survival data were analysed by the Kruskal–Wallis test. The met6-injected mice were sacrificed at 70 days post-infection, and their brains were removed and smeared onto Sabouraud agar containing chloramphenicol for culture.

Fluconazole, FK506, amphotericin B and CsA sensitivity assays. Sensitivity to antifungal drugs was determined for mutant and wild-type strains by the minimal inhibitory concentration (MIC) assay according to Galgiani & Lewis (1997) as modified by Vallim et al. (2004). Briefly, cells from each strain to be assayed were grown overnight at 30 °C in YEPD, washed in sterile distilled water, and diluted to 1–2 × 10⁶ cells ml⁻¹ in YEPD. The assay was performed in 96-well cell culture clusters (flat bottom, Corning) where a 90 μl volume of cells suspended in YEPD was dispensed in each well. The wells contained 10 μl volumes of twofold dilutions of fluconazole (Pfizer), FK506 (Fujisawa), amphotericin B (Sigma) or CsA (Novartis) diluted in YEPD. The concentrations of the compounds ranged from 16 to 0.0625 μg ml⁻¹ (fluconazole), 0–2 to
0·003 µg ml⁻¹ (FK506), 8 to 0·125 µg ml⁻¹ (amphotericin B), and 4 to 0·03 µg ml⁻¹ (CsA). A control with cells and without drugs was included, and a blank containing only YEPD medium was also used. The MIC was defined as the drug concentration in which growth was at least 80% less than the drug-free control following incubation for 72 h at 37°C. Growth was determined by measuring the turbidity of the cell suspension in a Multiskan Ascent (Lab System) at 620 and 540 nm. The experiment was performed in triplicate for fluconazole and amphotericin B, and in duplicate for FK506 and CsA. Once MICs were found, minimum fungicidal concentrations (MFCs) were determined by plating the entire contents from the wells containing the MIC and higher concentrations of drug onto YEPD, and incubating at 30°C for 72 h. MFCs were defined as the minimum concentration of drug in which there was a ≥99% reduction in the number of c.f.u. from the initial inoculum.

RESULTS

Identification of the \textit{C. neoformans} MET6 gene

The \textit{C. neoformans} methionine synthase gene (MET6) was identified by similarity at the predicted amino acid sequence level to the \textit{Saccharomyces cerevisiae} Met6p. The amino acid sequence of the \textit{Sacch. cerevisiae} MET6 gene was used to search the \textit{C. neoformans} serotype A database at the Broad Institute (http://www.broad.mit.edu/annotation/fungi/cryptococcus_neoformans/index.html). The search yielded one contig numbered 1,38 (e-value of −175). This region contained a partial ORF of 1304 bp with high similarity to the methionine synthase sequences of several organisms deposited in GenBank at NCBI, with the highest similarities being found to the \textit{Sacch. cerevisiae} and \textit{Schizosaccharomyces pombe} methionine synthases.

Expression analysis of MET3 and MET6 – homocysteine induces MET6 transcription

We performed Northern analyses to determine the response of both MET3 and MET6 to the presence of exogenous methionine and homocysteine. As shown in Fig. 1, the mRNA levels of the ATP sulfurylase gene MET3 showed a small decrease (0·6-fold) in response to exogenous methionine and a slight induction (2·6- and 2·9-fold) in the presence of 2 and 5 mM homocysteine, respectively. In contrast, we detected a sevenfold increase of MET6 mRNA when cells were grown in the presence of 200 µM methionine, and the amount of MET6 mRNA increased 9·2- and 19·2-fold in the presence of 2 and 5 mM of homocysteine, respectively. These results are consistent with other reports where homocysteine, the substrate of homocysteine, respectively. These results are consistent

Deletion and reconstitution of the \textit{C. neoformans} MET6 gene

Using fusion PCR (Davidson et al., 2002), we made an insertion–deletion construct in which 1304 bp of the MET6 partial ORF was deleted and replaced with the NAT marker (Fig. 2a). By comparing the amino acid translation of the C. neoformans MET6 region against Met6ps of other fungi, the \textit{C. neoformans} MET6 ORF, including introns, was predicted to be 2664 bp. Therefore, by deleting 1·3 kb, we removed approximately 49% of the MET6 ORF.

The met6Δ::NAT1 construct, which had 1 kb of homology flanking both sides of the NAT1-selectable marker to promote recombination with the chromosomal MET6 locus, was introduced into the \textit{C. neoformans} serotype A strain H99 by biolistic transformation (Toffaletti et al., 1993). Transformants were selected on YEPD + Nat. Several Nat-resistant transformants were screened for the inability to grow on SDC minus methionine; those that did not grow on this medium but grew on SDC were tested by PCR and Southern analysis for homologous integration of the deletion cassette at the MET6 locus (Fig. 2b). One of the transformants, which had the expected banding pattern in the confirmation PCR and the expected restriction digestion pattern in the Southern blot, designated TG076 (met6Δ::NAT1), was used for further analysis. The met6Δ::NAT1 allele was also replaced by the MET6 gene by introducing a 3858 bp DNA fragment containing MET6. The selection of the reconstituted strain was made on SDC minus methionine medium, where several Met⁺ colonies grew. Four of these Met⁺ and Nat-sensitive colonies were selected and tested by Southern analysis to confirm the restoration of the wild-type restriction profile (Fig. 2b). One of the MET6 reconstituted strains, designated TG077, was selected and used in further analysis.

The \textit{C. neoformans} met6 mutant is avirulent

Using the murine nasal inhalation model, we determined the virulence phenotypes of the met6, reconstituted and wild-type strains. As shown in Fig. 3, wild-type (H99)-infected
mice survived for a mean of 21 days post-infection (mean survival 21.1 ± 1.9 days; \( P < 0.001 \)), with 100% mortality occurring by day 25 post-infection. Similarly, mice infected with the MET6-reconstituted strain (TG077) survived a mean of 25 days post-infection (mean survival of 25 ± 2.4 days; \( P < 0.001 \)), with 100% mortality occurring on day 30 post-infection. In contrast, all ten mice infected with the met6 mutant (TG076) survived to day 70 post-infection. Therefore, the met6 mutant is avirulent.

At day 70 post-infection, the met6 mutant-infected mice were sacrificed, and their brains were dissected and smeared on Sabouraud agar containing chloramphenicol. Seven of the ten brain smears yielded between 1 and 27 C. neoformans met6 colonies; the mean for all ten mice was 2 ± 8 colonies. These C. neoformans met6 colonies were subcultured and their phenotypes were determined; all were Met2 and Nat resistant, demonstrating that some TG076 cells in fact remained viable in the brains and were not contaminants. However, all of the mice were healthy.

**C. neoformans met6 melanin production**

The biosynthesis of melanin, an important virulence factor in C. neoformans, was tested in the met3 and met6 mutants on Niger seed agar medium supplemented with 200 μM methionine. After 24 h of growth at 30 °C, there was little melanin production by the met3 or met6 mutants. However, after 48 h, the met3 and met6 mutants produced as much melanin as the reconstituted (TG077) and wild-type (H99)
strains in a qualitative plate assay (data not shown). Consistent with our previously published met3 results (Yang et al., 2002), we conclude that the delay in melanin production is indirect, and likely to be due to the slow growth of the met3 and met6 mutants, rather than to a direct effect on the melanin biosynthetic pathway.

**Capsule production by C. neoformans met6 mutant**

Capsule production is an important virulence factor in C. neoformans (Bulmer et al., 1967; Fromtling et al., 1982; Kwon-Chung & Rhodes, 1986). We tested capsule formation for met6 (TG076), met3 (H99-4), reconstituted (TG077 and H99-6) and wild-type (H99) strains in DMEM supplemented with 22 mM NaHCO3, 25 mM sodium MOPS and 200 μM methionine, as well as in 10% heat-treated fetal bovine serum in PBS buffer. The met3 mutant, the MET3 and MET6 reconstituted strains, and the wild-type all produced capsule to a similar extent in both capsule-inducing conditions. However, in contrast to the met3 mutant, the met6 mutant produced little or no visible capsule (Fig. 4). Determination of the number of c.f.u. after 48 h incubation in supplemented DMEM medium revealed that the met6 mutant did not grow in this medium and, in fact, exhibited a slight reduction in the number of c.f.u. (data not shown). Supplementation of the capsule induction medium with additional methionine did not increase growth of the met6 mutant. However, incubation of the met6 mutant for 24 h in fetal bovine serum and PBS buffer did not result in any loss of viability. Therefore, the met6 mutation results in substantially reduced capsule formation.

**Growth of met3 and met6 mutants with different sulfur and nitrogen sources**

Our analysis of C. neoformans ilv2 and spe3-lys9 mutants showed that satisfaction of auxotrophy is highly influenced by nitrogen source (Kingsbury et al., 2004a, b), consistent with uptake of free amino acids, peptides and spermidine all being subject to nitrogen repression. Therefore, we compared the growth of met3 and met6 mutants on medium containing ammonium sulfate versus proline as the sole nitrogen source. In addition, we determined the ability of met3 and met6 mutants to satisfy their auxotrophic requirements with methionine, cysteine and homocysteine.

As shown in Fig. 5, the met3 mutant can satisfy its auxotrophic requirement with methionine, cysteine or homocysteine. The met3 mutant showed substantially better growth when proline was the sole nitrogen source, suggesting that uptake of these amino acids is subject to nitrogen repression.

In contrast, while it can satisfy its auxotrophy with methionine, the met6 strain (TG076) cannot satisfy its auxotrophy with either cysteine or homocysteine, similar to Sacch. cerevisiae met6 (Thomas & Surdin-Kerjan, 1997). Also in contrast to met3, the met6 mutant did not exhibit

![Fig. 4. Capsule production by H99 (wild-type), TG076 (met6) and H99-4 (met3) following incubation in 10% (v/v) fetal bovine serum and PBS buffer for 24 h at 37 °C. Capsule production by the MET3- and MET6-reconstituted strains was indistinguishable from wild-type capsule production (data not shown).](image-url)
better growth on medium containing proline as the sole nitrogen source. This result suggests that the poor growth of \textit{met6} mutant in synthetic medium supplemented with methionine is not due to low methionine uptake. Instead, the poor growth of the \textit{met6} mutant may be due to the accumulation of the toxic intermediate homocysteine, which cannot be converted to methionine in methionine-synthase-deficient (\textit{met6}) mutants.

\textbf{\textit{met3} and \textit{met6} mutant starvation phenotypes}

The \textit{met3} and \textit{met6} methionine mutants, the reconstituted strains and the wild-type were grown in SD without methionine supplementation for up to 96 h at 30°C. At time zero and at every 24 h, samples of each strain were collected, diluted, and plated onto YEPD for c.f.u. counts. Both reconstituted and wild-type strains grew and reached the stationary phase of growth by 24 h (data not shown).

The number of \textit{met3} mutant c.f.u. increased to about 150 ± 4.9 \% (mean ± SD, \(n = 3\)) of the initial c.f.u. count, and it remained at this level for up to 96 h without loss of viability. The \textit{met6} mutant showed a slight increase in the number of c.f.u. in the first 24 h (117 ± 8 \%). However, after 24 h, the \textit{met6} mutant lost viability, and by 96 h the number of c.f.u. declined to 58 \% (± 9.8 \%) of the initial c.f.u. count. Therefore, in contrast to the cytostatic effect of methionine starvation on the \textit{met3} mutant, methionine starvation of the \textit{met6} mutant is cytoidal.

\textbf{Thermotolerance of the methionine-auxotrophic mutants}

We previously showed that \textit{C. neoformans} and \textit{Sacch. cerevisiae} \textit{met3} mutants are highly thermotolerant (Yang \textit{et al.}, 2002). The \textit{met6} and \textit{met3} mutants, reconstituted strains and wild-type were heat shocked at 42°C for 3 h, and the viability of the cells was assayed by determining the c.f.u. counts of heat-shocked cells versus those of non-heat-shocked cells. Consistent with our previous results (Yang \textit{et al.}, 2002), we observed a high degree of thermotolerance for the \textit{met3} mutant; the \textit{met3} mutant had a mean survival of 105 ± 10 \% (mean ± SD, \(n = 3\)) after heat shock, compared with the reconstituted and wild-type strains (4 ± 5 \% and 2 ± 0.2 \%). The \textit{met6} mutant was also thermotolerant (48 ± 10 \%) compared with its reconstituted strain (1 ± 6 ± 0.4 \%). However, the \textit{met6} mutant was significantly less thermostolerant than the \textit{met3} mutant.

\textbf{\textit{C. neoformans} \textit{met6} mutant is hypersensitive to fluconazole, FKS06 and CsA}

Previous reports indicated that homocysteine and/or \textit{S}-adenosylhomocysteine inhibit sterol biosynthesis (Hatanaka \textit{et al.}, 1974; McCammon & Parks, 1981; Parks & Casey, 1995). Therefore, since a \textit{met6} mutant is predicted to accumulate homocysteine, we tested the sensitivity of \textit{met6}, \textit{met3}, reconstituted and wild-type strains to the sterol synthesis inhibitor fluconazole.
We determined the MIC_{80} of the five strains for a range of fluconazole concentrations (16–0.0625 μg ml\(^{-1}\)) at 37 °C in YEPD for 72 h. The choice of YEPD was based on the fact that the met6 mutant does not grow in cell culture media such as DMEM or RPMI (data not shown), which are more commonly used in micro-dilution tests (Ghannoum et al., 1992). We found that 4 μg fluconazole ml\(^{-1}\) inhibits the growth of the met3 mutant, both the reconstituted strains and H99. This MIC result is similar to those previously reported for H99 (6.25 μg ml\(^{-1}\) in RPMI at 30 °C, Del Poeta et al., 2000; 2 μg ml\(^{-1}\) in RPMI at 37 °C, Vallim et al., 2004). However, consistent with our hypothesis, the met6 mutant was two- to fourfold more sensitive to fluconazole, with an MIC of 1–2 μg ml\(^{-1}\). The met6 mutant also had a lower fluconazole MFC (4 μg ml\(^{-1}\)) compared with the met3, wild-type and reconstituted strains (8 μg ml\(^{-1}\)).

Others have shown that calcineurin mutants and calcineurin inhibitors exhibit synergistic and cytocidal interactions with sterol synthesis inhibitors (Cruz et al., 2000, 2002; Del Poeta et al., 2000; Marchetti et al., 2000a, b, 2003; Odom et al., 1997a, b; Onyewu et al., 2003). Since the met6 mutant may have a sterol synthesis defect, we determined the MICs of the met6 mutant, the met3 mutant and H99 for the calcineurin inhibitor FK506. A range (0–2–0.003 μg ml\(^{-1}\)) of twofold serial dilutions of FK506 was tested for met6, met3 and H99 in an MIC assay at 37 °C. The wild-type (H99) and met3 mutant were both inhibited by ~0.05 μg FK506 ml\(^{-1}\). However, the met6 mutant was four times more sensitive to FK506 (~0.0125 μg ml\(^{-1}\)). We performed a viability test to check the cytostatic versus cytocidal effect of FK506 on the met6 mutant, and we found that at the met6 MIC, FK506 is cytocidal.

Under the rationale that CsA also inhibits calcineurin by binding to cyclophilin A, we tested a range of CsA concentrations (4–0.03 μg ml\(^{-1}\)) for met6, met3 and wild-type strains, predicting that the met6 strain would be hypersensitive to this drug, similar to its sensitivity to FK506. As predicted, the met6 mutant was two to four times (~0.25 μg ml\(^{-1}\)) more sensitive to CsA than the met3 and the wild-type H99 strains (~0.5 and 1 μg ml\(^{-1}\), respectively), which is comparable to the MIC determined by Cruz et al. (2000) for the serotype A H99 strain (0.39 μg ml\(^{-1}\)). The CsA MFC coincided with the MIC for all three strains tested.

Any reduction in sterol synthesis by the met6 mutant may result in an increase in resistance to the ergosterol-binding drug amphotericin B. Therefore, we also compared the sensitivity of the met3, met6 and wild-type strains to amphotericin B. While the MICs of this drug were the same for each strain (0.25 μg ml\(^{-1}\)), the amphotericin B MFC for the met6 mutant was reproducibly twice as high as for the met3 and wild-type strains (0.5 μg ml\(^{-1}\) versus 0.25 μg ml\(^{-1}\)). Therefore, the met6 mutant was modestly more resistant than the met3 and wild-type strains to amphotericin B.

**DISCUSSION**

In this study we identified the *C. neoformans* methionine synthase gene and characterized a *C. neoformans* met6 mutant with the purpose of evaluating a novel antifungal drug target. We chose to examine *MET6* for two reasons. First, there are two types of methionine synthases: (i) those that are cobalamin (vitamin B\(_{12}\))-dependent, such as human methionine synthase, and (ii) those that are cobalamin-independent, such as the fungal methionine synthases (Banerjee & Matthews, 1990; González et al., 1992; Thomas & Surdin-Kerjan, 1997). In *E. coli*, which contains both cobalamin-dependent (*metH*) and cobalamin-independent (*metE*) methionine synthases, a comparison of the MetE and MetH amino acid sequences revealed no sequence similarity, consistent with their having evolved independently (González et al., 1992). Consistent with the two types of methionine synthase having evolved independently, the cobalamin-dependent methionine synthases from *E. coli* and *Homo sapiens* (GenBank accession numbers AAC43113 and Q99707, respectively) were not similar to anything in the *C. neoformans* sequence database; that is, there was no similarity with *C. neoformans* Met6p. However, the cobalamin-independent methionine synthase from *E. coli* (GenBank accession number AAA23544) was highly similar to *C. neoformans* Met6p (e value −171). Such functional (co-factor requirements) and sequence differences between human versus fungal gene products are highly desirable in antifungal drug targets because they enhance the likelihood of selective toxicity; that is, the creation of drugs that affect the pathogen but not the host. The search for such targets is very important and has been especially challenging for targets affecting the eukaryotic pathogenic fungi (Barrett, 2002; Debono & Gordee, 1994).

The second reason for choosing to examine methionine synthase in *C. neoformans* is that the absence of methionine synthase not only causes growth inhibition in the absence of methionine, but also causes the accumulation of the toxic metabolic intermediate homocysteine in *A. nidulans* and *Sacch. cerevisiae* (Jakubowski, 2002; Marzluf, 1997; Pieniazek et al., 1973; Thomas & Surdin-Kerjan, 1997). Homocysteine accumulation may have multiple deleterious effects on the cell, such as the formation of highly reactive homocysteine thiolactone (Jakubowski, 1991, 2002, 2004) and ergosterol biosynthesis inhibition (Hatanaka et al., 1974; McCammon & Parks, 1981; Parks & Casey, 1995); such deleterious effects are desirable for an antifungal drug target. Therefore, we compared several phenotypes of met3 and met6 mutants. Since the met3 mutant is deficient in the first step of sulfate assimilation and does not accumulate any intermediate, this comparison allowed us to differentiate between phenotypes caused by methionine auxotrophy (both met3 and met6) versus homocysteine accumulation (met6 only). We also tested for synergistic interactions between antifungal drugs and the methionine-auxotrophic mutants to evaluate the potential of Met6p as an antifungal drug target, as well as determining the
transcriptional response of MET6 to homocysteine and methionine.

**MET6 transcription is induced by homocysteine and methionine**

One of the differences between the MET3 and MET6 genes is the transcriptional induction of MET6 by methionine and homocysteine. In this study we found a low transcript level for MET3, which was only slightly affected by exogenous methionine or homocysteine, consistent with our previous results (Yang et al., 2002). In contrast, we found that MET6 has a very strong transcriptional response in the presence of homocysteine, which can be seen as increasing levels of this compound are added to the medium (Fig. 1). This is consistent with the results found in other organisms in which homocysteine induces the methionine synthase gene (Kacprzak et al., 2003). The strong induction may not only be due to the availability of the substrate, but may also be interpreted as a way to efficiently detoxify homocysteine (Kacprzak et al., 2003).

When cells were grown in the presence of methionine, we observed an unexpected sevenfold increase in MET6 mRNA levels. The induction of MET6 mRNA levels by methionine suggests that MET6 transcription may be induced directly by methionine; however, induction by methionine would mean that the product of the Met6p reaction (methionine) would be inducing transcription, which would be unusual. Alternatively, increased levels of methionine may lead to increased levels of a methionine metabolite, such as homocysteine, which would in turn induce MET6.

**The role of MET3 versus MET6 in C. neoformans**

We constructed a met6 mutant and a reconstituted strain and compared the phenotypes of the *C. neoformans met6* and met3 mutants. Our results showed that the met6 mutant grows more slowly than the met3 mutant and, in contrast to met3, the met6 auxotrophy can only be satisfied by methionine, similar to *Sacch. cerevisiae met6*. Also, we found that the uptake of methionine and cysteine can be improved by the use of proline as the nitrogen source; this provides more robust growth for the met3 mutant, but not for the met6 mutant. The met3 and met6 mutants were both resistant to heat shock, although the met3 mutant was significantly more resistant than the met6 mutant. Regarding survival during methionine starvation, our results showed that the met3 mutant maintains viability more efficiently than the met6 mutant, which loses viability over time. Our results suggest that the more severe (relative to met3) phenotypes of the met6 mutant are unlikely to be due to the methionine auxotrophy per se, but instead are likely to be due to the accumulation of homocysteine.

**MET6 is essential for virulence**

Our virulence studies showed that the met6 mutant is avirulent in the inhalation mouse model. While the mice infected with the reconstituted strain (TG077) and the wild-type (H99) died between days 21 and 25, none of the mice infected with met6 mutant (TG076) died at up to 70 days post-infection, indicating avirulence. This result is similar to that observed for met3 mutant, which was completely avirulent in the mouse inhalation model (Yang et al., 2002).

Both met6 and met3 mutants showed a delay in melanin production *in vitro*, consistent with our previous results (Yang et al., 2002), which is likely to be due to the slow growth of both mutants. However, since melanin production is essential for virulence *in vivo* (Alspaugh et al., 2002; Gàomez & Nosanchuk, 2003), the delayed melanin formation of met3 and met6 mutants may be relevant for *in vivo* virulence. The met6 and met3 mutants differed in the production of capsule, another important virulence factor (Bulmer et al., 1967; Fromtling et al., 1982; Kwon-Chung & Rhodes, 1986). While the met3 mutant produced a similar amount of capsule to the wild-type, the met6 mutant produced no appreciable capsule in two types of capsule induction medium. Based on our met3 and met6 results, we conclude that methionine biosynthesis is critical for *C. neoformans* virulence. Based on the more severe phenotypes of the met6 mutant, we suggest that homocysteine accumulation may be deleterious both *in vitro* and *in vivo*.

**The met6 mutant is hypersensitive to antifungal drugs**

We presented evidence that the met6 mutation is more deleterious than the met3 mutation in *C. neoformans*, which is likely to be due to homocysteine accumulation. Also, in our studies we found a synergistic effect between the met6 mutation and fluconazole, which is important from the perspective of antifungal drug targets. The fact that the met3 mutant was as sensitive to fluconazole as the wild-type and reconstituted strains excludes the possibility that the hypersensitivity of the met6 mutant may be due to the generally slower growth rate of methionine-auxotrophic mutants or to methionine limitation. Therefore, this result indicates that the hypersensitivity is specific to the met6 mutant and that it is likely to be due to the synergistic action of homocysteine accumulation and fluconazole on ergosterol biosynthesis. Consistent with homocysteine accumulation resulting in reduced ergosterol biosynthesis in the met6 mutant, we found that the met6 mutant was slightly more resistant than the met3 mutant and the wild-type to amphotericin B. Since this drug destroys membrane integrity by binding ergosterol, any reduction in ergosterol concentration decreases the amount of amphotericin B binding, and hence results in increased drug resistance.

In a search for additional synergistic fungicidal interactions, we tested for and observed synergism between the met6 mutation and the calcineurin inhibitors FK506 and CsA. The observed synergisms are calcineurin- and met6-specific, with fourfold lower MIC values for FK506, and two- to fourfold lower MIC values for CsA, for the met6 mutant. These FK506 and CsA synergistic interactions were
fungal drug target. These reasons, methionine synthase is an attractive anti-
fluconazole, as well as calcineurin inhibitors. For all of synthase) inhibitor would interact synergistically with avirulent. Our data also suggest that a Met6p (methionine deficient in capsule formation (a key virulence factor), and is target. The synthase of C. neoformans spp. and Candida combination with calcineurin inhibitors (Cruz et al., 2000).

As the immunocompromised population increases, there is continuous need for new compounds to inhibit C. neoformans, as well as calcineurin inhibitors. For all of these reasons, methionine synthase is an attractive anti-fungal drug target.

ACKNOWLEDGEMENTS

We thank M. A. Vallim for assistance with C. neoformans biolistic transformation and MIC determination as well as helpful suggestions and discussions, J. A. Alspaugh for assistance with CaA MIC, and R. Marra for kindly supplying Niger seed agar plates. We also thank J. Heitman for critical reading of the manuscript. This work was funded by NIH grant PO1-AI4975.

REFERENCES


sulfometuron methyl and is required for survival at 37 °C and in vivo. Microbiology 150, 1547–1558.


