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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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 **Error! Reference source not found.**.

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.