Dissertation

Molecular understanding of the sulfur amino acid metabolic pathway in a human pathogen *Cryptococcus neoformans*

(ヒト病原菌 *Cryptococcus neoformans* の硫黄アミノ酸合成経路に関する分子遺伝学的研究)

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CONTENTS

Lists of Tables

List of Figures

Supplements

List of Abbreviations

APS	Adenosine phosphosulfate
ATP	Adenosine triphosphate
CFU	Colony forming unit
FPKM	Transcript per million mapped reads
FBS	Fetal bovine serum
OAH	O-acetyl-homoserine
OAS	O-acetyl-serine
ORF	Open reading frame
PBS	Phosphate buffered saline
SH	Silkworm hemolymph
WT	Wild type

DEDICATION

To my great supervisor Prof. Kiminori Shimizu and my family for their continuous support and encouragement during my Ph.D life!!

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ABSTRACT

Background

Pathogenic fungi, such as *Candida*, *Aspergillus* or *Cryptococcus* species, generally cause diseases in immunocompromised individuals. Cryptococcal meningitis is caused by a basidiomycete yeast *Cryptococcus neoformans* (*C. neoformans*). This disease occurs on people, such as AIDS patients, whose immune system is attenuated. Currently, drugs available for treatment is still limited, and novel drug target is in great demand. Amino acid biosynthetic pathways have been proposed as targets for antifungal drugs. While sulfur amino acid biosynthetic pathway of non-pathogenic fungi such as *Saccharomyces cerevisiae* (*Sa. cerevisiae*), *Aspergillus nidulans* (*A. nidulans*), *Schizosaccharomyces pombe* (*Sc. pombe*) have been well studied, there are only few genes of this pathway have been analyzed in *C. neoformans.* Therefore, the study on function of these genes will not only fulfil the knowledge on the sulfur metabolisms of this organism but also provide the promising target candidates for developing anti-*Cryptococcus* agents. The proposed sulfur amino acid metabolic pathway in *C. neoformans* is showed in **Figure 7**.

Identification of *MET5* gene in *Cryptococcus neoformans*

A wild type (WT) strain of *C. neoformans*, KN3501α, was transformed by *Agrobacterium tumefaciens*-mediated transformation (AtMT) and about 10,000 transformants were obtained. Using TAIL-PCR method, the T-DNA was found inserted into the locus tagged as *CNL05500* on chromosome 12. The predicted amino acid sequence of *CNL05500* contains a highly conserved pattern of the known sulfite reductase and was most similar

to the *MET5* gene of *Sa. cerevisiae*. Based on the sequence homology, the *CNL05500* gene was designated as *MET5*.

The *met5*Δ mutant could grow well on medium containing cysteine (Cys) as a sole sulfur source, while the *met5*Δ complement strain exhibited growth recovery to the level of the WT strain. The *C. neoformans met5*Δ mutant grew well under the presence of Cys but grew poorly on methionine (Met), which is not the case in *Sa. cerevisiae*, in which a *met5*Δ mutant grows equally well under the presence of either Met or Cys. Further, the *met5*Δ mutant grew on sulfide, but not on either sulfate or sulfite in *C. neoformans*. These results indicate that the *MET5* gene encodes a sulfite reductase involved in the sulfate assimilation pathway in *C. neoformans*. In *Sa. cerevisiae*, sulfite reductase, which catalyzes the direct reduction of sulfite into sulfide, is a heterodimer enzyme encoded by *MET5* and *MET10*; therefore, *MET5* and *MET10* exhibited an identical phenotype. Based on a BLAST search against the *C. neoformans* genome database, a *MET10* (*CNG03990*) was identified. The *met10*Δ mutant also grew on sulfide but not on sulfate or sulfite as a *met5*Δ mutant. It was also true that the *met10*Δ mutant grew well on Cys but not on Met as seen for a *met5*Δ mutant. Taken together, *MET5* and *MET10* genes of *C. neoformans* code for a sulfite reductase in a sulfate assimilation pathway.

Metabolism of sulfur amino acids in *Cryptococcus neoformans*

The metabolic pathway of sulfur amino acids is well-understood in fungi such as *Sa. cerevisiae*, *A. nidulans* and *Sc. pombe*. However, the knowledge on this pathway in *C. neoformans* remains still limited. Thus, the metabolism of sulfur amino acids in *C. neoformans* once again was reviewed to build up a complete model for this pathway.

In sulfate assimilation pathway, to date, only *MET3* gene (encoding an ATP

sulfurylase) has been shown to be involved in *C. neoformans*. *MET5* and *MET10* were confirmed as components of the sulfate assimilation pathway in *C. neoformans*. In addition, it was confirmed that *MET14* gene product converts adenosine phosphosulfate (APS) to phosphoadenosine phosphosulfate (PAPS) in the sulfate assimilation pathway in *C. neoformans*. However, all of the mutant strains of these genes grew better on Cys than they did on Met as a sole sulfur source, as seen for the *met3*Δ strain described previously. In contrast, in *Sa. cerevisiae*, these mutants grow well on either Met or Cys. The difference might be attributable to the presence of the reverse transsulfuration pathway (from homocysteine to Cys) in *C. neoformans*.

In a transsulfuration pathway, the conversion of homocysteine to Cys seems to occur. The *cys1*Δ mutant grew well under the presence of cystathionine and Cys, but slightly on Met and homocysteine, while the *met17*Δ did not. The *cys1*Δ*cys3*Δ double mutant grew on Cys but not on cystathionine. These results suggest that *C. neoformans* synthesizes homocysteine to Cys by a transsulfuration pathway, but not the opposite does not via a reverse-transsulfuration pathway. The *CYS3*, *CYS4*, and *MST1* genes were found in *C. neoformans* based on their sequence homology with those of *Sa. cerevisiae*. In *Sa. cerevisiae*, both *CYS3* and *CYS4* have been reported to cleave Cys and release sulfide *in vitro*. Sulfide synthesis via the function of *CYS3*, *CYS4*, and/or *MST1* was also supported by the experiment using the *met3*Δ*mst1*Δ*cys3*Δ*cys4*Δ quadruple mutant strain. This strain grew poorly on any single sulfur source, potentially because the all the sulfide synthetic pathways were blocked.

To identify genes that function in the transsulfuration pathway from homocysteine to Cys in *C. neoformans*, gene expression profiles of WT strain grown with different sulfur sources were analyzed when grown. Genes showed expression greater than two-

fold in homocysteine and cysteine than other sulfur sources were selected. Among 7881 genes of *C. neoformans* genome, 602 genes showed strong expression in homocysteine while in Cys there were 58 genes. These genes might be the candidate genes that involve in transsulfuration pathway. However, in order to determine the accurate one, the candidate genes are needed to be compared with the homologous genes in other organisms which have available function; and further experiments are needed to conduct on the filtered genes.

Involvement of *MET5* gene in virulence of *Cryptococcus neoformans*

To identify gene, influence the virulent factor, it is essential to evaluate the virulence of mutant strain in animal infection models. Recently, silkworm *Bombyx mori* has been introduced/used as an infection model which is not only as efficient as mice but also much more accessible. In this section, the virulence factor of *MET5* was tested by using silkworm as infection model. Surprisingly, there is no significant difference in mortality rate between silkworm larvae groups infected with the WT, *MET5* complement and the *met5*Δ mutant strains. However, the blood of silkworm contains several amino acids such as 5.2mg/100ml methionine and 22.2 mg/100ml cystathionine which could be sufficient to allow the *met5*Δ mutant strain to be virulent as the WT strain. Results on the incubation of WT, *MET5* complement, and *met5*Δ mutant strains with silkworm hemolymph supported this hypothesis. Therefore, further investigate is required to confirm the virulence factor of *MET5* gene.

This study has shown a molecular understanding of sulfur amino acid metabolic pathway in a human pathogen *C. neoformans*. The observed of *MET5* gene in sulfate

assimilation pathway and the existing of reverse transsulfuration pathway would be promising candidates for drug targets of this pathogenic yeast.

Chapter 1. General introduction

Pathogen fungi

Pathogenic fungi are fungi that causes disease in human or other organisms. Certain fungal species could become mycotoxin due to their ability to suppress hormonal immunity and cause tissue breakdown in many forms of allergies, cancer, or even death (Enyiukwu et al. 2014). Pathogenic fungi, such as *Candida*, *Aspergillus* or *Cryptococcus* species, generally cause diseases in immunocompromised human. Cryptococcal meningitis is caused by a basidiomycete yeast *Cryptococcus neoformans* (*C. neoformans*), a fungus lives in the environment throughout the world. People who have weakened immune systems such as AIDS patients can have *C. neoformans* infection by fungal breathing (Lin and Heitman 2006; Zaragoza 2019). *C. neoformans* can cause mortality up to 30% of AIDS patients (Idnurm et al. 2005). Globally, approximately one million cases of cryptococcal meningitis occur each year, resulting in about 600.000 deaths by 3 months after infection, in which Sub-Saharan Africa had the highest infection 3.2% (Park et al. 2009).

Cryptococcus neoformans life cycle

C. neoformans has five serotypes: A, B, C, D, and AD hybrid and includes two varieties: var. *neoformans* (serotypes A, D, and the AD hybrid) and var. *gattii* (serotype B and C). Currently, this complex group is classified into *C. neoformans* var. *neoformans* (serotype D), *C. neoformans* var. *grubii* (serotype A), and *C. gattii* (serotypes B, C) (Lin and Heitman 2006; Kwon-Chung et al. 2017). *C. neoformans* species generally caused

diseases in immunocompromised patients while *C. gatti* species mostly affects immunocompetent ones (Zhao et al. 2019).

C. neoformans has a growth form by two distinct differentiation pathways: mating and monokaryotic fruiting (**Figure 1**). The mating involves fusion of haploid cells of **a** and **α** to produce dikaryotic filaments, and the two parental nuclei migrate in the hyphae to develop basidium. The nuclei fuse and undergo meiosis followed by mitosis and production of haploid basidiospores by budding on four characteristic chains. During monokaryotic fruiting, cell and nuclear fusion of same mating type become diploid monokaryotic hyphae. Meiosis occurs during basidium development, haploid basidiospores are produced. Mature basidiospores are dispersed into the environment and germinate to haploid yeast cells (Idnurm et al. 2005; Voelz 2010).

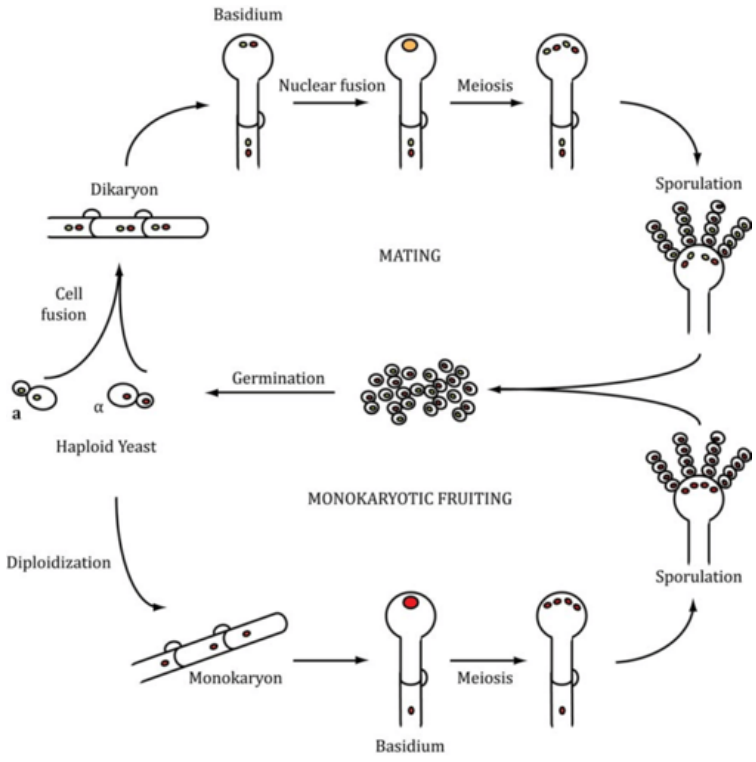


Figure 1. Schematic life cycle of *Cryptococcus neoformans* (Idnurm et al. 2005; Voelz 2010)

Virulence factors of *Cryptococcus neoformans*

Although multiple virulence phenotypes associated with *C. neoformans* had been known, there are three well established virulence factors: a capsule, melanin and the ability to grow well at 37ºC.

The extracellular polysaccharide capsule produced by *C. neoformans* is essential for its pathogenicity (Chang et al. 1996). The capsule plays an important role not only in the inhibition of phagocytosis but also in the inhibition of killing by macrophages (Poeta 2004). The products of four *CAP* genes (*CAP10*, *CAP59*, *CAP60*, and *CAP64*) are essential for both capsule formation and virulence. Deletion of these genes causes *C. neoformans* to lose its capsule and virulence, and the complement restores the virulent phenotype (Chang and Kwon-Chung 1994; Chang et al. 1996).

Melanin is another virulence component of *C. neoformans*. It has high physical and chemical strength that make it resistant to degradation, bind into many substances such as antibiotics, heavy metals and a number of proteins. Melanin contributes to the virulence by protecting *C. neoformans* against host effector mechanisms, such as macrophage-mediated phagocytosis, oxidants, and microbicidal peptides (Rosas et al. 2001). An enzyme, diphenol oxidase, encoded by two genes *LAC1* and *LAC2* involves in the production of melanin synthesis (Zaragoza 2019).

C. neoformans is thermotolerant, and its ability to grow at 37ºC is considered to be a virulence factor (Steen et al. 2002). Twenty genes have been validated to be necessary for high-temperature growth and pathogenesis in *C. neoformans*. The pathobiological importance of these genes has been studied and confirmed in animal model studies (Perfect 2006).

Cryptococcal pathogenesis

Immunosuppressed patients have high exposure to the infection of *C. neoformans*. The infection occurs through inhalation of cryptococcal spores. After inhalation, *C. neoformans* survive and evade the immunity in patients' lung then spreads through the bloodstream or lymphatic system to other organs, including brain (**Figure 2**) (Zaragoza 2019). The symptoms are often similar to other illnesses including fever, cough in lung and sensitivity to light, confusion or changes in behavior in brain. There is no evidence for human transfer to human or environment (Idnurm et al. 2005).

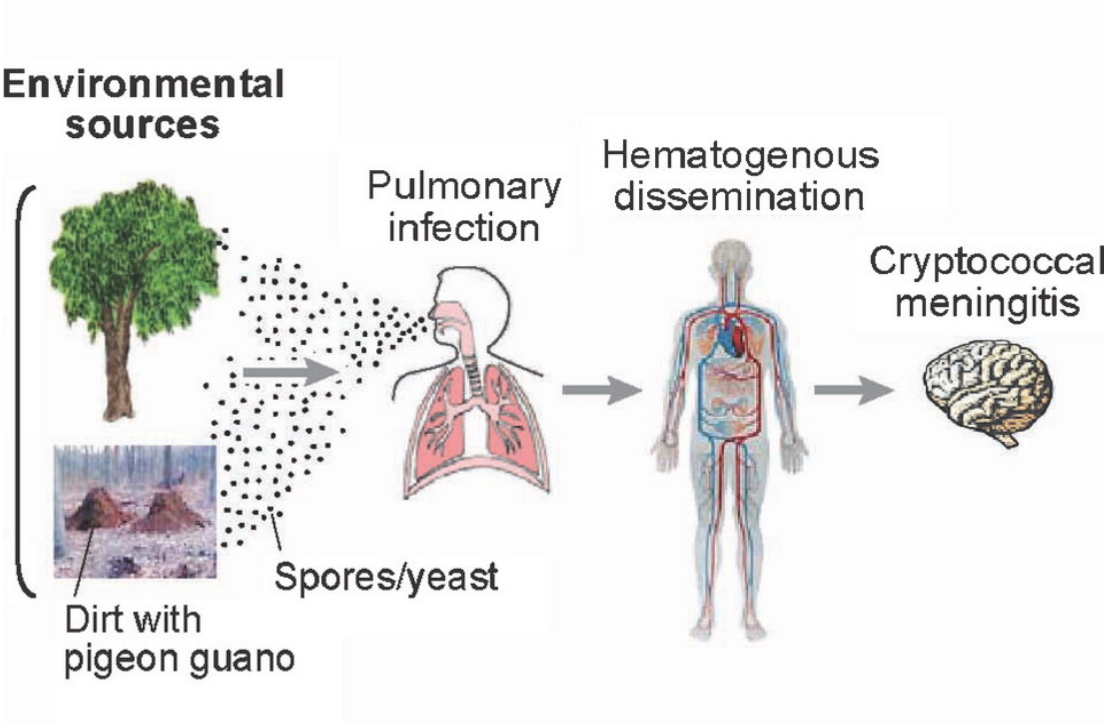


Figure 2. Infection cycle of *Cryptococcus* (Lin and Heitman 2006)

Current management and perspectives on *Cryptococcus neoformans* species

The standard therapy for cryptococcal meningitis disease consists of amphotericin B, flucytosine and fluconazole (Jarvis and Harrison 2007). Amphotericin B and the triazole fluconazole both target the fungal membrane and disturb membrane integrity. Flucytosine, after conversion to 5-fluorouacil, function as pyrimidine antagonist inhibiting RNA

biosynthesis (Vermes et al. 2000). The length of primary therapy with amphotericin B and flucytosine is suggested to be prolonged to at least two weeks in HIV-infected patients and four to six weeks with non-HIV infected patients (Perfect et al. 2010). To date, treatment of cryptococcosis remains a challenge. Even with immediate treatment, patients show high mortality rates and toxic side effects (Voelz 2010). The understand on pathogenesis mechanisms applied by *C. neoformans* intact with human host is the goal to prevent infection and improve the treatment methods.

Metabolism of sulfur amino acids

Targeting the metabolism of pathogenic fungi seems to be an important strategy against these agents, as it is essential to the survival of those fungi (Jakubowski 2004; Bachhawat and Yadav 2010; Kaltdorf et al. 2016). Among the few currently antifungal metabolic pathways (**Table 1**), the sulfur assimilatory pathways of the pathogenic yeast appear to be suitable for antifungal development (Bachhawat and Yadav 2010), due to their fundamental requirement of sulfur and significant differences from the corresponding pathways in humans.

Table 1. Pathways targeted by currently used antifungals in human (Bachhawat and Yadav 2010)

Pathway	**Target enzyme**	**Drug class**	**Example**
Ergesterol biosynthesis pathway	14α-Demethylase	Azoles (imidazole and triazoles)	Ketoconazole, Fluconazole
	Squalene epoxidase Δ^{14} Reductase and $\Delta^{7,8}$ isomerasae	Phenylmorpholine	Amorolfine
	Ergesterol	Polyene	Amphotericin B
Cell wall biosynthesis	β-1,3 Glucan polysaccharides	Echinocandins	Micafungin, caspofungin, anidulafungin
Nucleic acid biosynthesis	Nucleic acid biosynthesis		Flucytosine
Mitosis	Microtubule assembly		Griseofulvin

The sulfur metabolic pathway of humans is significantly different from the pathway in yeasts and fungi. Most of fungi is able to utilize inorganic sulphates and other different organic sulfur compounds such as cysteine, methionine and glutathione as sources of sulfur. In contrast, methionine as an essential amino acid has to be consumed to meet the requirement in human. Thus, sulfur pathways have the potential to be exploited for developing new antifungals.

Metabolism of sulfur amino acids in *Cryptococcus neoformans*

Among metabolic pathway, amino acid biosynthetic pathways have been proposed as target for development of antifungal drugs (Bachhawat and Yadav 2010). While in *Sa. cerevisiae*, the sulfur amino acid biosynthetic pathway has been well studied, few studies have been done to characterize the sulfur pathways of pathogenic *C. neoformans*. *MET2, MET3* and *MET6* have been confirmed for their roles in sulfur pathway of this yeast and provided some insights for attenuating the virulence of this pathogen. *MET3* gene (encoding ATP sulfurylase) takes the first role in sulfate assimilation pathway and *MET6* (methionine synthase) responses for Met and homocysteine synthesis. The mutant strains of these genes are avirulent in the mice infection model and their production of melanin, a well-known virulent factor is slower compare to WT and complement strains (Yang et al. 2002; Pascon et al. 2004), *MET2* (homoserine transacetylase) commits the first step in Met biosynthesis is the acylation of homoserine (Hse) by the enzyme homoserine transacetylase (HTA) (Nazi et al. 2007). Recently, *CYS2* and *CYS1* genes were found in the O-acetyl-serine (OAS) pathway that consists of serine-O-acetyl transferase and cysteine synthase. *MET17* encoding O-acetyl-homoserine (OAH) sulfhydrylase catalyzes the reaction from homoserine to methionine had been identified in *C. neoformans*. Mutant of *cys1*Δ, *met17*Δ showed avirulent in the intravenous infection mouse model. Further, *met17*Δ mutant produced melanin while *cys1*Δ mutant did not (Toh-e et al. 2017). Bzip protein *CYS3* controls the inhibition of sulfur assimilation pathway (de Melo et al. 2019). As these genes have been known in the contribute for the sulfur amino acid biosynthetic pathway of *C. neoformans*, the tentatively proposed sulfur amino acid biosynthesis pathway in *C. neoformans* has been established (**Figure 7**). However, there are still some genes and sub-pathways in this biosynthesis pathways remain unclear. Study on the

function of these genes in *C. neoformans* need to be considered for expanding knowledge on the sulfur metabolisms of this organism and the promising candidates target for developing anti *Cryptococcus* agents.

Evaluate cryptococcal pathogenesis using a silkworm infection model with *C. neoformans*

To identify virulence factor of microorganisms, it is essential to evaluate their virulence on animal infection models. The most common used models for studying microbial infection is the murine model (Mei et al. 1997; Kaito 2016). However, mammal models get ethical problems, expensive cost and long reproduction times which slow the progress of the experiment. More recently, *Galleria mellonella* (honey comb moth), *Bombyx mori* (silk worm) have been introduced as a model to study microbial infections (Kaito 2016; Tsai et al. 2016) due to their efficiency and low cost. Using silkworm as baculovirus infection model, a new antiviral compound, cynzeilanine was found from Maoutou, an herbal medicine (Orihara et al. 2008). The gene *gpa1*, *pka1*, and *cna1* are known to be necessary for the pathogenesis in mammals and the mutant strains of these genes showed the decrease of virulence in silkworm compare to WT (Matsumoto et al. 2012). Further, antifungal drugs, amphotericin B, flucytosine, fluconazole, and ketoconazole showed therapeutic effect on silkworm infected with *C. neoformans* (Matsumoto et al. 2012). Accordingly, silkworm larvae could be a useful model for testing the virulence factors of *C. neoformans* and the capability of developing antifungal agents.

Goal of this study

This study was focused on understanding of sulfur amino acid metabolic pathway of human pathogen *Cryptococcus neoformans*. First, the Chapter 1 is an overview general introduction on the sulfur metabolic pathway of *C. neoformans* and its current stage. In Chapter 2, the involvement of the novel *MET5* (*CNL05500*) gene, which encodes a sulfite reductase in the sulfate assimilation pathway of *C. neoformans* was identified. Continuously into Chapter 3, the genes related to sulfur amino acid pathway of *C. neoformans* were reviewed and analyzed. *MET10* (*CNG03990*) and *MET14* (*CNE03880*) genes, which are related to the *MET5* gene in sulfate assimilation pathway were also examined. The results showed that Cys was synthesized not only from OAS, but also from homocysteine, thus suggesting the existence of a transsulfuration pathway in *C. neoformans* (**Figure 7**). However, genes which active in reverse transsulfuration pathway still remain unclear. To evaluate the role of *MET5* gene in the virulence factors of *C. neoformans*, in Chapter 4, silkworm was used as infection model to examine killing ability of the WT KN3501α, *met5*Δ mutant and *MET5* complement strains. The virulence between these strains were not significantly different. The possible reason could be the amount of sulfur sources in silkworm hemolymph might be sufficient to allow the *met5*Δ mutant strain to be virulent as the WT control. This hypothesis was also tested here. Finally, a general discussion on all the results along with comparison to the corresponding pathways in other fungi and human was addressed in Chapter 5.

Chapter 2. Identification of *MET5* gene in *Cryptococcus neoformans*

INTRODUCTION

The amino acid biosynthetic pathways have been proposed as promising target for development of antimicrobial drugs (de Melo et al. 2019). Several enzymes have been validated as novel targets for antifungal agents. Enzymes involves in the sulfur amino acids metabolic pathways are well known in *Sa. cerevisiae* (Thomas and Surdin-Kerjan 1997). Once sulfate is internalized, the assimilation is carried out by ATP sulfurylase (*MET3*), yielding adenosine phosphosulfate (APS) which in turn is phosphorylated by APS kinase (*MET14*), producing 3'-phosphoadenonosine-5'-phosphosulfate (PAPS), a key intermediate in sulfurylation pathway. From this point, PAPS reductase (*MET16*) and sulfite reductase (*MET5* and *MET10*) catalyze the production of sulfide. In *Sa. cerevisiae*, **α** subunit and **β** subunit of sulfite reductase is encoded by *MET10* and *MET*5, respectively. Cells bearing a mutation in *MET5* gene exhibit the same phenotype as *met10*Δmutants (Thomas and Surdin-Kerjan 1997). In genome of *C. neoformans,* homologues of *MET5* and *MET10* were found but their function had not been verified. During my research to identify the genes involves in sulfur amino acid metabolic pathway of *C. neoformans*, *MET5* and *MET10* mutant strains were generated. The function of these genes in sulfate assimilation pathway of *C. neoformans* were proved by the growth of mutations on different sulfur sources.

MATERIALS AND METHODS

Strains, medium composition, and growth conditions

Serotype D, *C. neoformans* KN3501α (Nielsen et al. 2005) and strains generated using KN3501α were used throughout this work (**Table 2**). Strains were grown on solid YPD medium (1% yeast extract, 2% polypepton, 2% glucose, and 2% agar) or minimal YNB medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco), 0.5% ammonium sulfate, 2% glucose, and 2% agar). Sulfur-free B medium was prepared according to the description of Cherest and Surdin-kerjan (Cherest and Surdin-Kerjan 1992). The B medium was solidified by adding 2% agarose (Invitrogen/ ThermoFisher Scientific).

Additionally, a spot assay was used to examine the growth of each strain on a medium containing different sulfur sources. First, overnight cultures in YPD liquid were washed twice with sterile distilled water. Cells were counted using a counting chamber and serially diluted to 10^3–10^6 cells/ml. Five µl of each dilution was spotted onto plates containing 5 mM of different sulfur sources. The plates were incubated at 30°C for 4 days. Each experiment set was replicated thrice.

Agrobacterium tumefaciens-mediated mutagenesis

The WT KN3501α strain was transformed via *Agrobacterium tumefaciens*-mediated transformation (AtMT), as described by Walton et al. (Walton et al. 2005). The *A. tumefaciens* strain EHA105 carrying pPZP-HYG2 was used to introduce a hygromycin B resistance cassette into *C. neoformans*. The location of the T-DNA insertion was identified by thermal asymmetric interlaced PCR (TAIL-PCR) (Liu and Huang 1998) followed by sequence analysis, and the sequences obtained were compared to the *C.*

neoformans genome database using the BLASTn algorithm (Altschul et al. 1990).

Genetic manipulation

Gene disruption cassettes were generated using the double-joint PCR method reported by Kim et al. (Kim et al. 2012). The open reading frame (ORF) was deleted and replaced with a neomycin-resistance marker gene (NEO). Deletion constructs were introduced into the *C. neoformans* KN3501α strain using biolistic transformation, as described by Toffaletti et al. (Toffaletti et al. 1993). Transformed cells were grown on YPD medium containing 200 mg/ml G418-sulfate (Wako). The successful transformants were confirmed by PCR using appropriate primers (**Table 3**). For PCR analysis, *C. neoformans* genomic DNA was extracted as described by Hoffman and Winston (Hoffman and Winston 1987).

The complementation strains were constructed from the deletion strains as hosts. An ORF of the desired gene was amplified from *C. neoformans* genomic DNA and cloned into the pKIS612 plasmid (Shimizu, unpublished), which harbors a hygromycin-resistance gene (HYG), followed by introduction into a deletion strain by biolistic transformation. Transformed cells were grown on YPD medium containing 200 mg/ml hygromycin B (Wako). The successful transformants were confirmed by PCR using appropriate primers (**Table 3**).

RESULTS

Identification of the *C. neoformans MET5* gene

A WT strain of *C. neoformans*, KN3501α, was transformed by AtMT and about 10,000 transformants were obtained. Out of the 10,000 random T-DNA-inserted mutants, those that were able to grow on rich nutrition medium (YPD) but not on minimal YNB medium were selected. To identity the nutrients that were necessary for the growth of the mutants, 20 single amino acids were separately added to the YNB medium, which led to the discovery that the mutants grew on YNB medium supplemented with Cys (**Figure 3**a). Using TAIL-PCR, we found that T-DNA was inserted into the locus tagged as *CNL05500* on chromosome 12 (**Figure 3**b, c). The predicted amino acid sequences of *CNL05500* contain the highly conserved pattern of the known sulfite reductase and were most similar to the *MET5* gene of *Sa. cerevisiae* (GenBank accession AJR72581; 44.75% identity) and *A. nidulans* (GenBank accession CBF79717; 49% identity) (Supplementary figure 1). Based on sequence homology, the *CNL05500* gene was designated as *MET5* and further analyzed. We constructed the *met5*Δ mutant using the NEO marker (**Figure 4**a). The successful mutant was termed *met5*Δ*::*NEO and confirmed using PCR (**Figure 4**c). The complement of the *met5*Δ mutant (*met5*Δ+*MET5*) was constructed using the HYG marker and was confirmed by PCR (**Figure 4**b, d). The characteristics of these strains were further analyzed.

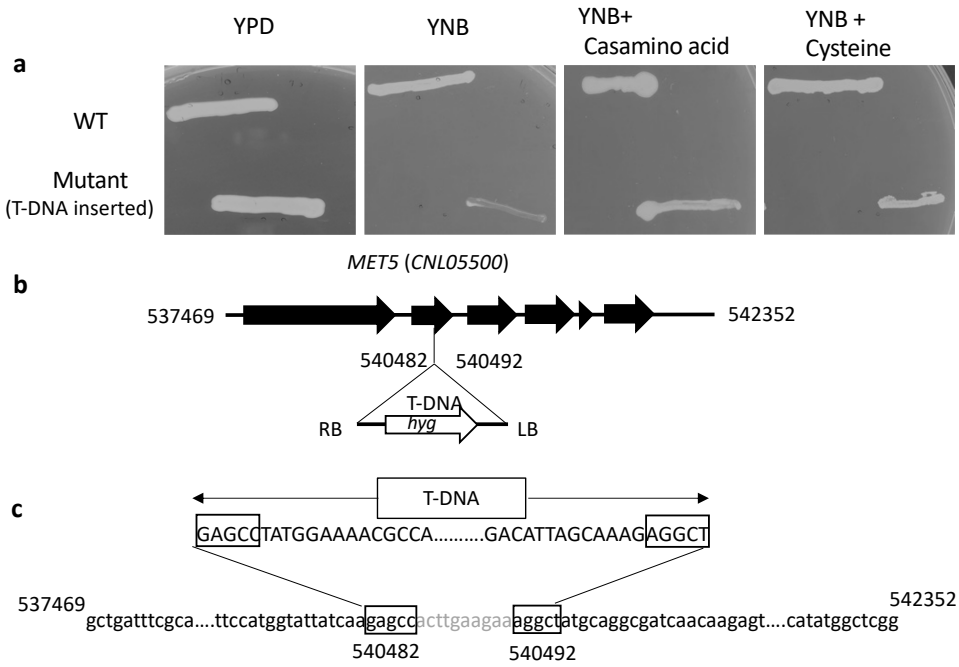


Figure 3. Identification of *MET5*, the gene encoding sulfite reductase in *C. neoformans*

a Growth of the T-DNA mutant strain on YPD, YNB, and YNB supplemented with 5 mM Casamino acid or 5 mM cysteine, respectively (top line: WT KN3501α; second line: T-DNA-inserted mutant). **b** Structure of the *CNL05500* locus. The arrows indicate the coordinates of the six exons on chromosome 12. **c** TAIL-PCR showed that the T-DNA was inserted between nucleotides 540482 and 540492 within the second exon of the *CNL05500* gene.

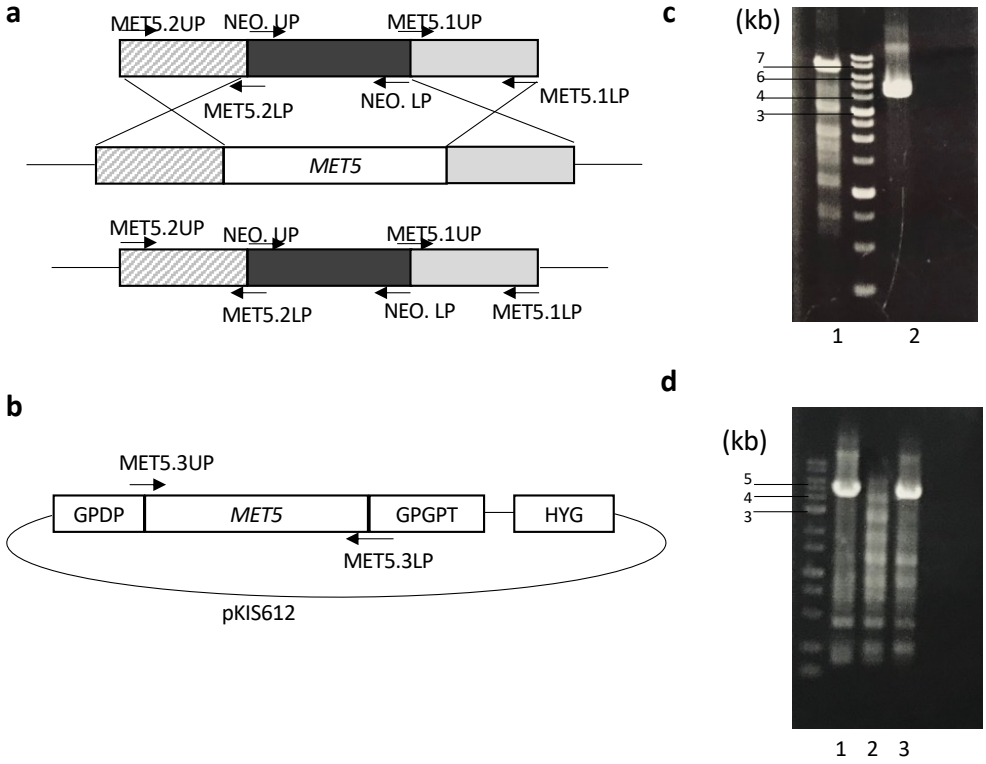


Figure 4. Construction of the *met5*Δ mutant using a NEO marker and of its complement using a HYG marker

a The *MET5* gene (open box) was replaced with the neomycin-resistance cassette (NEO, closed box). **b** Reconstitution of *MET5* with the Hygromycin B marker (HYG). **c** *MET5* gene (4884 bp with 2000 bp of flanking sequences) and *met5*Δ mutant with the NEO marker (3887 bp) (1: WT; 2: *met5*Δ). The primer pair, MET5.1LP and MET5.2UP, was used to confirm the WT *MET5* and *met5*Δ mutant genes. **d** Reconstitution of the *MET5* gene was confirmed by reintroducing the gene into *met5*Δ mutant (1: WT; 2: *met5*Δ, and 3: *met5*Δ +*MET5*) with primers, MET5.3UP, and MET5.3LP.

Role of the *MET5* gene in the sulfur-containing amino acid metabolism of *C. neoformans*

Compared with the WT strain, the *met5*Δ mutant could not grow on YNB medium but grew well on medium containing Cys as a sole sulfur source, while the *met5*Δ complement

strain exhibited growth recovery, to the level of the WT strain (**Figure 5**). This was similar to the results obtained for the T-DNA-inserted mutant (**Figure 3**a). The *C. neoformans met5*Δ mutant grew well in the presence of Cys but grew poorly on Met (**Figure 5**). In *Sa. cerevisiae*, the *MET5* gene encodes a sulfite reductase and the *met5*Δ mutant grows well in the presence of either Met or Cys (Masselot and de Robichon-Szulmajster 1975). Thus, to confirm whether the *MET5* gene is involved in the sulfate assimilation pathway of *C. neoformans*, the *met5*Δ mutant was grown on different sulfur sources. As shown in **Figure 5**, the *met5*Δ mutant grew on sulfide, but not on either sulfate or sulfite, while the *met5*Δ+*MET5* strain grew at a level similar to that of the WT strain. These results confirmed that the *MET5* gene encodes a sulfite reductase involved in the sulfate assimilation pathway of *C. neoformans*.

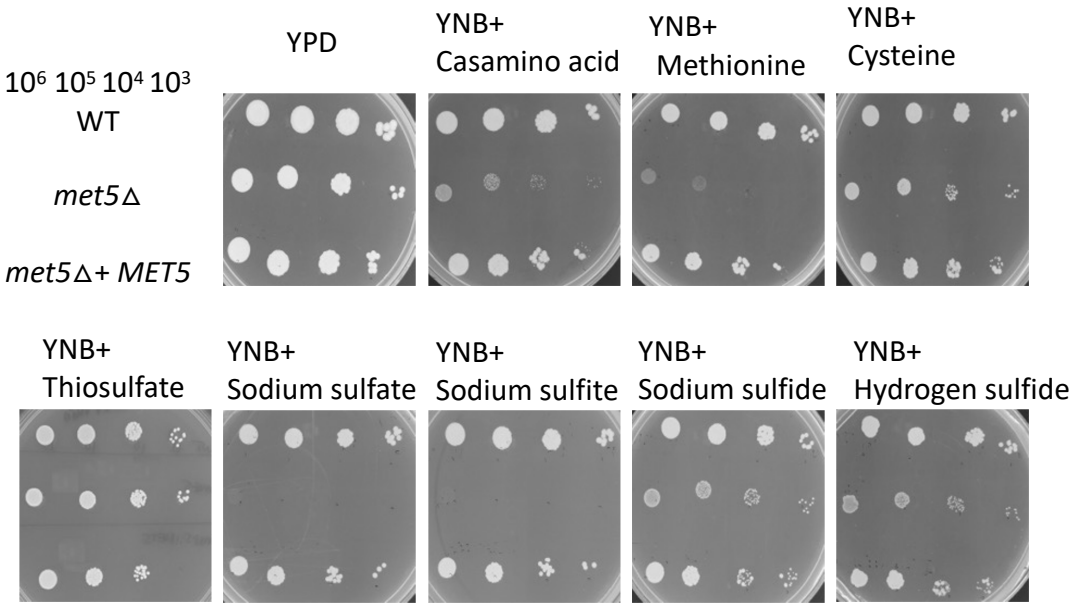


Figure 5. Phenotypes associated with the sulfite reductase encoding *MET5* gene

The growth of the WT, *met5*Δ mutant, and *met5*Δ+*MET5* complement strains were examined on YPD and YNB supplemented with the indicated supplements (5 mM). Plates were incubated for 4 days at 30°C.

In *Sa. cerevisiae*, sulfite reductase, which catalyzes the direct reduction of sulfite into sulfide, is a heterodimer enzyme encoded by β subunit *MET5* and α subunit *MET10*; therefore, *MET5* and *MET10* exhibited an identical phenotype (Thomas and Surdin-Kerjan 1997). Furthermore, based on a BLAST search against the *C. neoformans* genome database, a *MET10* (*CNG03990*) homolog was identified. A *MET10* mutant (*met10*Δ) was constructed and tested for sulfur source requirement. As shown in **Figure 6**, the *met10*Δ mutant required the same sulfur sources as the *met5*Δ mutant.

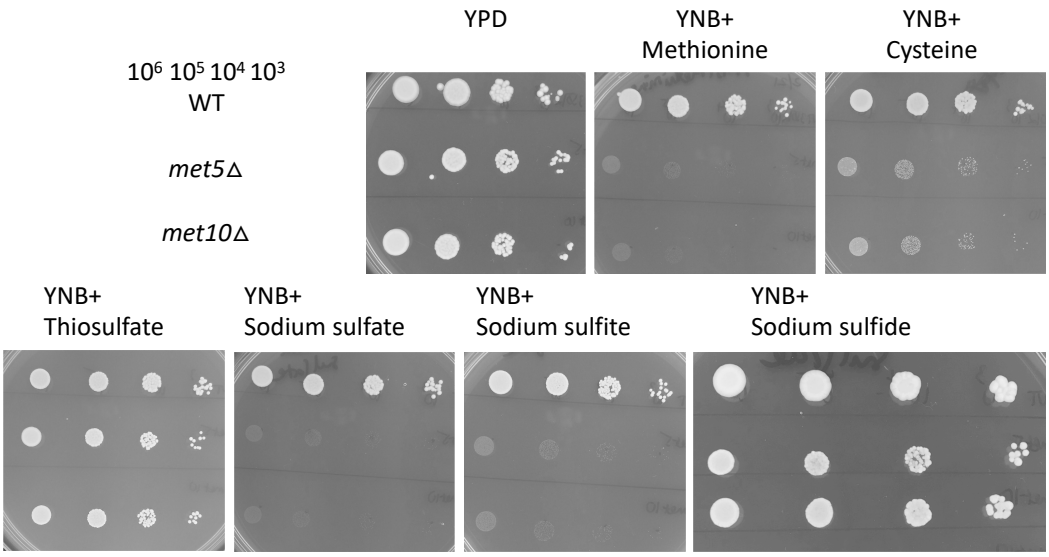


Figure 6. Phenotypes associated with the *MET5* and *MET10* genes

Growth of the WT, *met5*Δ, and *met10*Δ strains on YNB medium supplemented with different sole sources of sulfur (5 mM). Plates were incubated for 4 days at 30°C.

DISCUSSION

This work described the cloning *MET5* gene of *C. neoformans, met5*Δ mutant genes showed the grow ability on sulfide but not on sulfate or sulfite. This gene showed same phenotype and high similarity with sulfite reductase sequences *MET5* **β** subunit of *Sa.*

cerevisiae. By using the homologous sequence, *MET10* **α** subunit of *Sa. cerevisiae* was found in *C. neoformans*. As predicted, mutant strain showed the grow on sulfide but not on sulfate and sulfite similar to *met10*Δ mutant of *Sa. cerevisiae*. Therefore, the function of *MET5* and *MET10* genes in sulfate assimilation pathway were recognized in *C. neoformans* as in *Sa. cerevisiae*. In addition, according to Chet at al. (2018), thiosulfate is converted into sulfite and sulfide by the genes RDL1/2 in *Sa. cerevisiae*. Therefore, I also tested *met5*Δ and *met10*Δ mutant on the use of thiosulfate as the sole sulfur source for growth. As in *Sa. cerevisiae*, *C. neoformans* also showed the growth of *met5*Δ and *met10*Δ mutant on the growth of supplement of thiosulfate (**Figure 5**, **Figure 6**).

Currently, in sulfate assimilation pathway of *C. neoformans* only *MET3* encodes ATP sulfurylase, which converts sulfate to APS in the inorganic sulfur assimilation was found. The *met3*Δ mutant has been observed to grow well on cysteine and methionine (Yang et al. 2002). However, the *C. neoformans met3*Δ mutant did not grow well as *Sa. cerevisiae met3*Δ mutant in the presence of exogenous methionine, suggesting poor transport for methionine in this yeast compared to *Sa. cerevisiae* (Bachhawat and Yadav 2010). The slow grow on methionine also observed with *met5*Δ and *met10*Δ mutants in *C. neoformans* even through these strains grow well in both methionine and cysteine in *Sa. cerevisiae* (Thomas and Surdin-Kerjan 1997). The different responses of these two species could be due to the methionine/cysteine uptake systems. The characterization of amino acid auxotroph genetically evaluates the potential of genes products as antifungal drug targets. By this criteria, *MET5* gene of *C. neoformans* is a good candidate for developing antifungal drug target on this species.

Chapter 3. Metabolism of sulfur amino acids in *Cryptococcus neoformans*

INTRODUCTION

Cryptococcus neoformans is a pathogenic yeast with worldwide distribution (Lin and Heitman 2006; Li and Mody 2010) that has been recognized as the most common serious infectious agent causing meningitis in patients with acquired immunodeficiency syndrome (AIDS) (Dismukes 1988; Hajjeh et al. 1995). Despite the high threat posed by this yeast, the number of drugs available for the management of cryptococcal meningitis in patients with HIV infection is limited (Saag et al. 1999). Therefore, the investigation of new antifungal agents for *C. neoformans* is urgent.

C. neoformans has been extensively used as a model organism to study virulence in animals and has emphasized the idea that sulfur assimilation may be an important feature of fungal pathogenicity (Yang et al. 2002; Pascon et al. 2004; Toh-e et al. 2017). Contrary to microorganisms and plants, humans do not have an assimilatory mechanism for inorganic sulfur; thus, they require methionine (Met) as an essential amino acid (Sohn et al. 2014). There is a link between sulfur uptake, biosynthesis of Met and cysteine (Cys), and the survival of *C. neoformans* in the host (de Melo et al. 2019). Previous studies of the sulfur metabolic pathway of *C. neoformans* have focused on the effects on the metabolism and virulence of this pathogen. The *MET2*, *MET3*, and *MET6* genes have been shown to be involved in methionine biosynthesis, and the disruption of these genes leads to Met auxotrophy (Yang et al. 2002; Pascon et al. 2004; Nazi et al. 2007). The involvement of the *MET17*, *CYS1*, and *CYS2* genes in the O-acetyl-serine (OAS) and

homocysteine synthase (O-acetyl-homoserine sulfhydrylase (OAH) pathways was described by Toh-e et al. (Toh-e et al. 2017), which provided added knowledge on sulfur metabolism in *C. neoformans*. Recently, the *CYS3* (*CNAG04798*) gene, which confers Met/Cys auxotrophy, was identified as a major regulator of inorganic sulfur uptake in *C. neoformans* (de Melo et al. 2019).

Overall, five sub-pathways have been tentatively proposed for sulfur amino acid biosynthesis in *C. neoformans* (Toh-e et al. 2017; de Melo et al. 2019): The sulfate assimilation pathway, OAS pathway, OAH pathway, transsulfuration pathway, and methyl cycle (**Figure 7**). Although the sulfur metabolic pathway has been extensively studied in other yeasts, such as *Sa. cerevisiae* and *Sc. pombe* (Thomas and Surdin-Kerjan 1997; Yamagata 2007), the knowledge on the sulfur metabolic pathway of *C. neoformans* remains incomplete, especially the transsulfuration pathway between homocysteine and Cys. The transsulfuration pathway occurs in both directions from homocysteine to Cys (reverse transsulfuration pathway) and vice versa (forward transsulfuration pathway) in *Sa. cerevisiae* (Thomas and Surdin-Kerjan 1997), whereas the forward transsulfuration pathway has been exclusively identified in *Sc. pombe* (Brzywczy and Paszewski 1994) (**Figure 8**). In *C. neoformans*, however, the transsulfuration pathway remains controversial (Toh-e et al. 2017; de Melo et al. 2019).

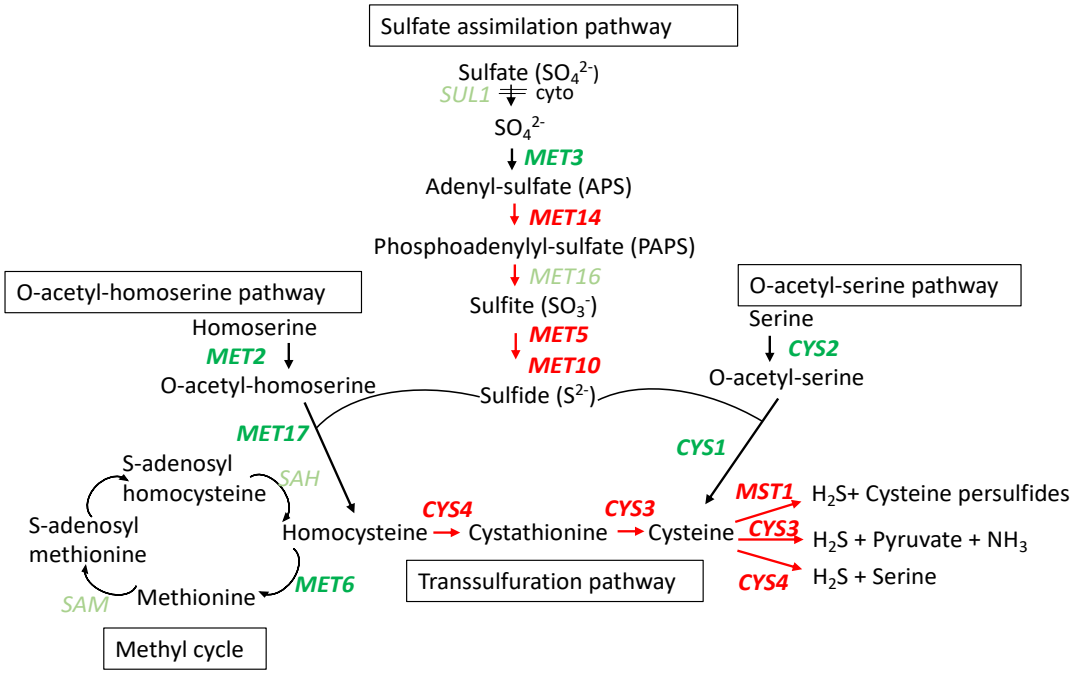


Figure 7. Sulfur-containing amino acid metabolic pathways proposed for *C. neoformans*

The genes were named after the homologous genes identified in *Sa. cerevisiae*. The red color indicates genes for which the function was identified in this study, the bold green color indicates those identified in previous studies, and the light green color indicates the proposed gene in the pathway. The arrows indicate the pathways that were proposed in this study (red color) or in previous studies (black color).

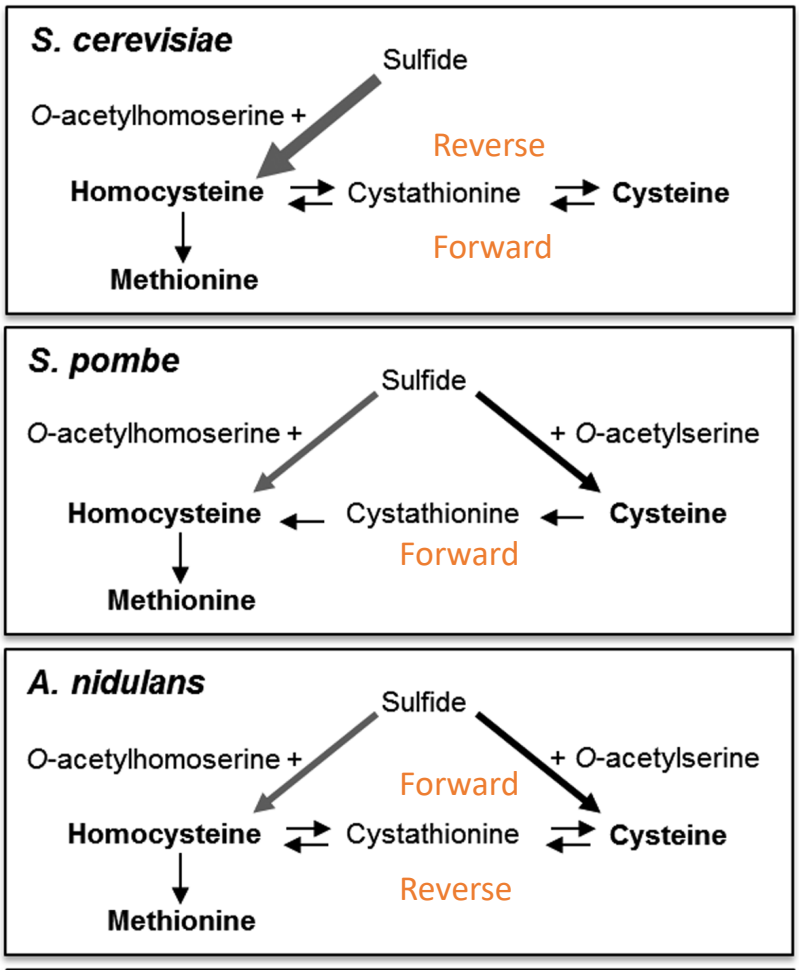


Figure 8. Different pathways in yeast and filamentous fungal species (Sohn et al. 2014)

MATERIALS AND METHODS

Strains, medium composition, and growth conditions

Serotype D *C. neoformans* KN3501α (Nielsen et al. 2005) and strains generated using KN3501α were used throughout this work (**Table 2**). Strains were grown on solid YPD medium (1% yeast extract, 2% polypepton, 2% glucose, and 2% agar) or minimal YNB medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco), 0.5% ammonium sulfate, 2% glucose, and 2% agar). Sulfur-free B medium was prepared according to the description of Cherest and Surdin-kerjan (Cherest and Surdin-Kerjan

1992). The B medium was solidified by adding 2% agarose (Invitrogen/ ThermoFisher Scientific).

Additionally, a spot assay was used to examine the growth of each strain in a medium containing different sulfur sources. First, overnight cultures in YPD liquid were washed twice with sterile distilled water. Cells were counted using a counting chamber and serially diluted to 10^3–10^6 cells/ml. Five ml of each dilution was spotted onto plates containing 5 mM of different sulfur sources. The plates were incubated at 30°C for 4 days. Each experiment set was replicated thrice.

For the liquid culture, each strain was examined in 5 ml of liquid YNB-based medium with different sulfur sources at 30°C with shaking at 150 rpm. OD_{600nm} of the suspensions was monitored using an initial value of 0.1 until a plateau was reached. The experiment was replicated thrice.

Table 2. Strains used in this study

Strain	**Genotype**	**Source**
KN3501 α	*MATα* Wildtype	Nielsen et al. 2005
KN3501 a	*MATa* Wildtype	Nielsen et al. 2005
CAT706	*MATα ura5 met3::URA5*	Toh-e et al. 2017
CAT789	*MATα ura5 cys1::URA5*	Toh-e et al. 2017
CAT915	*MATα ura5 met2::URA5*	Toh-e et al. 2017
CAT1972	*MATα ura5 met17::URA5*	Toh-e et al. 2017
CAT594	*MATα ura5 cys4::HYG cku70::NEO*	Toh-e et al. 2017
CAT2685	*MATα ura5 cys3::HYG*	Toh-e et al. 2017
CAT1603	*MATa ura5 met6::URA5*	Toh-e et al. 2017
CAT2283	*MATa ura5cys1::URA5 cys3::HYG*	Toh-e et al. 2017

CAT2741	*MATα ura5 met3::URA5 mst1::NAT cys3::HYG cys4::HYG*	Toh-e et al. 2017
*met5*Δ	*3501α met5::NEO*	This study
*met5*Δ*+MET5*	*3501α met5::NEO+MET5*	This study
*met10*Δ	*3501α met10::NEO*	This study
*met14*Δ	*3501α met14::NEO*	This study
*cys3*Δ*met5*Δ	*ura5 cys3::HYG, met5::NEO*	This study
*cys4*Δ*met5*Δ	*MATα ura5 cys4::HYG cku70::NEO, met5::NEO*	This study

Table 3. Primers used in this study

Gene name	**Primer name**	**Sequence (5'->3')**
	NATNEOHYG. UP	GAAGAGATGTAGAAACTAGC
	NATNEOHYG. LP	AGGATGTGAGCTGGAGAGCG
	URA5-5	CTTGGTGGACTGATTGTGAT
	URA5-3	CTCCCCACCTTCCCACTTCC
	TAILKS3	cctgtgtgaaattgttatccgctca
	TAILKS4	tcacattaattgcgttgcgctcact
	TAILKS5	agaggcggtttgcgtattggctaga
	TAILKSO	gcacactgcgaattcgagacagaca
	TAILKS1	aaccaagggcgaattccagcacact

	TAILKS2	tcccaacagttgcgcagcctgaatg
	AD1	ntcgastwtsgwgtt
	AD2	ngtcgaswganawgaa
MET5	MET5.1LP	gaaccacagccaagtctgctgtctc
	MET5.2UP	gctcatcatagttcaataagccgag
	MET5.1UP	GCGCCGCTCTCCAGCTCACATCCTatgattcaagttgtaatggc
	MET5.2LP	GGAAGCTAGTTTCTACATCTCTTCctgaataaaaaaaagtcagc
	MET5.3UP	atgtctgtcctcgccgccatctcct
	MET5.3LP	ctatgccgtcacagttgtgcctgcc
MET10	CNG03990R	taacgatccctggtatcaaacacca
	CNG03990F	ttactctggagcgtagcgtggtgct
	CNG03990KO-R	GGAAGCTAGTTTCTACATCTCTTCtttgcgagaaagaattgcgg
	CNG03990KO-F	GCGCCGCTCTCCAGCTCACATCCTacacgggtttcaagagacgc
MET14	CNE03380F	ccgcacctgctccacgaaggatgat
	CNE03380R	agtaaacatggaatcgcgtagatgg
	CNE03380KO-F	GGAAGCTAGTTTCTACATCTCTTCaaaggaaaagagaaaaaaga
	CNE03380KO-R	GCGCCGCTCTCCAGCTCACATCCTttttcaaacgttttatggat
MET17	CNC01220R	acaaaagcgccatgcaattccttgg
	CNC01220F	cttcccagaccactcatgcgaacga
	deltamet17F	tgtttcgtcacgtatggctgagagg
	deltamet17R	cgaaatgtcgtgcgcgtgccaaatg
CYS1	CNL05880F	accaaatagccagcgaggaaagatc

	CNL05880R	ctaattcctaacacgcagtaccacc
	deltaCYS1R	tggctgctggatgtctagaagtgaa
	deltaCYS1F	ggacaaggtcgagtcaccaacaacc
CYS3	cys3-5F	gtccgtgagcagacattcattacct
	cys3-4R	catccatcatacacacatagcccta
	cys3-6R	gacaataaaggcctgatctgcgaga
	cys3-7F	ggctggaaattccgaccaatccaac
CYS4	cys4-6	tccgtcgtgtattttattcgcctcg
	cys4-5	attagttgcgagacattgcgacagc
	delta cys4F	gtaaggcagcactcaatacctaaag
	delta cys4R	ctgctatggtgtatatctacgtagc

RNA extraction, RNA-Seq, and quantification analyses

The WT KN3501α strain was grown in liquid B medium supplemented with four different sulfur sources, i.e., Met, Cys, homocysteine, or sodium sulfate, for 24 h at 30°C with shaking at 150 rpm by rotary shaker NR20. Cells were collected by centrifuge Hitachi CT15RE, frozen in liquid nitrogen, and lyophilized overnight. Total RNAs were purified using the Fast Gene Premium Kit (Nippon-Genetics) according the protocol provided by the manufacturer. The purity of the RNA was measured on a NanoDrop 2000 instrument (Thermo Fisher). The RNA levels and integrity were determined using the RiboGreen RNA Assay Kit on a Victor X2 Fluorometer (Life Technologies, USA) and the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, USA), respectively. One library was prepared using the TruSeq Stranded mRNA Library Prep Kit (Illumina), and cDNA was sequenced on an Illumina NovaSeq 6000 instrument in a 2 × 150 paired-end protocol. Read merging, adapter trimming, and quality control were

performed by cutadapt ver. 2.5 (Martin 2011). HISAT v.2.1.0 (Kim et al. 2015) was used to map the short reads to the annotated genome sequence of *C. neoformans* JEC21. Subsequently, the transcripts for each sample were assembled and integrated using Stringtie v2.0.4 (Pertea et al. 2015) and Cuffmerge v2.2.1 (Trapnell et al. 2010), respectively. The gene expression levels were measured in fragments per kilobase of transcript per million mapped reads (FPKM) using Cuffdiff v2.2.1 (Trapnell et al. 2010) and then converted to transcripts per kilobase million. Genes related to the sulfur-containing amino acid biosynthesis pathway were selected and indicated on the heatmap. A heatmap of these genes was prepared using R package gplot version 3.0.1 (Warnes et al. 2016) in R studio (RStudio Team 2016).

RESULTS

Sulfur-containing amino acid metabolic pathways of *Cryptococcus neoformans*

Sulfate assimilation pathway

In *Sa. cerevisiae*, sulfate is absorbed into cells to yield adenosine phosphosulfate (APS) via ATP sulfurylase (*MET3*), followed by APS kinase (*MET14*), to produce 3`-phosphoadenosine-5` phosphosulfate (PAPS), which is then converted to sulfide by PAPS reductase (*MET16*) and sulfite reductase (*MET5/MET10*) (de Melo et al. 2019). To examine the relationship between *MET5* and other genes involved in the sulfate assimilation pathway of *C. neoformans*, a series of mutant strains involved in this pathway were grown on various sulfur sources. To date, only the ATP sulfurylase gene *MET3* has been analyzed as a Met auxotrophy-conferring gene in *C. neoformans* (Yang et al. 2002). A BLAST search using the amino acid sequences of *MET14* of *Sa. cerevisiae* and *A.*

nidulans showed that *CNE03880* was its homolog in *C. neoformans* with 67% and 61% identity respectively (**Supplementary Figure 1**). The *met14*Δ mutant of *C. neoformans* could grow with sulfite or sulfide, but not with sulfate (**Figure 9**). This suggests that the *MET14* gene functions as an APS kinase. In *Sa. cerevisiae*, mutant strains of the *MET3*, *MET14*, and *MET5* genes grow well in the presence of Cys or Met (Thomas and Surdin-Kerjan 1997). However, the *C. neoformans met3*Δ, *met14*Δ, and *met5*Δ mutants showed better growth in the presence of Cys vs. Met (**Figure 9**). Growth conditions for these strains were confirmed by similar results in liquid cultures (**Figure 10**).

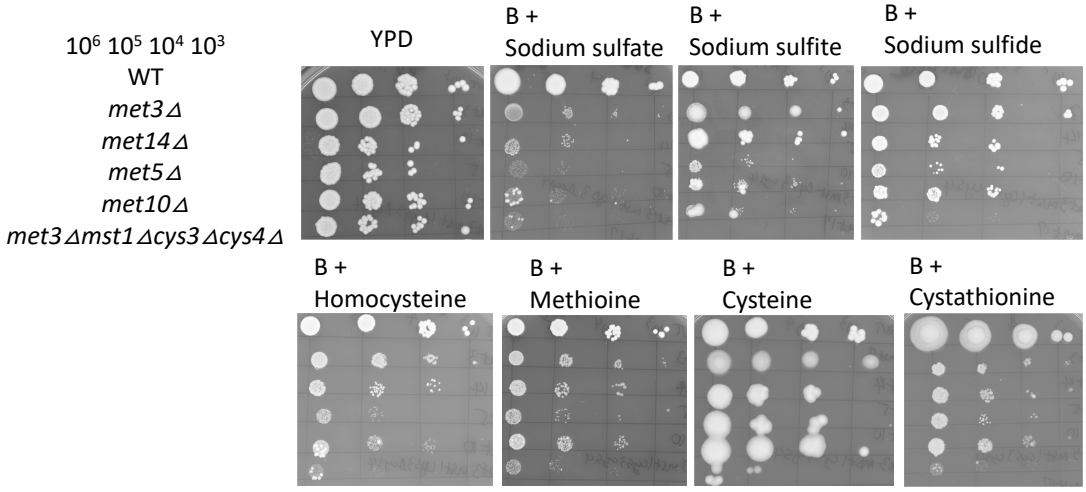


Figure 9. Sulfate assimilation pathway in *C. neoformans*

The WT, *met3*Δ*, met14*Δ, *met5*Δ, and *met10*Δ, and *met3*Δ*mst1*Δ*cys3*Δ*cys4*Δ quadruple mutant strains were inoculated on B medium supplemented with different sulfur sources at 5 mM. Plates were incubated for 4 days at 30°C.

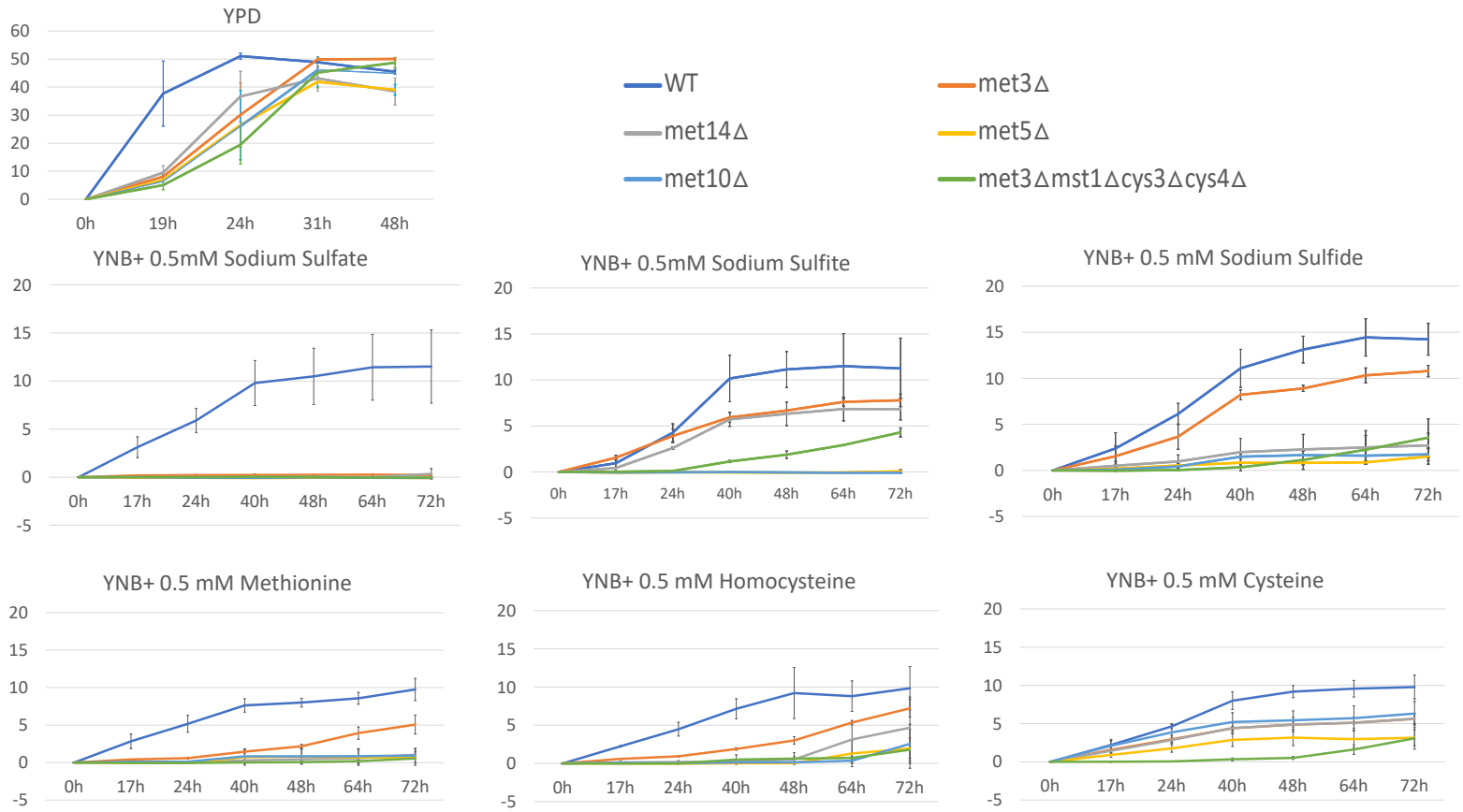


Figure 10. Growth rates

Cells of indicated strains were measured at OD_{600mm} in YPD or YNB media supplemented with different sulfur sources (5 mM) and incubated at 30°C. The data are represented as mean ± standard deviation of three determinations.

Biosynthesis of cysteine and homocysteine

The filamentous fungus *Aspergillus nidulans* possesses two pathways for Cys biosynthesis, one from the OAS pathway and the other from homocysteine through the transsulfuration pathway. *Sa. cerevisiae* synthesizes Cys from homocysteine via the transsulfuration pathway because the OAS pathway is not present in this yeast. The fission yeast *Sc. pombe* produces homocysteine from OAH; however, Cys is synthesized only from the OAS pathway, as this yeast lacks the reverse transsulfuration pathway (Brzywczy and Paszewski 1994; Thomas and Surdin-Kerjan 1997; Sohn et al. 2014). It has been shown that *C. neoformans* possesses both the OAS and OAH pathways (**Figure 7**) (Toh-e et al. 2017). In *C. neoformans*, *MET2* encodes homoserine transacetylase (Nazi et al. 2007), and the *met2*Δ mutant grows on Met or homocysteine, but not on Cys or cystathionine (**Figure 11**). It was suggested that the *CYS1* gene is required for Cys synthesis and that Cys is synthesized solely through the OAS pathway (Toh-e et al. 2017). The *cys1*Δ mutant grew well on Cys, but slowly on Met or homocysteine (**Figure 11**). In fact, while the mutant was hardly observed on the plates with serial dilution spotting, it could be detected when the strain was streaked and grown for 10 days (**Figure 11**b). However, the use of cystathionine, which is an intermediate metabolite between Cys and homocysteine in the transsulfuration pathway, as a sole sulfur source yielded *cys1*Δ mutant growth after 10 days of incubation at 30°C (**Figure 11**a). This observation indicates that the conversion from cystathionine to Cys is active in *C. neoformans*. In contrast, the *met17*Δ mutant grew on B medium with homocysteine or Met, but not on Cys or cystathionine (**Figure 11**). These results indicate that the conversion from homocysteine to Cys via the reverse transsulfuration pathway is exclusively active, and that the forward transsulfuration pathway is absent in *C. neoformans*.

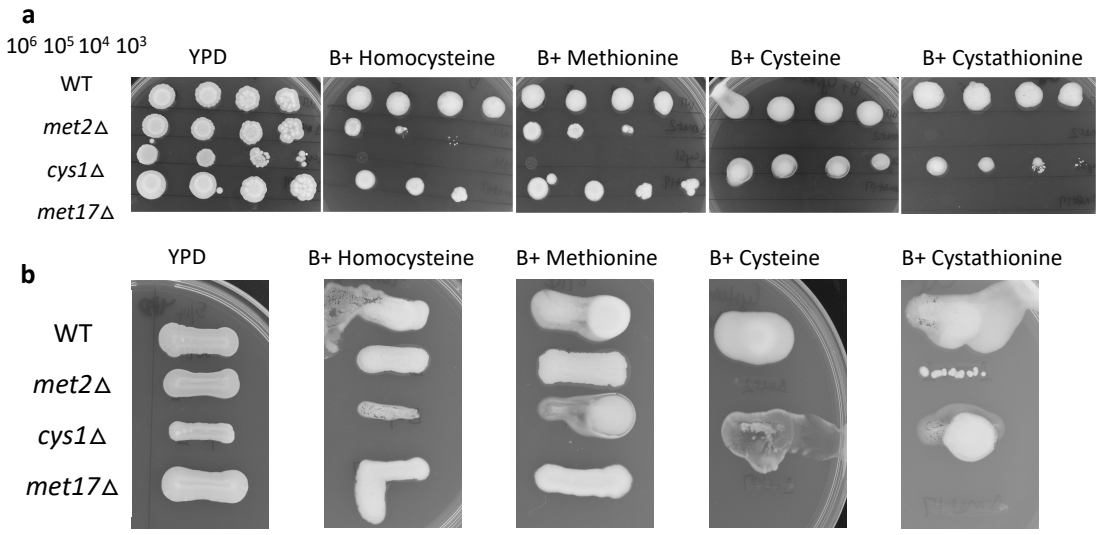


Figure 11. Cysteine–homocysteine synthesis pathway in *C. neoformans*

a WT, *met2*Δ, *cys1*Δ, and *met17*Δ strains were inoculated on B medium supplemented with different sulfur sources at 5 mM, followed by incubation for 4 days at 30°C. **b** WT, *cys1*Δ, *met2*Δ, and *met17*Δ strains were streaked on B medium supplemented with different sulfur sources at 5 mM, followed by incubation for 10 days at 30°C.

Transsulfuration pathways

The transsulfuration pathway consists of reactions that allow the interconversion between homocysteine and Cys via the cystathionine intermediate. Both the forward and reverse transsulfuration pathways exist in *Sa. cerevisiae* and involve two different sets of enzymes (Thomas and Surdin-Kerjan 1997). The synthesis of Cys from homocysteine is catalyzed by cystathionine β-synthase (*CYS4*) and cystathionine γ-lyase (*CYS3*), while the synthesis of homocysteine from Cys is catalyzed by cystathionine γ-synthase (*STR2*) and cystathionine β-lyase (*STR3*) (Santiago and Gardner 2015; Huang et al. 2016). The amino acid sequence encoded by *CNK01740* in *C. neoformans* was found to most related to *Sa. cerevisiae STR3* and *A. nidulans METG* when using the BLAST search with identity was 45% and 58%, respectively (**Supplementary Figure 1**). Therefore, mutant *str3*Δ was created and inoculated on different sulfur sources. As showed in **Figure 12**, if

CNK01740 functions as cystathionine β-lyase (*STR3*) then I can predict that the deletion of the *CNK01740* gene results in the Met auxotroph. However, the mutant of *str3*Δ grew well on any sulfur sources tested. Thus, the gene *STR3* may not be involved at all in the transsulfuration pathway of *C. neoformans.* In addition, mutant *met2*Δ and *met17*Δ could not grow on cysteine and cystathionine (**Figure 11**). These results indicate the forward transsulfuration pathway from cysteine to homocysteine could be absent in *C. neoformans*.

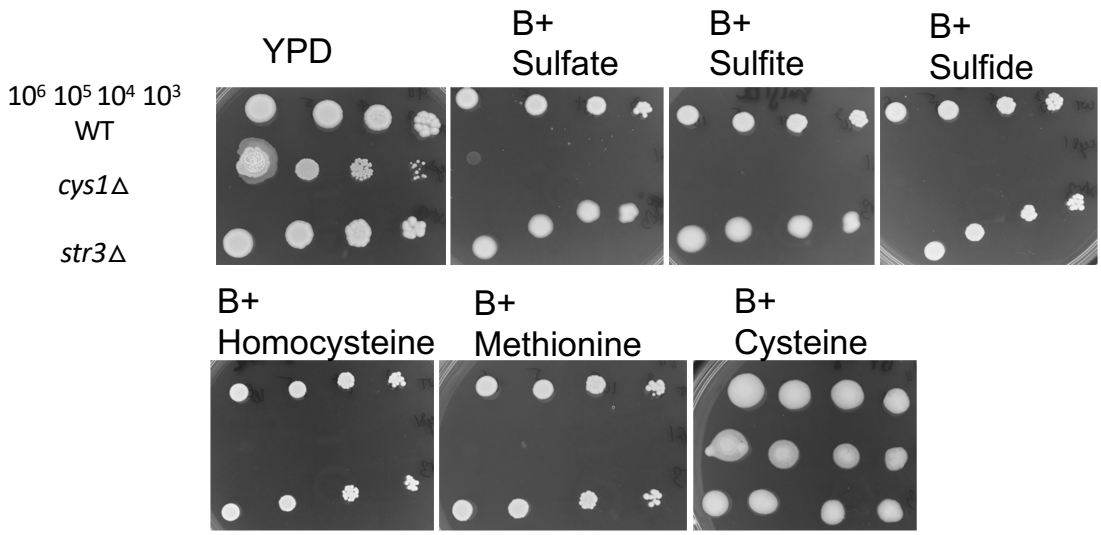


Figure 12. Evaluation of the forward transsulfuration pathway from cysteine to homocysteine

WT, *cys1*Δ, and *str3*Δ strains were inoculated on B medium supplemented with different sulfur sources at 5 mM.

The *cys1*Δ mutant grew on medium containing cystathionine or Cys, whereas the *cys1*Δ*cys3*Δ double mutant grew only on Cys, and not on cystathionine (**Figure 13**a), even after a longer incubation (10 days) (**Figure 13**b). This suggests the participation of the *CYS3* gene in the conversion from cystathionine to Cys. In addition, the active reverse transsulfuration pathway from homocysteine to Cys in *C. neoformans* was confirmed.

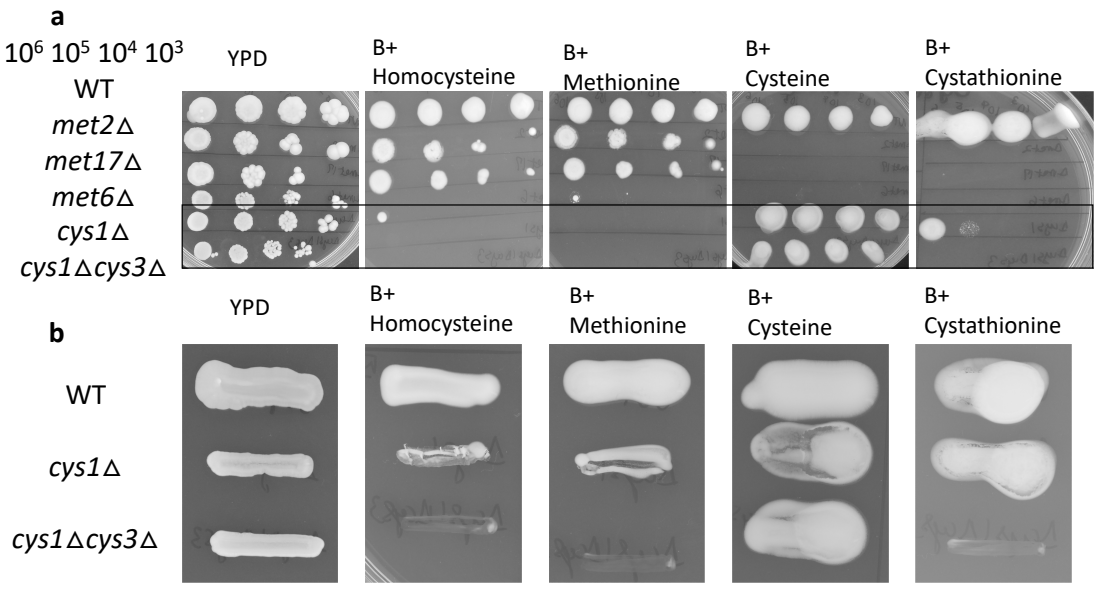


Figure 13. Conversion from cystathionine to cysteine by the enzyme encoded by *CYS3*

a WT, *cys1*Δ, and *cys1*Δ*cys3*Δ strains (square box) were inoculated on B medium supplemented with different sulfur sources at 5 mM. **b** WT, *cys1*Δ, and *cys1*Δ*cys3*Δ strains were streaked on B medium supplemented with different sulfur sources at 5 mM, followed by incubation for 10 days at 30°C.

The active reverse transsulfuration pathway (from homocysteine to Cys) was proposed in *C. neoformans* by the functional analysis of the *CYS3* and *CYS4* genes (Toh-e et al. 2017). To confirm the function of the *CYS3* and *CYS4* genes, *cys3*Δ and *cys4*Δ mutants were tested on medium containing different sulfur sources. The *cys4*Δ mutant grew well on B medium supplemented with Met, but poorly in the presence of Cys. In contrast, the *cys3*Δ mutant grew in the presence of any of the sulfur sources (**Figure 14**). Hence, I hypothesized that the *CYS3* and *CYS4* genes have other functions in the sulfur-containing amino acid biosynthetic pathways. In *Sa. cerevisiae*, both *CYS3* and *CYS4* cleave Cys and release sulfide in vitro (Hopwood et al. 2014; Huang et al. 2017). In addition, the *TUM1* gene of *Sa. cerevisiae* produces sulfide from Cys, similarly to the 3-mercaptopyruvate sulfurtransferase (*3-MST*) gene in humans (Flannigan and Wallace

2015; Huang et al. 2016, 2017). A homolog of *TUM1* was identified in *C. neoformans* and was termed *MST1* (*CND03690*). Therefore, the *cys3*Δ*met5*Δ and *cys4*Δ*met5*Δ double mutants and the *met3*Δ*mst1*Δ*cys3*Δ*cys4*Δ quadruple mutants were constructed to block all possible pathways that produce sulfide in *C. neoformans* and tested on minimal medium supplemented with different sulfur sources. The *cys3*Δ*met5*Δ and *cys4*Δ*met5*Δ double mutants grew well on Cys (**Figure 14**a). These results were confirmed by longer incubation (10 days) (**Figure 14**b). However, the *met3*Δ*mst1*Δ*cys3*Δ*cys4*Δ quadruple mutant could not grow on any of the sulfur sources (**Figure 9** last line). These results suggest that, in addition to *MET5*/*MET10*, *CYS3*, *CYS4*, and *MST1* participate in sulfide production from Cys in *C. neoformans*.

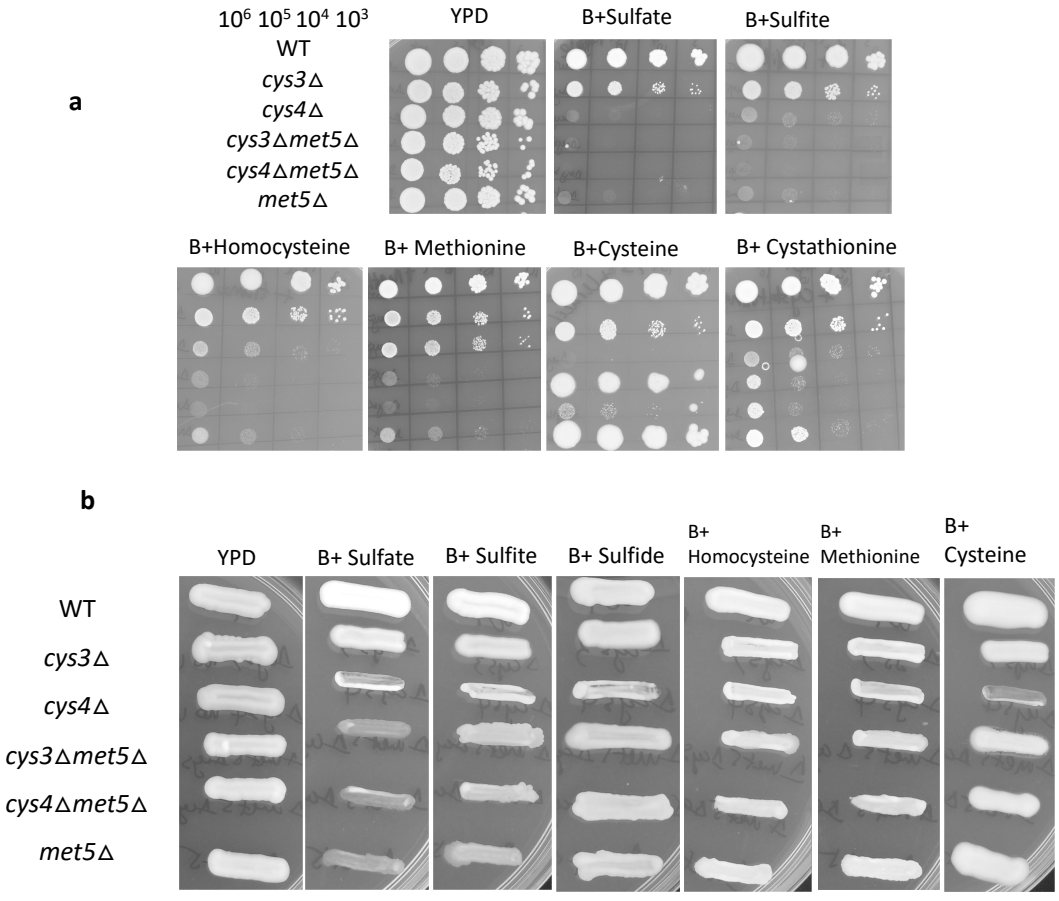


Figure 14. Transsulfuration pathway in *C. neoformans*

WT, *cys3*Δ, *cys4*Δ, *cys3*Δ*met5*Δ, *cys4*Δ*met5*Δ, *met5*Δ strains were inoculated on B medium supplemented with different sulfur sources at 5 mM. **a**; Plates were incubated for 4 days at 30°C. **b**; Plates were incubated for 10 days at 30°C

Transcription profiles of sulfur metabolic genes in *C. neoformans*

In expecting to identify the potential novel gene involved in reverse transsulfuration pathway from homocysteine to Cys. Transcriptional profiles of a *C. neoformans* WT strain under defined conditions in minimal B media supplemented with sole sulfur sources of Cys, homocysteine, Met, and sodium sulfate were examined. Genes showed expression greater than two-fold at homocysteine and cysteine than other were selected. Among 7881 genes of *C. neoformans* genome, 602 genes showed strong expression in homocysteine while there were 58 genes in Cys (**Figure 15**). Among the genes that were up-regulated in homocysteine and Cys, about half of them are currently classified as genes coding for hypothetical proteins with unknown function. Number of unknown function genes are too large to narrow down the gene expression between homocysteine and cysteine in transsulfuration pathway. Further experiments are needed to narrow down the number of genes in this transsulfuration pathway.

Data available for RNA sequences was uploaded on GenBank at GSE153693.

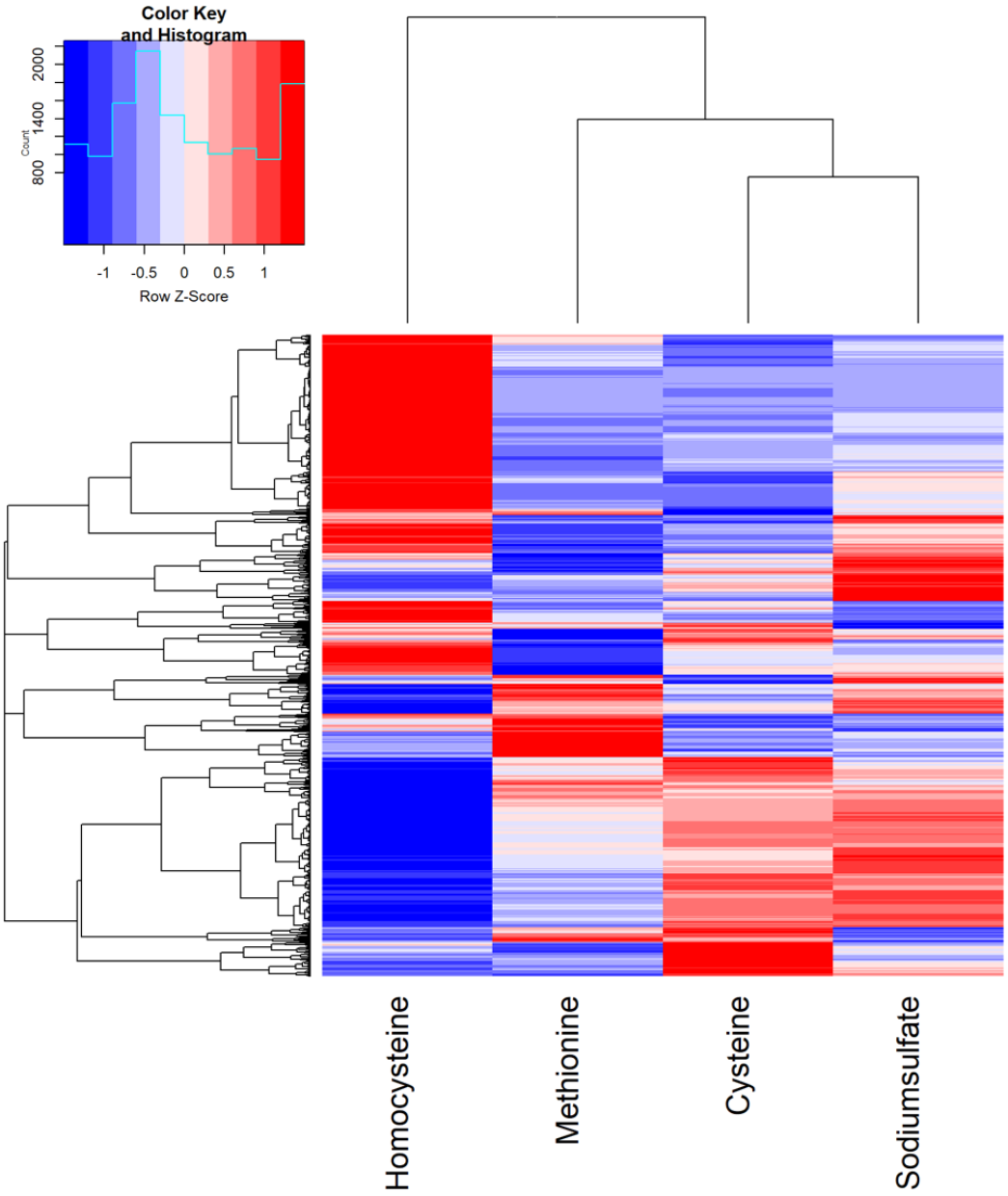


Figure 15. Gene expression in *C. neoformans* in different nutritional conditions

The WT KN3501α strain was grown on B medium supplemented with 5 mM sodium sulfate, homocysteine, methionine, and cysteine, respectively.

DISCUSSION

To date, only the *MET3* gene (encoding an ATP sulfurylase) has been shown to be involved in the sulfate assimilation pathway of *C. neoformans* (Yang et al. 2002). Based

on sequence homology in *C. neoformans*, the *CNL05500* gene is similar to sulfite reductase *MET5* of *Sa. cerevisiae* (44.75% identity). This gene was designated as *MET5* in *C. neoformans* and tested using the sulfur analysis. *MET5* was confirmed as a part of the sulfate assimilation pathway, as was *MET5* of *Sa. cerevisiae,* by the *met5*Δ mutant that grew on sulfide but not on either sulfate or sulfite. In addition, I demonstrated that the sulfite reductase *MET5* gene conferred Cys auxotrophy. The *met5*Δ mutant of *Sa. cerevisiae* can grow well in the presence of Met or Cys as a sole sulfur source (Thomas and Surdin-Kerjan 1997). In contrast, the *C. neoformans met5*Δ mutant grew well in the presence of Cys and homocysteine, and much slower in the presence of Met. The *MET14* and *MET10* genes were both involved in the sulfate assimilation pathway of *C. neoformans*. Once sulfate is internalized, these genes should operate together to produce sulfide. As expected, the transformation of sulfate was used by the active *MET3*, *MET14*, *MET5*, and *MET10* genes in the sulfate assimilation pathway of *C. neoformans*, as observed in *Sa. cerevisiae*. However, when the mutants of these genes were grown on Cys or Met as a sole sulfur source, all of the strains tested grew better on Cys than they did on Met, similar to the phenotype of the *met3*Δ strain described previously (Yang et al. 2002). In contrast, in *Sa. cerevisiae*, these mutants grow well in the presence of either Met or Cys (Thomas and Surdin-Kerjan 1997). This difference might be attributable to the presence of the reverse transsulfuration pathway (from homocysteine to Cys) alone in *C. neoformans*.

I showed that homocysteine is likely converted to Cys in *C. neoformans* because the growth of the *cys1*Δ mutant in the presence of cystathionine, while the *cys1*Δ*cys3*Δ double mutant grew on Cys but not on cystathionine. Suggested that the reverse transsulfuration pathway is functional and its activity is detectable because of the growth

of the *cys1*Δ mutant on B medium supplemented with Met and the loss of micro-colonies via the introduction of the *cys4*Δ and/or *cys3*Δ mutation into the *cys1*Δ mutant strain. In this study, the *cys1*Δ mutant grew well in the presence of cystathionine and Cys, but slightly on Met and homocysteine, while *met17*Δ did not. This result suggests that, in *C. neoformans*, the direction of conversion from homocysteine to Cys occurs, whereas the opposite does not.

The genes, *CYS3*, *CYS4*, and *MST1*, were found in *C. neoformans* based on their sequence homology with those of *Sa. cerevisiae* (Toh-e et al. 2017). Suggest that sulfide is also produced from Cys by these genes in *C. neoformans* (Hopwood et al. 2014; Flannigan and Wallace 2015; Huang et al. 2016, 2017). Similar to the synthesis of sulfide in mammalian systems, cystathionine β-synthase, which is encoded by *CYS4*, produces sulfide through a reaction involving the generation of cystathionine from homocysteine and Cys from cystathionine. Cystathionine γ-lyase, which is encoded by *CYS3*, produces sulfide through a reaction involving the generation of Cys from cystathionine (Moody and Calvert 2011; Flannigan and Wallace 2015). In *Sa. cerevisiae*, both *CYS3* and *CYS4* have been reported to cleave Cys and release sulfide in vitro (Hopwood et al. 2014; Huang et al. 2017). In addition, the *TUM1* gene from *Sa. cerevisiae* has been shown to produce sulfide from Cys, and this gene has a similar function as that of 3-mercaptopyruvate sulfurtransferase (*3-MST*) in humans (Flannigan and Wallace 2015; Huang et al. 2016, 2017). Sulfide synthesis via the function of *CYS3*, *CYS4*, and/or *MST1* was also supported by the experiment that used the *met3*Δ*mst1*Δ*cys3*Δ*cys4*Δ quadruple mutant strain. This strain could not grow on any single sulfur source, potentially because the whole sulfide synthesis pathway was blocked. Further study on the measurement of sulfide production will elucidate the involvement of these genes in the catabolism of Cys in *C. neoformans.*

Previously, various studies investigated the role of gene expression in the metabolic pathways of *C. neoformans* (Chen et al. 2014; Attarian et al. 2018; de Melo et al. 2019), but my study is the first to investigate the influence of different sulfur sources on the expression levels of genes involved in sulfur metabolism. The expression levels of genes associated with sulfur metabolism are partially different, further effort is required to identify genes controlling the transsulfuration pathway.

The yeast *Sa. cerevisiae* can generate sulfide from Cys (Jiranek et al. 1995; Huang et al. 2016). In mammals, enzymes of the transsulfuration pathway (encoded by *CYS3* and *CYS4*) and 3-mercaptopyruvate sulfurtransferase (encoded by *MST*) degrade Cys to release sulfide (Shibuya et al. 2009; Mikami et al. 2011; Kashfi and Olson 2013). Homologs of these enzymes were found in the *C. neoformans* genome. It remains unknown whether these proteins function in a similar fashion to that of human sulfurtransferase. Although *C. neoformans* shares some sulfur pathways with *Sa. cerevisiae*, the differences observed in the transsulfuration pathway broaden our basic understanding of the sulfur metabolic networks among organisms and might be informative for the development of antibiotics targeting the metabolism of amino acids.

Chapter 4. Involvement of *MET5* gene in virulence of *Cryptococcus neoformans*

INTRODUCTION

To prevent pathogen invasion, hosts develop the immune systems. The immune systems of mammals involve antibodies to recognize the specific invaders. Insects do not produce antibodies, although they do develop natural immune systems. To identify a potential antifungal drug target, evaluation of virulent character of auxotrophic mutant on animal is a commonly recommended approach. In *C. neoformans MET3* (encoding an ATP sulfurylase) has been suggested as a potential drug target, because a *met3*Δ strain was avirulent to mice in contrast to the WT strain and the reconstituted strain both of which completely killed mice (Yang et al. 2002). A similar result was also observed for a methionine synthase gene *MET6* of *C. neoformans*. While the mice infected with the WT or the reconstituted strain died within 25 days, none of the mice infected with *met6*Δ mutant died up to 70 days post infection, indicating that *MET6* gene is required for virulence (Pascon et al. 2004). Although mouse infection models are widely used to evaluate the virulence of pathogens, the use of large number of mammals for infection experiments have been argued because of the cost and ethical problems. Silkworms, larvae of *Bombyx mori*, have been recently introduced as a cost-effective animal model. They can reproduce in a short period of time and can be easily maintained in laboratories. This infection model has been used and tested for the capability to determine pathogenicity of a wide variety of pathogens (Kaito et al. 2002; Kaito 2016; Tsai et al. 2016). Further, *C. neoformans* has also been shown to be virulent against silkworm after

injection (Matsumoto et al. 2012). It was shown that the mutant strains with decreased virulence against mice were also less virulent against silkworms. These results suggest that the virulence of a human pathogen *C. neoformans* can be evaluated using silkworm infection models. To evaluate the necessity of *MET5* gene on the virulence of *C. neoformans*, silkworm infection model was established to examine the virulence of *met5*Δ mutant strain in comparison with the WT and the complement strains.

MATERIAL AND METHODS

Culture conditions and fungal strains

C. neoformans strains: WT KN3501α, *met5*Δ and the *met5*Δ+*MET5* complement was cultured in liquid YPD (1% yeast extract, 1% polypepton, and 1% glucose) medium at 30ºC with shaking at 150 rpm. Fungal cells were mixed with phosphate buffered saline (1x, pH 7.5) (PBS). The desired concentration of cells ($2x10^9$ cells/ml, $6x10^8$ cells/ml, and $2x10^8$ cells/ml) was counted using hemocytometer. 50 µl of yeast suspensions with a total of $1x10^8$ cells, 3×10^7 cells or $1x10^7$ cells respectively were injected to each larva.

Silkworm infection experiment

Silkworms (*Bombyx mori* larvae) were purchased from Kogensha, Nagano, Japan and maintained in plastic containers at 25ºC. Larvae were fed with an artificial diet silk mate 2S (Nihon Nosan, Tokyo, Japan) until they reach to fifth-instar. *C. neoformans* cell suspension (0.05 ml) was injected into the hemolymph of the larvae through the dorsal surface using a 27-gauge needle. The control group was injected with PBS. The injected larvae were maintained without feeding at 37ºC and inspected. Ten larvae were used for

each group to assess the virulence of *C. neoformans*. The experiment was conducted in triplicate.

Survival of *C. neoformans* under the presence of silkworm hemolymph

Hemolymph was collected from fifth-instar silkworms using 27-gauge needles. 50 μl of $2x10^9$ cells/ml of *C. neoformans* cell suspension of WT, *met5*Δ, and *met5*Δ+*MET5* strains was mixed with 100 μl hemolymph in a test tube containing 2 ml of PBS. A control experiment with only PBS were also prepared. These test tubes were shaken at 150 rpm at 37°C. 5μl of 10^3 dilution of each mixture was cultured on YPD medium every two days to exam the survival and growth of each strain after the treatment.

Statistical analysis

Statistical analysis of the survival curves was performed using log-rank tests (The PRISM software package). P-value of <0.05 was considered as statistically significant.

RESULTS

Virulence of *met5*Δ strain

The silkworm was used to test the involvement of *MET5* gene in virulence of *C. neoformans*. *C. neoformans* cell suspension with different concentrations of each strain were injected into silkworm body to examine *C. neoformans* virulence. As shown in **Figure 16**a, with injection of 10^8 cells, the *met5*Δ+*MET5* complement strain started to kill silkworms at the third day after injection, while the WT and the mutant *met5*Δ started at second day. However, with lower cell concentration of $3x10^7$ cells/larva and 10^7

cells/larva, in every treatment groups, silkworms started to die after 2 days of injection (**Figure 16**b, c). All silkworms died after 5 days of injection, while the silkworms in control group survived until the sixth day. The survival of silkworms shows no significant difference between the WT, *met5*Δ, and *met5*Δ+*MET5* complement treatments while the survival rate of control group is significantly higher than the other groups.

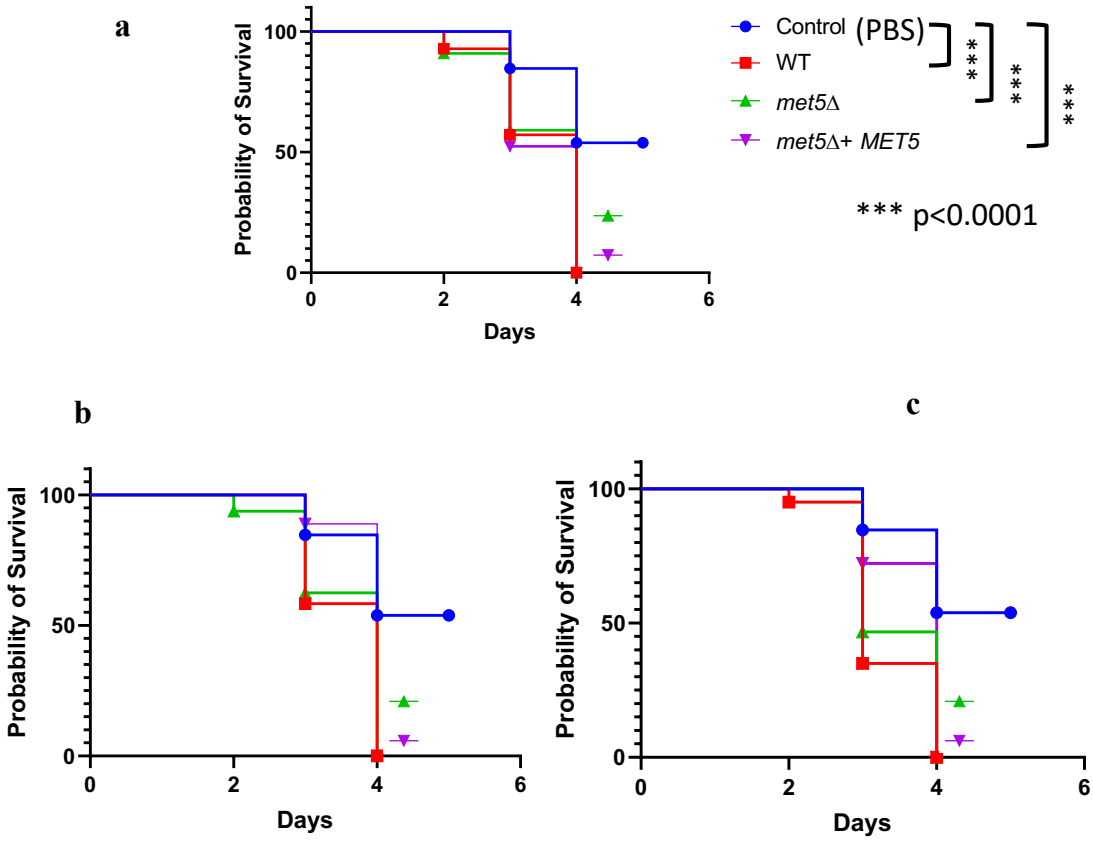


Figure 16. Virulence test

C. neoformans cell suspension in different concentration were injected into 10 fifth-instar larvae. The injected larvae were maintained without feeding at 37°C and daily inspected. Numbers of dead larvae were counted until all larvae were dead. Number of *C. neoformans* cells were injected into 10 larvae; **a**. $1x10^8$ cells/larva, **b**. $3x10^7$ cells/larva, and **c**. $1x10^7$ cells/larva.

Survival and growth of *C. neoformans* strains after culturing with silkworm hemolymph

As the results of silkworm infection showed insignificant difference among strains (see the results), and some known components of silkworm hemolymph could play as sulfur source for *C. neoformans*, I hypothesized that the mutant strain had been induced to perform at same level with WT and complement strains. In order to test how the silkworm hemolymph impacts the survival and growth of *C. neoformans,* I conducted the culture in silkworm hemolymph experiments. As shown in **Figure 17**, after five days of incubation, cells of WT, *met5*Δ mutant, and *met5*Δ+*MET5* complement strains in silkworm hemolymph showed the survival growth rate higher than the cells were incubated in only PBS. Especially the *met5*Δ mutant and *met5*Δ+*MET5* complement strains showed significantly much lower growth rate after only three days and almost lost their viability after five days in PBS treatment. In contrast, in the treatment with silkworm hemolymph, all the strains showed strong viability and growth even after five days of treatment. Further, the growth and survival of *met5*Δ mutant and *met5*Δ+*MET5* complement strains were relatively similar while WT showed somewhat faster.

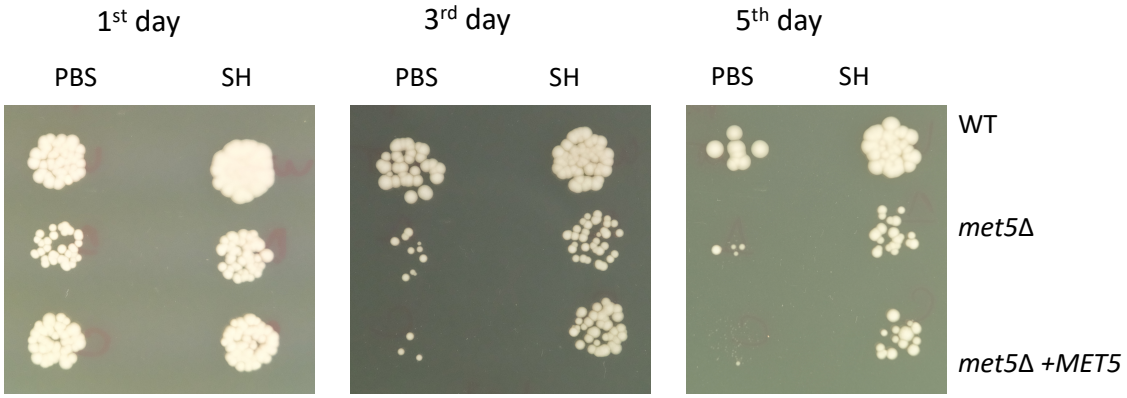


Figure 17. Growth of *C. neoformans* cells on silkworm hemolymph

One hundred μl of hemolymph was extracted from fifth-instar silkworm larvae mixed with 50 μl of $2x10^9$ cells/ml of WT, *met5*Δ, and *met5*Δ+*MET5* strains of *C. neoformans* cell in 2 ml of PBS. Tubes were shaken at 150 rpm at 37°C. After two days, 5 μl of $1x10^3$ dilution of each mixture was cultured on YPD medium to exam the survival of each strain after treatment. **PBS**: Phosphate buffer saline; **SH**: silkworm hemolymph.

DISCUSSION

To identify virulence factors of pathogens, it is essential to evaluate the virulence of gene-knockout mutants in animal infection models. Due to the cost and ethical problems, silkworm has been introduced and used as an alternative infection model which is more cost-effective and efficient (Kaito 2016). Silkworm has been shown as a suitable infection model for examination and identification of virulence factors of *C. neoformans*. Silkworms with injection of *gpa1*Δ, *pka1*Δ, and *cna1*Δ genes which contribute to the pathogenesis of *C. neoformans,* survival longer than those infected with the WT (Matsumoto et al. 2012). In order to determine whether the *MET5* gene is involved in the virulence of *C. neoformans*, I employed the silkworm infection model. However, *met5*Δ mutant strain showed no difference in the virulence of *C. neoformans* compared to the WT and the complement strains. According to Kawase (Kawase 1996), silkworm hemolymph contains several sulfur amino acids such as methionine (5.2 mg/100 ml), cystathionine (22.2 mg/100 ml). The amount of sulfur amino acids might be sufficient for mutant strain to be as virulent as the WT and the complement strains. Further experiment had supported this hypothesis. As shown in **Figure 17**, *met5*Δ mutant strain showed similar growth rate compared with the WT and the *met5*Δ+*MET5* complement strains on YPD after culturing in the silkworm hemolymph. Various studies have shown the bioactive effect of silkworm hemolymph on cells' growth and viability. For example, silkworm hemolymph has been used as a medium supplement in animal cell cultures for

the prevention of apoptosis (Choi et al. 2005), for the improved production of recombinant proteins (Ha and Park 1997), and for the stimulation of cell growth (Reddy 2017). Silkworm hemolymph has been shown to possess an inhibit ability not only on baculovirus-induced insect cell apoptosis (Rhee and Park 2000) but also on the apoptosis and detachment of the mammalian cell (Shin et al. 2002). Therefore, some components of silkworm hemolymph maybe able to support the growth of *C. neoformans* *met5*Δ mutant cells.

Further, according to the result from our collaborators' experiment on mice infection model, in tested the infection of different *C. neoformans* strains on mice. *met5*Δ strain which in injected into mice showed the mice survival as PBS injected in 40 days. While WT strain showed the survival of mice only 13 days and the *met5*Δ+*MET5* complement showed the survival within 30 days. Therefore, *met5*Δ strain is showed the less virulent than the WT and the *met5*Δ+*MET5* complement strains (personal communication). Therefore, even though *MET5* gene seems not essential for virulence of *C. neoformans*, it can be still a potential drug target.

Chapter 5. General discussion

Identification of sulfite reductase *MET5* gene in *C. neoformans*

Formerly in the sulfur pathways of *C. neoformans*, only *MET3* (encoding ATP sulfurylase) has been shown to be involved in sulfate assimilation pathway to synthesizes sulfide (Yang et al. 2002). In contrast, multiple genes that play roles in this pathway had been proposed and functionally confirmed in *Sa. cerevisiae*. Therefore, based on sequence homology, *CNL05500* and *CNG03990* in *C. neoformans* genome were identified as homologues of *MET5* and *MET10* of *Sa. cerevisiae* (44.75% and 38.37% identity respectively) which code for **α** and **β** subunits of sulfite reductase. As expected, the resulting mutant strains *met5*Δ and *met10*Δ were not able to grow in the presence of sulfate or sulfite but could grow on sulfide. The function of these genes was confirmed, which code for sulfite reductase to produce sulfide in sulfate assimilation pathway as in *Sa. cerevisiae*. Nonetheless, in *Sa. cerevisiae*, mutant of these genes grow well under the presence of either Met or Cys (Masselot and de Robichon-Szulmajster 1975; Thomas and Surdin-Kerjan 1997), pathogenic fungus *Aspergillus fumigatus*, the mutant *sF* gene encode **β** subunits of the sulfite reductase grew poorly on methionine-containing medium (Amich et al. 2016). In *C. neoformans*, *met5*Δ mutant strain grew better under the presence of Cys than Met. The characteristics of *MET5* gene provided more information to complete the sulfate assimilation pathway of *C. neoformans*.

Metabolism of sulfur amino acid in *C. neoformans*

Among the common sulfur amino acids, only Cys and Met are incorporated into protein in the biological metabolism (Brosnan and Brosnan 2006). In mammals, Met plays more crucial role than Cys does because Cys can be synthesized only from Met via transsulfuration (MacCoss et al. 2001). Mammals consume these amino acids from diet to live, while bacteria, plants, and fungi have developed sulfur amino acid biosynthetic pathways (Jastrzębowska and Gabriel 2015). In fungi, an overview of sulfur amino acid biosynthesis pathway is summarized in **Figure 7** which consists of sub-pathways: the sulfate assimilation pathway, OAS pathway, OAH pathway, transsulfuration pathway, and methyl cycle as in *A. nidulans* (Paszewski et al. 2016); but some sub-pathways might be missing in some organisms. *Sa. cerevisiae* does not have the OAS pathway and the transsulfuration pathway in *Sc. pombe* is only from cysteine to homocysteine (Brzywczy and Paszewski 1994; Thomas and Surdin-Kerjan 1997). *Hasenula polymorpha* does not have the OAH pathway which catalyzes the synthesis of homocysteine from homoserine by incorporation of sulfide (Sohn et al. 2014). Therefore, various genes were examined to understand the whole picture on the sulfur amino acid pathways of a human pathogenic fungus *C. neoformans*.

In sub-pathways sulfate assimilation pathway, *MET5*, *MET10*, *MET14* were found, these genes showed vital growth on minimal medium YNB supplemented with Cys and poorer with Met. This is consistent with *MET3* gene which was found in previous studies in *C. neoformans* (Yang et al. 2002). Similar to sulfate assimilation pathway in *Sa. cerevisiae*, genes involved in this pathway could assimilate sulfate to sulfite and sulfide (Thomas and Surdin-Kerjan 1997). Therefore, the mutant of genes did not grow well on sulfate but better on sulfide as in **Figure 9**. In *Sa. cerevisiae*, mutants of genes involved

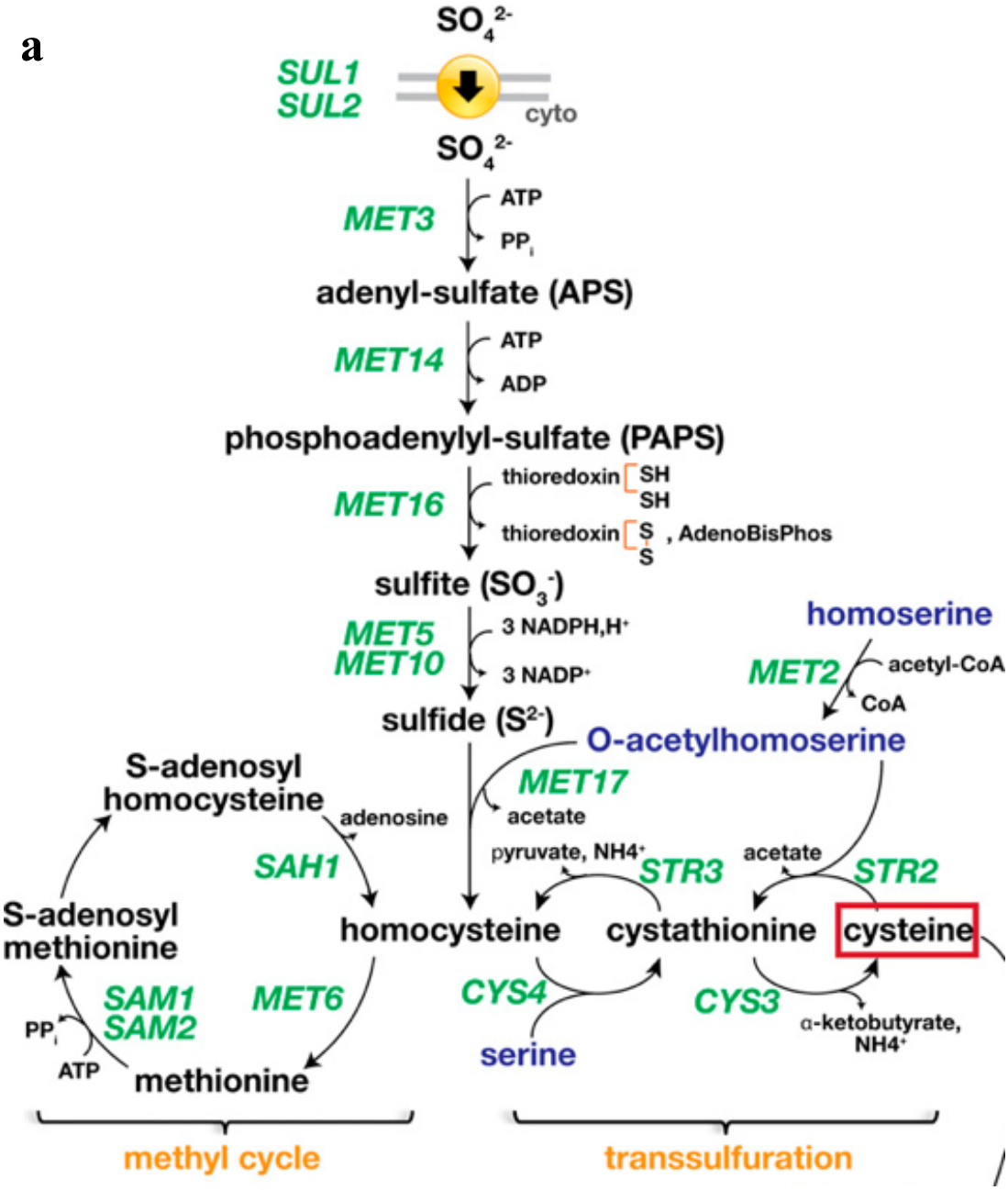
in sulfate assimilation pathway grow well on either Cys or Met (Thomas and Surdin-Kerjan 1997). However, in *C. neoformans*, these gene mutants grew well only on Cys but not on Met, suggesting that the conversion mechanism via transsulfuration pathway between Cys and Met in *C. neoformans* is different from that in *Sa. cerevisiae*.

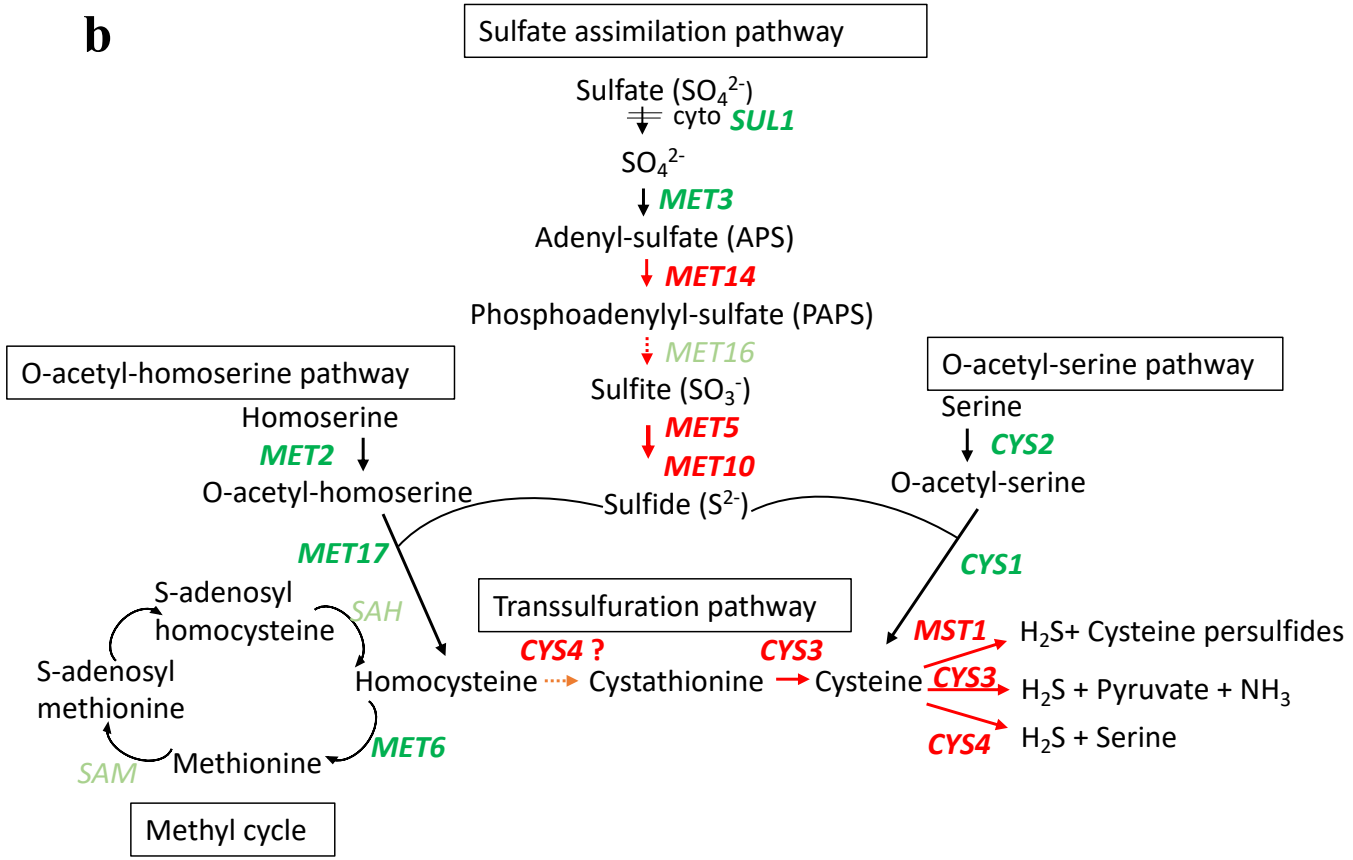
The transsulfuration pathway consists of reactions that allow the interconversion between Met and Cys. Both the forward and reverse transsulfuration pathways exist in *Sa. cerevisiae*, therefore the mutant of homocysteine synthase gene *met17*Δ showed the growth well on either sulfur-free medium supplemented with Met or Cys (Masselot and de Robichon-Szulmajster 1975). However, transsulfuration pathway of *C. neoformans* consist of only reverse direction from homocysteine to cysteine, mutant of homocysteine synthase gene *met17*Δ of *C. neoformans* grew on Met but not on Cys (**Figure 11**). In addition, the mutant of cysteine synthase gene *cys1*Δ showed the growth on both Cys and cystathionine while the double mutant of cysteine synthase gene and cystathionine lyase gene *cys1*Δ*cys3*Δ grew on Cys but not on cystathionine (**Figure 13**). These results confirmed that the transsulfuration pathway in *C. neoformans* consist of only reverse pathway from homocysteine to cysteine.

Sulfur amino acid metabolic pathway of *Sa. cerevisiae, C. neoformans* and human are summarized in **Figure 18**. In *Sa. cerevisiae* Cys can be synthesized only from homocysteine through reverse transsulfuration pathway. Human do not have sulfate assimilation pathway and amino acids are acquired only from diet. Met is an essential amino acid in human and Cys can only be synthesized from Met via reverse transsulfuration pathway. In *C. neoformans*, Cys is synthesized from both serine through OAS and homocysteine through the reverse transsulfuration pathway. These indicates that the Cys biosynthetic pathway of *C. neoformans* is unique and essential for the

survival of this fungus. For this reason, sulfur amino acid pathway of *C. neoformans* can be a promising target for screening of anti-*Cryptococcus* drug

a


SO4 2-
SUL1
SUL2
cyto
SO4 2-
MET3
ATP
PPi
adenyl-sulfate (APS)
MET14
ATP
ADP
phosphoadenylyl-sulfate (PAPS)
MET16
thioredoxin SH SH
thioredoxin S S , AdenoBisPhos
sulfite (SO3-)
MET5
MET10
3 NADPH,H+
3 NADP+
sulfide (S2-)
homoserine
MET2
acetyl-CoA
CoA
O-acetylhomoserine
MET17
acetate
S-adenosyl homocysteine
adenosine
SAH1
pyruvate, NH4+
STR3
acetate
STR2
S-adenosyl methionine
homocysteine
cystathionine
cysteine
SAM1
SAM2
PPi
ATP
MET6
CYS4
CYS3
α-ketobutyrate, NH4+
serine
methionine
methyl cycle
transsulfuration


b
Sulfate assimilation pathway
Sulfate (SO_4^{2-})
cyto SUL1
SO_4^{2-}
MET3
Adenyl-sulfate (APS)
MET14
Phosphoadenylyl-sulfate (PAPS)
MET16
Sulfite (SO_3^-)
MET5
MET10
Sulfide (S^{2-})
O-acetyl-homoserine pathway
Homoserine
MET2
O-acetyl-homoserine
MET17
O-acetyl-serine pathway
Serine
CYS2
O-acetyl-serine
CYS1
Transsulfuration pathway
CYS4 ?
CYS3
Homocysteine
Cystathionine
Cysteine
MST1
H_2S+ Cysteine persulfides
CYS3
H_2S + Pyruvate + NH_3
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H_2S + Serine
S-adenosyl homocysteine
SAH
S-adenosyl methionine
MET6
Methionine
SAM
Methyl cycle

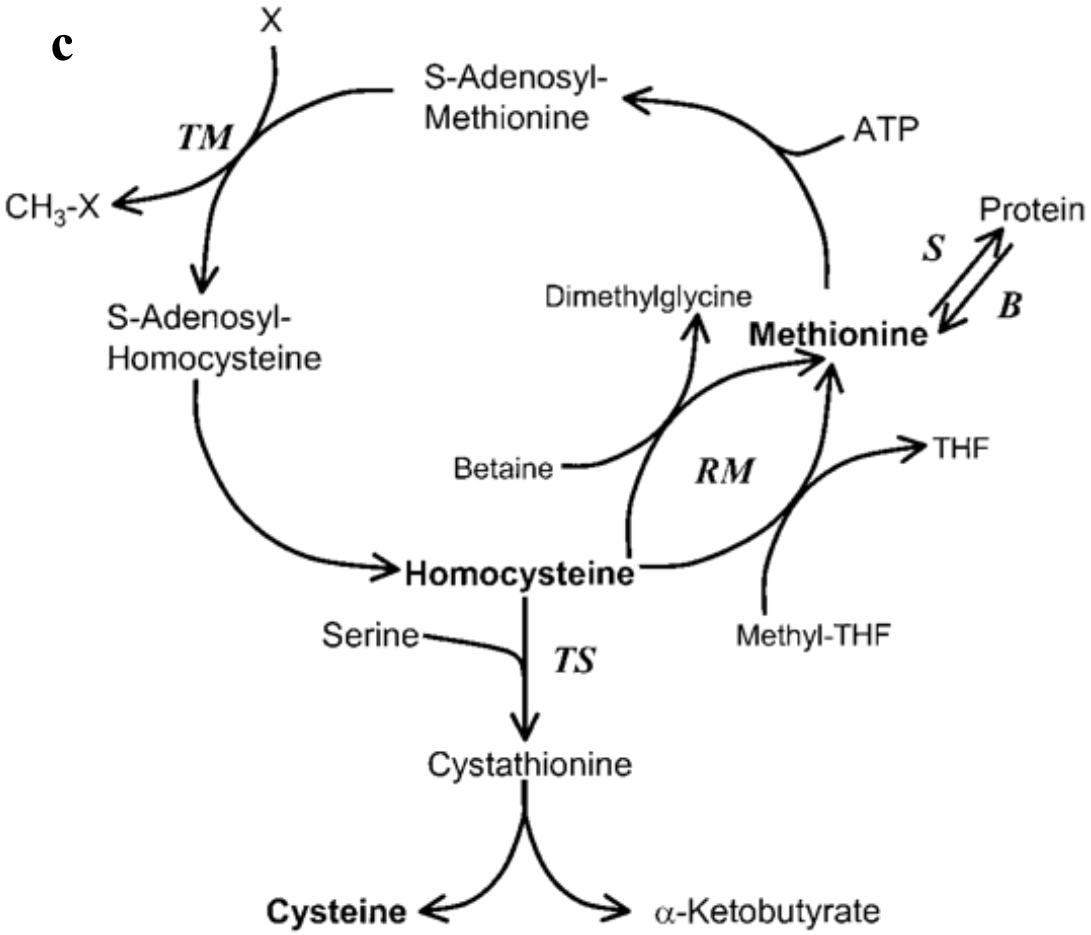

Figure 18. Sulfur amino acid metabolic pathway of *Sa. cerevisiae*, *C. neoformans*, and human

- **a.** Sulfur amino acid metabolic pathway *Saccharomyces cerevisiae* (Thomas and Surdin-Kerjan 1997)
- **b.** Sulfur amino acid metabolic pathway *Cryptococcus neoformans*
- **c.** Sulfur amino acid metabolic pathway in human (MacCoss et al. 2001)

Transcription profiles of sulfur metabolic genes in *C. neoformans*

The availability of the genome sequence data of *C. neoformans*, which has been enhanced by numerous sequencing effort (Loftus et al. 2005; Fraser et al. 2005; Srikanta et al. 2014),

has brought the possibility for large scale transcriptional study. Function of numerous genes have been determined and confirmed by inspecting their expression levels in response to various environmental conditions (Steen et al. 2002; Kim et al. 2012; Janbon et al. 2014). In this study, I attempted to find the genes which are potentially associated in reverse transsulfuration pathway between homocysteine and Cys by identifying differentially expressed genes from RNA-seq data of the WT strain cultured in different sulfur sources: sodium sulfate, homocysteine, Cys, and Met; genes up-regulated in homocysteine and Cys media than others were selected for further inspection. Homocysteine raised the most different expression profile, while expression profile of Cys is similar with the other two conditions (**Figure 15**). Further, multiple clusters of genes were highly expressed in homocysteine but low in Cys and vice versa. In detail, among 7881 genes of *C. neoformans* genome, 602 genes showed strong expression in homocysteine while there were 58 genes in Cys. Nonetheless, this high number of genes is hard to narrow down. This situation also occurred in several previous studies with similar approach. Janbon et al. (2014) identified two clusters of genes, which were more highly expressed when grown on pigeon guano compared to on YPD or on starvation media. These genes encode transporters, transcription factors and lipid metabolism which were explained to be necessary for the growth in ecologically diverse habitat like pigeon guano, but their specific roles were not further discussed. Similarly, genes expression profiles were determined using *C. neoformans* strains isolated from patients or environment to find genes which contribute to the pathogenicity of *C. neoformans*, Chen et al. (Chen et al. 2014) detected simply a group of potential genes. Among genes that were up-regulated in homocysteine and Cys, about half of them are currently classified as genes coding for hypothetical proteins with unknown function. Information from those

with known function is hard to link to the conversion between homocysteine and cysteine. The number of up-regulated genes in homocysteine is much greater than in Cys but further experiments may explain the reason why this difference was observed to reveal the sulfur metabolic pathway of *C. neoformans*.

Virulence of the *met5*Δ mutant strain

Silkworm has been used as an animal model of human infection with multiple pathogenic fungi including *C. neoformans* (Ueno et al. 2011; Ishii et al. 2016). Newly discovered *MET5* gene which is involved in sulfate assimilation pathway can be a potential target for screening anti-*Cryptococcus* drugs. Therefore, by using silkworm model, I aimed to test the virulence of the *met5*Δ mutant. Unexpectedly, the survival rate of silkworm showed no significant difference between WT, *met5*Δ and *met5*Δ+*MET5* complement strains. This was incongruent with the results from my collaborators who did the tests by mice infection model. From these outcomes, *met5*Δ mutant strain showed the less virulence compared with WT and *met5*Δ+*MET5* complement strains. These contrary results had been reported in other studies where both infection models were used. In the test of hypervirulent *Klebsiella pneumoniae* (hvKp) and classical *Klebsiella pneumoniae* (cKp) strains on *G. mellonella* larva infection assay, some *K. pneumonia* isolates were identified as hypervirulent strains which rapidly killed *G. mellonella* larva but did not kill mice (Li et al. 2020). Silkworm hemolymph has been known to inhibit baculovirus infection and increased the host cell longevity (Rhee and Park 2000). As silkworm hemolymph contains methionine (5.2 mg/100 ml), cystathionine (22.2 mg/100 ml) (Kawase 1996), I hypothesized that the sulfur sources in silkworm hemolymph could be sufficient for the

growth of *met5*Δ mutant strain and give a similar virulence as WT and *met5*Δ+*MET5* complement strains. This hypothesis was supported experimentally. The growth of *met5*Δ mutant which incubated with silkworm hemolymph was similar with the WT and the *met5*Δ+*MET5* complement strains while the growth of these strains in PBS were lost. Therefore, the sulfur sources in silkworm hemolymph could allow the growth of *met5*Δ mutant strain to kill silkworms.

Collectively, in this study I revealed: (1) a novel of *MET5* sulfate reductase gene involved in the sulfate assimilation pathway, (2) the sulfur amino acid pathway in *C. neoformans*, (3) *MET5* gene seems not essential for virulence in silkworm model, it still can be a potential drug target.

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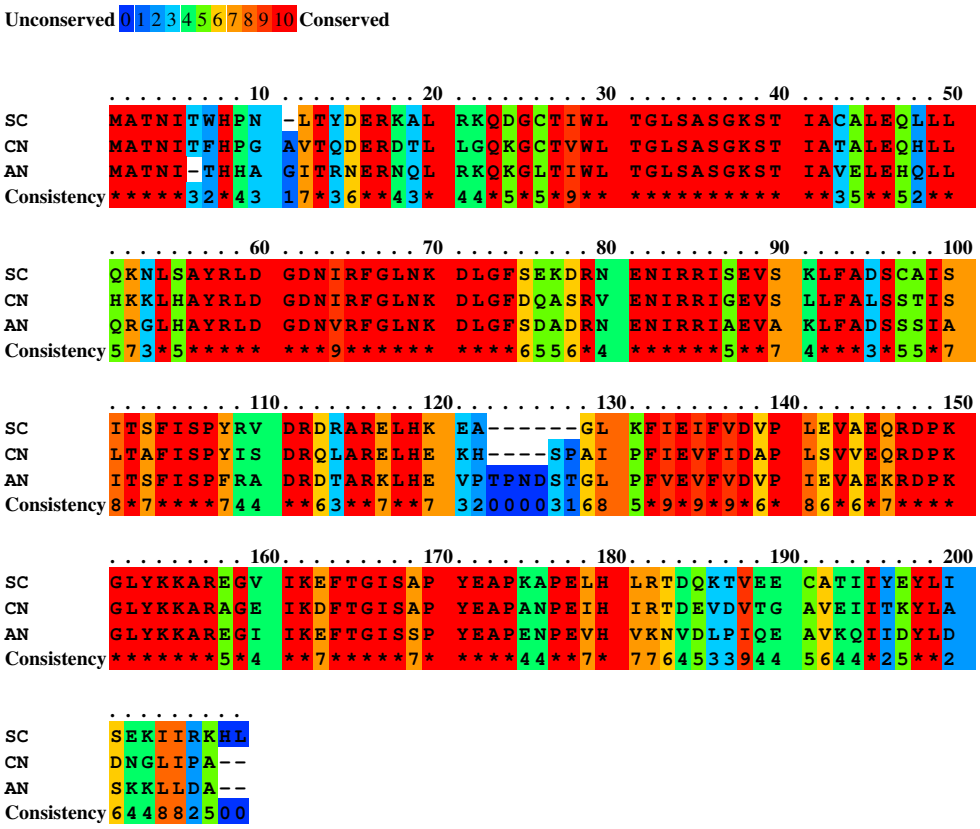
Supplements

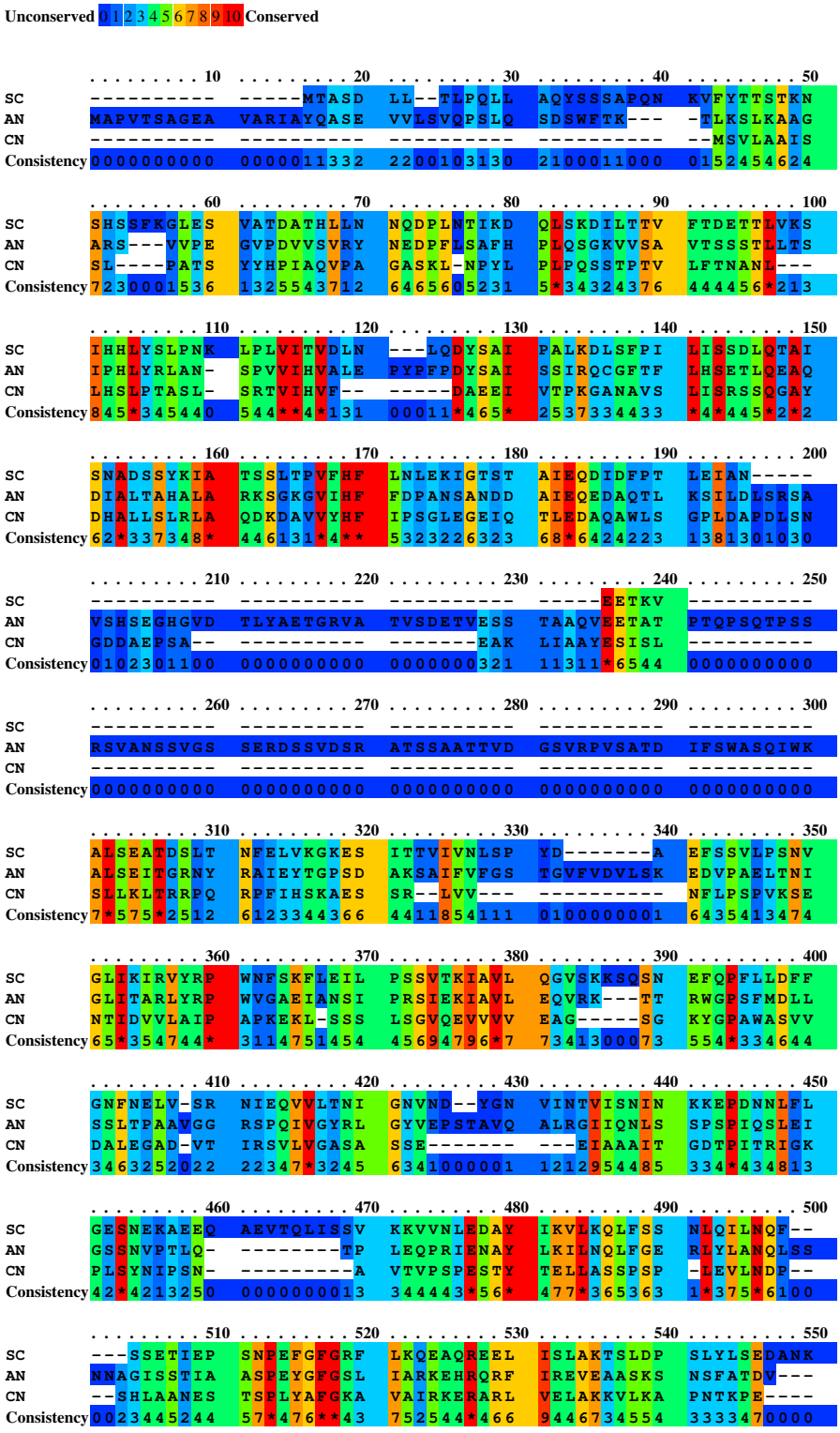
Supplementary Figure 1

Sequence amino acid alignment of APS Kinase *MET14* (**a**), Sulfite reductase *MET5* (**b**), and Cystathionine beta-lyase *STR3* (**c**) of *C. neoformans* (CN) with other yeast *Sa. cerevisiae* (SC) and filamentous fungal *A. nidulans* (AN). The conservation scoring is performed by PRALINE. The scoring scheme works from 0 for the least conserved alignment position, up to 10 for the most conserved alignment position. The colour assignments as in figure.

a (*MET14*)



b (*MET5*)



Results colour-coded for amino acid conservation

c (*STR3*)