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The Gene for Cobalamin-independent Methionine Synthase is Essential in *Candida albicans*: A Potential Antifungal Target

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Abstract

Methionine synthase catalyzes the transfer of a methyl group from tetrahydrofolate to homocysteine to produce methionine. Although mammalian enzymes are cobalamin-dependent, fungal methionine synthases are cobalamin-independent. The opportunistic pathogen *Candida albicans* is a diploid and carries two copies of the methionine synthase gene, *MET6*. Homologous recombination was used to disrupt a single *MET6* gene. *MET6/met6* knock-outs, deleted with either the *URA3* or *ARG4* cassette, grew as well as the wild-type strain. However, we were unable to obtain a viable *met6/met6* deletion strain, even on media supplemented with exogenous methionine. This suggests that methionine synthase is essential to *C. albicans*. To explore this further, a *C. albicans* strain was constructed in which one *MET6* locus was deleted and the second placed under a regulatable promoter. The conditional mutant grew well under inducing conditions, even in the absence of methionine. It would not grow under repressing conditions in the absence of methionine, but would grow when the media was supplemented with exogenous methionine. A western blot showed that a small amount of enzyme was expressed under repressing conditions. Taken together, these data reveal that methionine is necessary for growth of *C. albicans*, but not sufficient – a minimal level of methionine synthase expression is required, perhaps to limit homocysteine toxicity. Furthermore, these results suggest that cobalamin-independent methionine synthase is a plausible target for the design of antifungal agents.

Keywords

Methionine synthase; gene disruption; *Candida albicans*; cobalamin-independent; *MET6* gene; homologous recombination; conditional mutant

INTRODUCTION

Methionine synthases transfer a methyl group from 5-methyl-tetrahydrofolate to homocysteine, forming methionine. This is the last step in methionine biosynthesis, and is the point where the one-carbon metabolism pathway converges with the methionine metabolism pathway (Figure 1). There are two classes of methionine synthases, which do not share any sequence homology [1,2]. Cobalamin-dependent methionine synthases belong to one class; this form is present in humans, and other organisms that are able to obtain or synthesize cobalamin. These are very large proteins, about 140 kDa, with four distinct structural domains

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which function in binding homocysteine (Hcy), $\text{CH}_3\text{-H}_4\text{PteGlu}_n$, cobalamin, and AdoMet respectively [3]. X-ray structures of several of these domains are known [4–6].

Cobalamin-independent methionine synthases represent the second class, which are found in fungi (including yeast), higher plants, and some prokaryotes. The cobalamin-independent enzymes have a molecular mass around 86 kDa; X-ray structures of the enzymes from *Arabidopsis thaliana* [7] and from the thermophilic bacteria *Thermotoga maritima* [8] have recently been reported. Steady state kinetic analyses of the cobalamin-independent enzymes from *Saccharomyces cerevisiae* and *Candida albicans*, and detailed mechanistic studies on the *E. coli metE* enzyme, have recently been reported (Suliman *et al.*, 2005; Taurog *et al.*, 2006; Taurog & Matthews, 2006). The structural and mechanistic differences between methionine synthases from humans and those from fungi and yeast make the cobalamin-independent methionine synthase a potentially attractive antifungal drug target.

C. albicans is a major fungal pathogen, and is responsible for nosocomial infections in immunocompromised patients [9]. In recent years, there has been a steady increase in *C. albicans* infections due to the increase in immunocompromising diseases such as AIDS, and the increase in hospital procedures such as organ transplants. Toxicity of present drug therapies, and the increase in drug resistance, prompt the need to find new antifungal therapies.

In this study we describe the construction of a conditional cobalamin-independent methionine synthase (*MET6*) mutant in *C. albicans*, using a PCR-based gene disruption method. We also describe the phenotype of the resulting mutant, which reveals that *MET6* is essential. The findings from these experiments support the study of *MET6* as a potential antifungal drug target, and provide the basis for future analysis of its gene function.

MATERIALS AND METHODS

Strains, media, and plasmids

The *C. albicans* strain BWP17 (*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*), and the plasmids pGEM-URA3 and pRS-ARG4ΔSpeI were generously provided by Dr. Aaron Mitchell [10]. The plasmid pURA3-PGAL1-GFP was obtained from Dr. Judith Berman [11]. Cultures were grown at 30°C in YPD + Uri for routine nonselective growth, or YMD medium for selective growth. YPD + Uri medium consisted of 10 g yeast extract, 20 g peptone, 20 g glucose, and 80 mg of uridine per liter. YMD medium consisted of 6.7 g/L yeast nitrogen base without amino acids and 20 g/L glucose, and was supplemented with the necessary auxotrophic requirements including: 80 mg/L uridine, 20 mg/L L-histidine, 20 mg/L L-methionine, and 40 mg/L L-arginine. To induce the *GAL1* promoter, glucose was replaced with 20 g/L galactose. Agar plates contained 20 g/L agar.

MET6 gene deletion

A PCR-based gene disruption method [10] was used to attempt to create a *met6/met6* deletion mutant strain. The 80-mer primers used are listed in Table 1. The CaMET6-5DR and CaMET6-3DR primers were designed so that 20 nucleotides from each bind to either pRS-ARG4ΔSpeI or pGEM-URA3 plasmids, amplifying an ARG4 or a URA3 cassette, respectively. The remaining 60 nucleotides are homologous to the sequences that flank the *MET6* open reading frame (ORF). The deletion cassettes were amplified in PCR reactions containing 0.1 ng of pGEM-URA3 or pRS-ARG4ΔSpeI, 0.4 μM of each primer (CaMET6-5DR and CaMET6-3DR), 1X KOD polymerase PCR buffer (Novagen), 0.4 mM dNTPs, 1.5 mM MgCl_2 , and 1 U KOD Hot Start DNA polymerase (Novagen). The mixture was incubated at 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 sec, 50 °C for 1 min, and 72 °C for 3 min. After a final extension at 72 °C for 8 min, 5 μL of each sample was examined on a 0.8% agarose

gel to confirm the presence of the expected product. The remainder of each reaction was ethanol precipitated and resuspended in 5 μ L of TE buffer (10 mM Tris HCl pH 7.5, 1 mM EDTA pH 8.0), without further purification. The samples were prepared for transformation by adding 10 μ g of Herring-Testes DNA (Sigma) to the PCR products.

Overnight cultures of BWP17 were diluted 100-fold in 50 mL of YPD + Uri media, and incubated at 30 °C with shaking for four generations (approximately 6 hours). Cells were pelleted and washed with 5 mL sterile water, and suspended in 0.5 mL TELiOAc (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, and 100 mM Lithium Acetate). 100 μ L aliquots of cell suspension were dispensed into microcentrifuge tubes containing PCR product and Herring-Testes DNA, and incubated at room temperature for 30 minutes. Then, 0.7 mL of PLATE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 100 mM Lithium Acetate, 40% Polyethylene Glycol 3350) was added to each tube, mixed, and incubated at room temperature overnight (approximately 16 hours). The next day, the mixtures were heat-shocked for 1 hour at 42 °C. Cells were pelleted in a microcentrifuge for 30 seconds, suspended in 0.1 mL of sterile water, and plated on selective media. The plates were incubated for 3–5 days at 30 °C until transformants appeared.

Genomic DNA isolation and identification of integration events

Overnight cultures were grown in 3 mL selective media and the cells were spun down in a microcentrifuge, for 5 minutes. The cells were washed in 1.0 mL sterile water, and then suspended vigorously in 0.4 mL TENTS (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 100 mM NaCl, 2% Triton X-100, 1% Sodium Dodecyl Sulfate). 0.2 mL of 0.45 mm glass beads and 0.4 mL phenol-chloroform were added and the tubes were vortexed for 2 minutes. The tubes were then spun for 10 min at $15,000 \times g$ in a microcentrifuge. The upper aqueous layer was pipetted into a new tube, and 1 mL of ethanol was added. Nucleic acid was precipitated and pelleted in a microcentrifuge at 4 °C for 5 minutes. The pellet was dissolved in 200 μ L of TE containing 10 μ g/mL RNase A, and incubated at room temperature for 30 min. The DNA was then ethanol precipitated, and dissolved in 100 μ L TE. Analytical PCRs were carried out as previously described, except that 2 μ L of genomic DNA served as the template. The genomic DNA from the *URA3* cassette transformations was screened using the FL-5DR and FL-3DR primers, which annealed to the target gene locus outside of the altered region. The genomic DNA from the *ARG4* cassette transformations was screened using the ARG-4-1020-5DR primer, which annealed within the *ARG4* cassette, and FL-3DR primer, which annealed outside of the altered region.

Construction of the conditional *MET6* mutant

The Berman Laboratory has created plasmids containing cassettes for PCR-mediated construction of regulatable genes [11]. The plasmid pURA3-PGAL1-GFP contained a cassette composed of a *URA3* selection marker, a *GAL1* regulatable promoter, and a GFP gene. For our experiments the GFP tag was not needed, and only the portion of the cassette that contained the *URA3* selection marker and the *GAL1* regulatable promoter was used; this cassette was labeled *P_{GAL1}*. To integrate *P_{GAL1}* upstream of *MET6*, a forward primer was designed to include 70 nucleotides from the sequence immediately upstream of –15 from the *MET6* start codon, and 23 nucleotides that bind on the *P_{GAL1}* cassette (GAL1-5DR, Table 1). The reverse primer was designed to include the reverse complement of nucleotides 1-74 of the *MET6* ORF, and the reverse complement of 18 nucleotides that bind on the *P_{GAL1}* cassette (GAL1-3DR, Table 1). The *P_{GAL1}* cassette was amplified in PCR reactions containing 200 ng stock of plasmid pURA3-PGAL1-GFP, 0.4 μ M stock of each primer (GAL1-5DR and GAL1-3DR), 1X KOD polymerase PCR buffer (Novagen), 0.4 mM dNTPs, 3 mM MgCl₂, 20 μ g BSA, and 1 U KOD Hot Start DNA polymerase (Novagen). The mixture was incubated at 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 sec, 50 °C for 1 min, and 68 °C for 3 min. After a

final extension at 68 °C for 8 min, the PCR reactions were checked by fractionating 5 µL of each sample on a 0.8% agarose gel. The remainder of each reaction was ethanol precipitated and resuspended in 5 µL of TE without further purification. Ten of the samples were combined and used to transform the *MET6/met6* mutant that was previously deleted with the *ARG4* selection marker. Genomic DNA from the Arg+Uri+ transformants was screened using the ARG4-1020-5' primer, which annealed within the *ARG4* cassette, and the FL-3DR primer, which annealed outside of the altered region, to test for the presence of the *ARG4* cassette. The transformants were also screened using the URA3-517-5DR, which annealed within the *URA3* portion of the *P_{GALI}* cassette, and the FL-5DR primer, which annealed outside of the altered region, to test for the presence of the *P_{GALI}* cassette. Further screening reactions were set up with the FL-5DR and FL-3DR, which both anneal outside the altered region.

Characterization of *C. albicans* Met6p antibodies

Polyclonal antibodies were raised against purified CaMet6p protein [19] in rabbits at M.D. Anderson Cancer Center (Bastrop, TX). The antibodies were characterized using wild-type (DAY4 – a *ser1 ura3-52 trp1 leu2 his4*) and *met6* mutant (SDYa – a *ser1 ura3-52 trp1 leu2 his4 met6::URA3*) strains of *S. cerevisiae*. DAY4 and SDYa cells [19] were grown in YMD media supplemented with SLHWU or SLHWM, respectively, at 30 °C to an OD₆₀₀ of ~ 3.0 and the cells were harvested. The cell pellets were resuspended in lysis buffer (25 mM Tris, Cl (pH 7.5), 10 mM KCl, 10 mM BME, 1mM PMSF) and lysed using acid-washed glass beads (Sigma) in a Fastprep FP120 (Thermo Savant). The lysed cells were centrifuged at 12,000 × g for 20 min and the supernatants were collected. and twenty µg each were electrophoresed in duplicate on an 8 % SDS-polyacrylamide gel according to Laemmli (12). One half of the gel was stained with Coomassie Brilliant Blue and proteins on the duplicate half of the gel were transferred onto a nitrocellulose membrane (Millipore). Immunoblotting was performed using a 1:2000 dilution of polyclonal anti-Met6p as the primary antibody and a 1:2000 dilution of goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad) as the secondary antibody. The immunoblot was developed by chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

SDS-PAGE and Immunoblotting

C. albicans strains were grown under inducing and repressing conditions to an OD₆₀₀ of 1, and spun down in a centrifuge for 5 minutes at 5000 × g. The cell pellets were resuspended in 2 mL/gm wet weight of 50 mM KPO₄/10 mM BME/1 mM phenylmethylsulfonyl fluoride, pH 7.0. The cells were lysed using 450 µm glass beads (Sigma), and the cell debris was pelleted in a microcentrifuge. The protein concentrations in the whole cell lysates were estimated using absorbance at 280 nm. Equal amounts of protein from each of the samples were loaded on an 8% SDS-polyacrylamide gel, and SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [12]. The protein samples were transferred to a PVDF membrane (Millipore) using the Bio-Rad semi-dry blotter apparatus at 100 mA for 20 minutes, and the membrane was blocked with 5% non-fat milk. Immunoblotting with the polyclonal anti-Met6p antibody was carried out as described above.

RESULTS

MET6 Gene Deletion

The parental *C. albicans* cell strain used was BWP17, which is auxotrophic for uridine, arginine, and histidine [10]. The first gene deletion experiments were performed using the *URA3* cassette and the *ARG4* cassette separately. Each cassette had 60 nucleotides on each end that complement the sequences flanking the *MET6* ORF; through homologous recombination these cassettes should replace one *MET6* allele, as illustrated in Figures 2A and 3A. The PCR-amplified cassettes were introduced into BWP17, and transformants were selected on plates

that lacked either uridine or arginine. Colonies appeared within 3–5 days, and genomic DNA was purified from the resulting colonies and tested for the deletion using PCR. Figure 2B shows a 0.8% agarose gel of the PCR reactions performed using primers that flank the deleted region in the *URA3* cassette transformants. The undisrupted allele results in a 2500bp PCR product (lane 1), and the allele replaced with the *URA3* cassette gives an 1800bp PCR product (lane 2). The two bands in lane 2 show that one copy of *MET6* was deleted, creating a *MET6/met6* mutant. Figure 3B shows the PCR analysis of the *ARG4* transformants. The *ARG4* cassette is the same size as the *MET6* gene, and when using the primers that flank the deleted region, only one band appears (lanes 1, 4). To test whether the *ARG4* cassette had successfully replaced the *MET6* gene, additional PCR reactions were performed using a 5' primer that bound within the *ARG4* cassette and a 3' primer that bound outside of the deleted region. These primers give a 723 bp band (Figure 3B, lane 3), confirming that the *ARG4* cassette replaced one copy of the *MET6* gene. Both the *URA3* and *ARG4 MET6/met6* mutants grew as well as the wild type *C. albicans* strain BWP17 in minimal media lacking methionine (data not shown).

Because *C. albicans* is diploid, a second deletion is needed to create a *met6/met6* null mutant. The *URA3 MET6/met6* deletion mutant was therefore transformed with the *ARG4* cassette, and the *ARG4 MET6/met6* deletion mutant was transformed with the *URA3* cassette. All of the transformations were plated on agar plates that lacked uridine and arginine, but contained histidine and methionine. In repeated attempts, no colonies were ever observed from the second round of deletion experiments, consistent with the notion that the double knockout was lethal.

Analysis of a conditional *MET6* mutant

Since we were unable to obtain a *met6/met6* null mutant, we attempted to construct a conditional *met6* mutant using the *P_{GAL1}* cassette [11]. This cassette contains a *URA3* selection marker and a *GAL1* promoter, which was amplified from the plasmid pURA3-PGAL1-GFP. This *P_{GAL1}* cassette was then transformed into the *MET6/met6::ARG4* mutant strain where, through homologous recombination, the *GAL1* promoter was inserted in front of the remaining copy of *MET6* (Figure 4A). These transformants were plated on media containing galactose and histidine, where only those that integrated the *P_{GAL1}* cassette would grow. Colonies appeared after 7 days. The resulting transformants were tested using PCR to confirm that the cassette had integrated in front of the *MET6* gene (Figure 4B). When amplified with the FL-5DR and FL-3DR primers there should be two PCR products, one at 2500bp, which represents the *ARG4* cassette, and one at ~6000 bp which represents the *P_{GAL1}* cassette plus the *MET6* ORF. Only the 2500 bp product was observed (Figure 4B, lane 1), presumably due to inefficient amplification of the ~6000bp product. The presence of the *ARG4* cassette in the conditional mutant was tested for in the same way as in the *MET6/met6* mutants (723 bp product). Amplification with FL-5DR and URA3-517-5DR primers (545 bp predicted) confirmed the presence of the *URA3-P_{GAL1}* cassette in front of the remaining *MET6* ORF (Figure 4B, lane 3). The presence of the *P_{GAL1}* cassette was also confirmed by amplification using the GAL1-2918-5DR and CaMET6-518-3DR primers (1121 bp predicted; Figure 4B, lane 4).

The *GAL1* promoter is induced by galactose and repressed by glucose [13,14]. Under inducing conditions, in the presence of galactose, the conditional *MET6* mutant strain grows normally, and does not require methionine (Figure 5A, row 1). Under repressing conditions, in media containing glucose and histidine (the parental BWP17 strain is a *his1/his1* null), the conditional *MET6* mutant strain did not grow unless methionine was included (Figure 5A, compare rows 2 and 3). A Western blot revealed that even under repressing conditions (glucose), a protein of the same mobility as *Met6p* was still expressed, albeit at very low amounts (Figure 5B, compare lanes 1 and 2). Some of the lower molecular weight cross-reacting bands in lane 1 might be proteolytic fragments of *Met6p*, as they are more abundant in the extract prepared

from cells grown under inducing conditions. There is, however, one prominent cross reaction band, at about 50 kDa, which is equally intense in both lanes 1 and 2. The presence of cross reaction material raises the question as to whether the 86 kDa band in lane 2 is indeed Met6p. To help address this issue we carried out an immunoblot with extracts from *S. cerevisiae*, where the *met6* deletion strain is viable. As shown in Figure 6, Panel B, a strong signal at the position predicted for Met6p (~86 kDa) was detected in the wild-type DAY4 extract (lane 3), but there is no band in the *met6* mutant extract (lane 4). The antibodies exhibit weak cross-reactivity with other *S. cerevisiae* proteins (lanes 3 & 4), but none that interfere with detection of the authentic Met6p at 86 kDa. It is also evident that the *S. cerevisiae* extract contains a 50 kDa protein that cross reacts with the Met6p antibodies, even in the SDYa deletion strain. This strengthens the notion that the 86 kDa band in lane 2 of Figure 5B reflects a low level of Met6p expression.

As shown in Figure 7 the conditional mutant, under repressing conditions, exhibited methionine concentration-dependent growth, requiring 50 μ M methionine to achieve normal growth on solid media.

DISCUSSION

Initial attempts to create a *met6/met6* null mutant using the PCR-based gene disruption method were unsuccessful. After the first round of transformations, one copy of *MET6* was completely replaced by a selection cassette. This was successful with both the *URA3* and the *ARG4* cassettes (Figures 2 and 3). These *MET6/met6* heterozygotes grew normally. However, subsequent attempts to delete the second copy of *MET6* in the heterozygous mutants, using a different selection cassette, were unsuccessful. Transformation mixes from these experiments were plated, multiple times, on media supplemented with histidine and methionine, but no colonies appeared on the plates even when allowed to incubate for up to 10 days. The results from these experiments suggest that *MET6* is essential, and a double deletion is lethal since colonies did not grow even when supplemented with exogenous methionine.

We considered the possibility that our failure to obtain any *met6/met6* transformants might be due to the inability of the BWP17 strain to transport sufficient methionine to support growth. We view this an unlikely explanation since *C. albicans* is reported to possess an active amino acid permease that transports methionine [15] and other *C. albicans met* mutant strains can be rescued by methionine supplementation [16,17]. Thus, it appears that the lethality of *met6* nulls in *C. albicans* is not due simply to methionine starvation.

As an alternative strategy to create a *met6* null mutant, we constructed a conditional *MET6* strain. One copy of the *MET6* gene was replaced with an *ARG4* cassette, and a regulatable *GAL1* promoter was inserted in front of the second *MET6* copy. The conditional mutant grew well on galactose media lacking methionine (Figure 5A). However, under repressing conditions, the conditional mutant grew only in the presence of exogenously added methionine. These results confirm that the BWP17 strain can take up sufficient methionine to support normal growth. So why can't a homozygous *met6/met6* null mutant be rescued by methionine?

To help elucidate the essential nature of the *MET6* gene, Met6p protein levels were visualized by immunoblot in cells grown under inducing and repressing conditions. Met6p protein could be detected in cells grown in glucose (repressing conditions), although at a much lower level than under inducing conditions (Figure 5B). The Met6p signal under repressing conditions was so weak it could only be detected in an overloaded gel (300 μ g of total protein). This observation is consistent with previous reports that the *GAL1* promoter is leaky in glucose [13, 14]. Survival of the conditional mutant under repressing conditions is apparently due to the small amount of methionine synthase expression. This expression level is not enough to support methionine

synthesis at the levels required for growth, since the conditional mutant has an absolute methionine requirement under repressing conditions (Figure 5A). Thus, methionine is necessary for growth, but not sufficient – a minimal level of methionine synthase expression is required in addition. This would explain our failure to obtain a homozygous *met6/met6* strain of *C. albicans*.

In *S. cerevisiae* and *Aspergillus nidulans*, the gene encoding methionine synthase is conditionally required, as disruption mutants of these organisms can be fully rescued by methionine [18,19]. In contrast, our results show that the *MET6*-encoded methionine synthase is essential for viability in *C. albicans*, even when grown in media containing methionine. There are several other organisms in which *met6* mutants are not fully rescued by methionine supplementation. Pascon *et al.* [20] showed that a *met6* mutant strain of *Cryptococcus neoformans* is not viable without methionine, but does grow at a slow rate when supplemented with methionine. The *C. neoformans met6* mutant was also shown to be avirulent in the mouse inhalation model, and is more susceptible to antifungal drugs [20]. Seong *et al.* [21] observed that a methionine synthase gene (*msy1*) deletion mutant of *Fusarium graminearum* is defective in aerial hyphal growth even in presence of methionine. Likewise, Fujita *et al.* [22] reported that disruption of the methionine synthase gene (*met26*) in *Schizosaccharomyces pombe* leads to a requirement for both methionine and adenine.

In all of these cases, the phenotype of a methionine synthase mutant is more complicated than just methionine auxotrophy, suggesting that the *MET6* gene has some other role(s) in addition to the synthesis of methionine. An accumulation of intracellular homocysteine would be predicted to occur in methionine synthase mutant cells, and indeed this is observed in both *S. cerevisiae* and *S. pombe* [22]. Thus, the additional growth defects, not rescued by methionine, could be caused by toxicity of intracellular homocysteine [20,23]. Homocysteine has been reported to interfere with purine biosynthesis and sterol biosynthesis [24,25]. Neither supplementation with adenine nor serine, which might be expected to lower homocysteine levels by stimulating its metabolism to cysteine via transsulfuration [26] (Figure 1), allowed us to recover *met6/met6* disruptants (data not shown). On the other hand, in the conditional mutant grown under repressing conditions, the low expression of the *MET6* protein might catalyze enough homocysteine remethylation to avoid the affects of homocysteine toxicity, thereby allowing growth, as long as sufficient methionine is provided in the medium.

Regardless of mechanism, the *MET6* gene appears to be essential in *C. albicans*, suggesting that methionine synthase is a potential antifungal drug target in this and other pathogenic fungi. It might be expected that inhibition of methionine synthase would be negated by the methionine typically present in mammalian interstitial fluids and plasma. However, human tissues are actually quite low in methionine --normal plasma methionine in humans is 3–40 μ M [27]. Manning *et al* [28] reported that methionine auxotrophy did not alter the pathogenicity of *C. albicans* in mice. More recently, however, a methionine auxotrophic mutant of *C. albicans* has been shown to exhibit decreased virulence in a mouse model [29]. Indeed, under conditions that approximate the low methionine levels typically found in humans, the conditional mutant grew about two to three times more slowly than wild type cells (data not shown). The repressed conditional mutant does express a low amount of methionine synthase enzyme (Figure 5B), and may thus be a good model for the activity of the enzyme in the presence of an inhibitory drug. Increasing antifungal drug resistance, together with increasing numbers of immunocompromised patients, emphasizes the need for new antifungal drugs. Some of the present drug therapies are toxic, and not effective for certain types of *Candida* infections. Because fungal methionine synthases are cobalamin-independent, while human methionine synthase is cobalamin dependent, it is likely that specific inhibitors of cobalamin-independent methionine synthase would be effective, broad range antifungal agents.

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Figure 1.

Methionine metabolism in *Candida albicans*. The remethylation, transsulfuration, and sulfur assimilation pathways are shown. Genes and enzymes catalyzing individual reactions are: *SHM1* and *SHM2*: serine hydroxymethyltransferase (SHMT; EC 2.1.2.1); *MET13*: methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20); *MET6*: methionine synthase (EC 2.1.1.14); *SAM2*: S-adenosylmethionine synthetase (EC 2.5.1.6); *SAH1*: S-adenosylhomocysteine hydrolase (EC 3.3.1.1); *CYS4*: cystathionine β -synthase (CBS; EC 4.2.1.22); *CYS3*: cystathionine γ -lyase (EC 4.4.1.1); *orf19.2092*: Cystathionine beta-lyase (EC 4.4.1.8); *STR2*: Cystathionine gamma-synthase (EC 2.5.1.48); *MET2*: homoserine O-acetyltransferase (EC 2.3.1.31); *MET15*: O-acetylhomoserine (thiol)-lyase (EC 2.5.1.49); *orf19.7152*: cysteine synthase (EC 2.5.1.47). "X" represents any methyl group acceptor. Gene and open reading frame (orf) names are taken from the *Candida* Genome Database (<http://www.candidagenome.org/>).

Figure 2.

A. Schematic of homologous recombination occurring after transformation with the *URA3* cassette, and primer binding locations of detection primers listed in Table 1. B. Analysis of *URA3* transformants. Lane 1, PCR products of genomic DNA from BWP17 cell strain amplified with FL-5DR and FL-3DR; lane 2, PCR products of genomic DNA from BWP17/*URA3* transformant amplified with FL-5DR and FL-3DR. Lane M, DNA size markers (bp). PCR amplification of the *MET6* gene with FL-5DR and FL-3DR results in a 2500 bp product, and amplification of the *URA3* cassette results in a 1800 bp product.

Figure 3.

A. Schematic of homologous recombination occurring after transformation with the *ARG4* cassette, and primer binding locations of detection primers listed in Table 1. B. Analysis of *ARG4* transformants. PCR products of genomic DNA from BWP17 amplified with FL-5DR and FL-3DR (lane 1) or ARG4-1012-5DR and FL-3DR (lane 2). PCR products of genomic DNA from BWP17/*ARG4* transformants amplified with ARG4-1012-5DR and FL-3DR (lane 3) or FL-5DR and FL-3DR (lane 4). Lane M, DNA size markers (bp). PCR amplification of the *MET6* gene with FL-5DR and FL-3DR results in a 2500 bp product; amplification of the *ARG4* cassette also results in a 2500 bp product. PCR amplification of the *ARG4* cassette with ARG4-1012-5DR and FL-3DR results in a 723 bp product in the *ARG4* transformant, and no product in the wild-type strain.

Figure 4.

A. Schematic of homologous recombination occurring after transformation with the *P_{GAL1}* cassette into the heterozygous *MET6/met6::ARG4* mutant and primer binding sites. B. PCR Analysis of *P_{GAL1}* transformants. Genomic DNA from the conditional mutant amplified with *FL-5DR* and *FL-3DR*, lane 1; *ARG4-1012-5DR* and *FL-3DR*, lane 2; *URA3-517-5DR* and *FL-5DR*, lane 3; *GAL1-2918-5DR* and *CaMET6-518-3DR*, lane 4. Lane M, DNA size markers (bp).

Figure 5.

MET6 is essential. A. Serial 10-fold dilutions of a liquid culture containing the conditional mutant was plated onto solid media containing galactose and 129 μ M histidine (row 1); glucose, 129 μ M histidine, and 134 μ M methionine (row 2); medium containing glucose and 129 μ M histidine (row 3) Histidine is required because the parental BWP17 strain is a *his1/his1* null. B. Immunoblot analysis of cellular extracts. 300 μ g of total protein was loaded per lane and the positions of molecular weight markers, in kDa, are shown to the left. Primary antibody was 1:1000 dilution of anti-Met6p. Conditional mutant induced with galactose, lane 1. Conditional mutant repressed with glucose, and supplemented with methionine, lane 2.

Figure 6.

Characterization of *C. albicans* Met6p antibodies. Panel A is a coomassie stained SDS-PAGE of whole cell extract from wild type (lane 1) and the met6 mutant (lane 2) of *S. cerevisiae*. Panel B is the corresponding immunoblot using a 1:2000 dilution of the *C. albicans* Met6p antibodies. Twenty μ g of total protein was loaded per lane and the positions of molecular weight markers, in kDa, are shown to the left.

Figure 7. Methionine concentration-dependent growth of conditional mutant under repressing conditions. Strains were streaked onto yeast minimal glucose plates supplemented with histidine, uridine, arginine and either 0, 5, or 50 μM methionine. *Sector 1*, *MET6/met6::URA3*; *Sector 2*, *MET6/met6::ARG4*; *Sector 3*, BWP17 (wild-type); *Sector 4*, conditional *met6* mutant. Plates were photographed after 3 days incubation at 30°C.

Table 1

Primers used in gene deletion experiments.

PRIMER	SEQUENCE 5' - 3'	Purpose
CaMET6-5DR ^d	TGTTAAGATTTATCTACAAATCAAAAACTTTTATATCCCCCTTATATCAACA TGTGGAATGTGAGCGGGATA	Deletion of MET 6.
CaMET6-3DR ^a	TCAACATTTCAAGAAATGCAAAATAAATCAATGAATGCTTTGAAATCATAAAAGAT TTTCCCAAGTCACGACGTT	Deletion of MET 6.
GAL1-5DR ^b	CCTTTTCTTATCTCTCTCTAAATTTTGTAAAGATTTTATCTACAAATCAAAAACCTTTTATATCCCTCTAGAAAGGACCA CCCTTTGATTG	GAL1 promoter introduction.
GAL1-3DR ^b	GTAGGCCTCAGTGATTTTCTCTTTGACCACCAATACGTGGGAAACCTAAGACGGGAAGATTGA ACCA7TTTATAAACGCGGGATCC	GAL1 promoter introduction.
FL-3DR	TATCCCCCTATCTGAAA	Detection of deleted alleles.
ARG4-1012-5DR	GTCGTCATATAAAACATAAAAAAGAAA	Detection of deleted alleles.
ARG4-517-5DR	CCATTATTGATGCATTGAC	Detection of deleted alleles.
URA3-517-5DR	GTTGAAATTGCTAAATCCG	Detection of deleted alleles.
GAL1-2918-5DR	GGGAAAAAAATTTTAAAGCAGTTC	Detection of modified alleles.
CaMET6-518-3DR	GCTTTACCTAAATACAAAGTAAG	Detection of modified alleles.

^a Boldface sequences in CaMET6-5DR and CaMET6-3DR primers are segments that anneal to plasmids pGEM-URA3 and pRS-ARG4ΔSpeI for amplification of the *URA3* and *ARG4* deletion cassettes.

^b Boldface sequences in GAL1-5DR and GAL1-3DR primers are segments that anneal to plasmid pURA3-PGAL1-GFP for amplification of the *PGAL1* cassette.

^d Primer binding locations are illustrated in Figures 2A, 3A, and 4A.