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学位論文題目　**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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論文内容の要旨

Background

Pathogenic fungi, such as *Candida*, *Aspergillus* or *Cryptococcus* species, generally cause diseases in immunocompromised individuals. Crytococcal 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*□ 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.

Virulence of *MET5* gene *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 blood 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.

論文審査の結果の要旨

現在，真菌感染症治療に利用できる薬剤は限られており，新しい薬剤標的の発見が強く望まれている。本研究では，抗真菌薬の標的候補として病原菌 *Cryptococcus neoformans* の硫黄アミノ酸生合成経路の解明を試みた。*C. neoformans* の野生型株 KN3501αを *Agrobacterium tumefaciens* 形質転換（AtMT）によって形質転換し，約 10,000 の形質転換体を得た。このうち，1 株は硫黄アミノ酸の一つシステイン（Cys）を要求する変異株であった。TAIL PCR 法によって，T-DNA がパン酵母 *Saccharomyces cerevisiae* の亜硫酸レダクターゼをコードする *MET5* の相同遺伝子（以後，*MET5* とする）に挿入されていることが分かった。遺伝子破壊実験により，*MET5* 遺伝子欠損株（met5Δ株）を作出した。遺伝子破壊株の栄養要求性について検討したところ，ポリペプトン，酵母エキスのようなアミノ酸混合物に加え，システイン（Cys）を含む培地で良好に増殖した。また，硫酸塩または亜硫酸塩を含む培地では増殖が認められず，硫化水素を培地に加えることで増殖した。これらのことから，*MET5* 遺伝子は亜硫酸レダクターゼをコードすることが確認された。*MET5* 遺伝子に加え，*MET10* 遺伝子（亜硫酸レダクターゼの二量体ヘテロダイマーを MET5 と構成する），*MET14* 遺伝子（アデノシンホスホ硫酸（APS）をホスホアデノシンホスホ硫酸（PAPS）に変換する酵素をコード）が硫黄アミノ酸の硫酸同化経路に関わることを見出した。硫黄転移経路の構成を確かめるため，*CYS3*, *CYS4*, *STR2*, *STR3* および *MST1* 遺伝子破壊株を構築し，各種硫黄源を含む培地で培養したところ，逆向き硫黄転移経路をもつものの，順向き硫黄転移経路はもたないことが示唆された。これらの結果から，これまでに知られている真菌類の硫黄代謝経路とは異なる本菌特有の硫黄アミノ酸代謝経路が存在することが提案された。*MET5* 遺伝子ひいては硫黄アミノ酸合成経路の病原性への寄与を評価するため，カイコを用いた病原性試験を行ったところ，野生型株と met5Δ株の病原性に有意な差は認められなかった。カイコの血リンパ液を用いて met5Δ株を培養したところ野生型株と同様の生育を示したことから，カイコの血中には met5Δ株の生育に十分な硫黄アミノ酸が含まれることが示唆された。

以上のように，*C. neoformans* の硫酸同化経路に関わる新たな遺伝子 MET5 を見出し，さらには本菌における硫黄アミノ酸代謝経路は，他の真菌類のものとは異なる独特な構造であることを示した。

本研究により得られた知見は新規性に富み，真菌類のアミノ酸合成経路の解明に大いに貢献する成果であり，本学博士（工学）の学位に相応しいものと判定された。