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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

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 (Fig. 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 which function in binding homocysteine (Hcy)[1], 5-methyl-tetrahydrofolate cobalamin, and *S*-adenosylmethionine, 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 *Escherichia 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.

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[1] *Abbreviations used:* Hcy, homocysteine; ORF, open reading frame.

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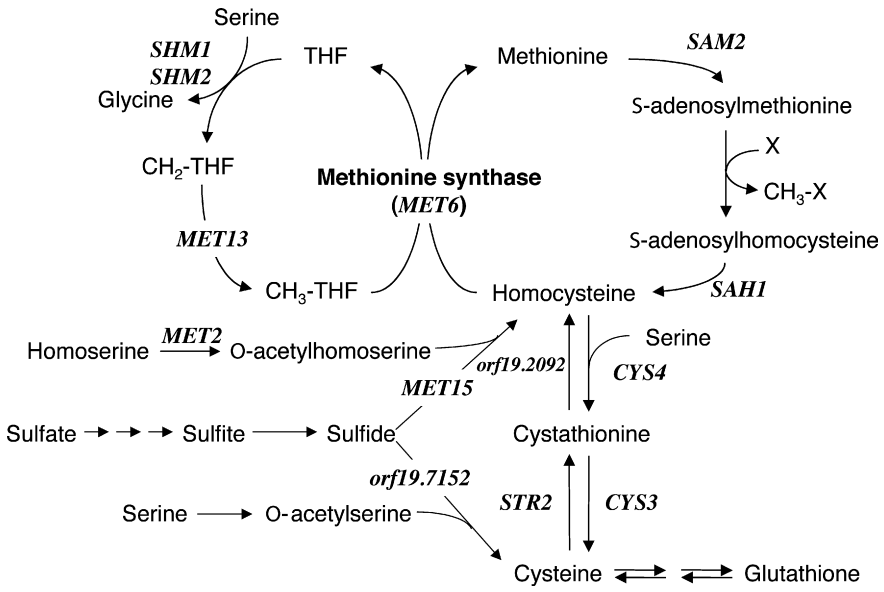

Fig. 1. Methionine metabolism in *Candida albicans*. The remethylation, transulfuration, and sulfur assimilation pathways are shown. Genes and enzymes catalyzing individual reactions are: *SHM1* and *SHM2*: serine hydroxymethyltransferase (SHMT; EC 2.1.2.1); *MET13*: methylenetetrahydrofolate reductase (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 β-lyase (EC 4.4.1.8); *STR2*: Cystathionine γ-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/).

Candida 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 1U 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 s, 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 μL 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 μg 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 h). 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). Hundred microliters of aliquots of cell suspension were dispensed into microcentrifuge tubes containing PCR product and Herring-Testes DNA, and incubated at room temperature for 30 min. 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