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Short Report

Cryptococcus neoformans MET5 Gene is not Essential for Virulence in the Silkworm Infection Model

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ABSTRACT

The involvement of the *MET5* gene in virulence of *Cryptococcus neoformans* was examined using the silkworm *Bombyx mori* infection model. In the virulence assay, the *met5Δ* mutant showed virulence not significantly different from the wild-type strain, suggesting that the *MET5* gene is not essential for full virulence of *C. neoformans*. The effect of silkworm hemolymph on the survival of the *met5Δ* mutant was also tested. The *C. neoformans met5Δ* strain incubated in the silkworm hemolymph for five days remained viable, suggesting that silkworm hemolymph supports survival of the *met5Δ* strain.

Key words : antifungal, *Bombyx mori*, sulfur assimilation pathway

Introduction

Cryptococcus neoformans is a basidiomycetous yeast that is distributed worldwide and causes cryptococcosis[1-3]. *C. neoformans* causes a high mortality rate for patients with acquired immunodeficiency syndrome in developing countries[4]. Despite the high risk, the standard treatment for cryptococcal meningitis is still limited, while resistant strains are becoming more abundant and widespread[5, 6]. Therefore, the discovery and development of new antifungal agents for *C. neoformans* has become an urgent matter.

The methionine-cysteine biosynthetic pathway has been proposed as a promising target for antifungal drug development[7-9]. In our previous study, the *MET5* gene was identified to encode sulfite reductase along with *MET10*, which is responsible for the conversion of sulfite to sulfide in the sulfate assimilation pathway[10]. While other genes involved in sulfur-containing amino acid metabolic pathways had been tested and proven to be required for virulence of *C. neoformans* infection[7, 8, 11], this newly discovered gene has not yet been examined for its effect on *C. neoformans* virulence. As using mammalian animals as experimental models for human pathogens leads to various issues including cost and ethical problems, the silkworm *Bombyx mori* was proposed as a model to evaluate the anti-infectious agents that were used in tests with multiple pathogenic bacterial and fungal species including *C. neoformans*[12-16].

In this study, we used the silkworm model to examine the involvement of *MET5* gene in the virulence of *C. neoformans*. Although the *met5Δ* mutant grew poorer than the wild-type strain on various growth conditions, the former unexpectedly showed a similar virulence against the silkworm. From this, we hypothesized that the silkworm hemolymph might provide sufficient nutrition to enable the mutant strain to grow as well as the wild-type. Our hypothesis was further confirmed by the viability assay using medium containing silkworm hemolymph.

Materials and methods

Culture conditions and fungal strains

The *met5Δ* and *met5Δ+ MET5* complemented strains of the wild type KN3501α *C. neoformans* were created and maintained from the previous study[10]. Along with the wild-type, these strains were cultured in liquid YPD (1% (w/v) yeast extract, 1% polypepton, and 1% glucose) medium at 30 ℃ with shaking at 150 rpm. The overnight cultures were collected, and the fungal cells were counted using a

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Table 1. Detailed comparison of survivability of silkworms injected with *C. neoformans* strains

		P value of comparison of survival curve			
Cell concentration	Strain	Control	Wild type	*met5Δ*	*met5Δ+ MET5*
2×10^9 CFU/ml	Control		—	—	—
	Wild type	0.0004		—	—
	met5Δ	0.0009	0.9366		—
	met5Δ+ MET5	0.0006	0.9105	0.8561	
6×10^8 CFU/ml	Control		—	—	—
	Wild type	0.0006		—	—
	met5Δ	0.0028	0.9145		—
	met5Δ+ MET5	0.0095	0.0321	0.0684	
2×10^8 CFU/ml	Control		—	—	—
	Wild type	0.0002		—	—
	met5Δ	0.0012	0.4127		—
	met5Δ+ MET5	0.0037	0.0204	0.1407	

hemocytometer. The desired concentration (2×10^9 CFU/ml, 6×10^8 CFU/ml, 2×10^8 CFU/ml) of cells was prepared by mixing and diluting the yeast suspensions with phosphate buffer saline (PBS, pH 7.5).

Virulence assay

The silkworm infection experiment was performed according to the previously established method[12]. The third-instar silkworm *Bombyx mori* larvae were purchased from Kougensha, Nagano, Japan and maintained in plastic containers at 25 ℃. The larvae were fed an artificial diet (SilkMate 2S, Nihon-Nosan, Yokohama, Japan) until the fifth-instar stage. Yeast suspensions (0.05 ml) were 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 at 37℃ without feeding and were inspected daily. For each strain, ten larvae were used for infection to assess the effect of the *MET5* gene on the virulence of *C. neoformans*. The experiment was conducted with three different cell concentrations as described above and repeated three times.

Survivability of *C. neoformans* strains in silkworm's hemolymph

For each *C. neoformans* strain, 50 µl of 6×10^8 CFU/ml were cultured in a 20 × 150 mm test tube with 2 ml of minimal YNB medium (0.17% yeast nitrogen base without acids and ammonium sulfate (Difco), 0.5% ammonium sulfate, 2% glucose, and 2% agar) with 100 µl of hemolymph of the silkworm. The test tubes with only YNB were treated as control. These test tubes were shaken at 150 rpm at 30℃ for two days. To observe the effect of the silkworm hemolymph on the viability of each strain, we spotted 5 µl of 10^{-3} dilution of the cultures on YPD medium on the first and third day of the incubation then incubated for three days at 37 ℃. The experiment was repeated three times.

Statistical analyses

The significance of differences between the survival curves of the silkworms injected with different *C. neoformans* strains was evaluated by log-rank (Mantel-cox) test using PRISM 8 (GraphPad Software, LLC, San Diego, CA, USA; https://www.graphpad.com/scientific-software/prism/). $P < 0.05$ was considered indicative of a statistically significant difference.

Results and discussion

Virulence of the *met5Δ* strain

To examine the involvement of the *met5* gene of *C. neoformans* in virulence, we injected the wild-type, *met5Δ,* and *met5Δ + MET5* strains into the silkworm. In every cell concentration, the silkworms started to be killed by *C. neoformans* strains from the second day, and all died on the fourth day after injection. In contrast, half of the silkworms in control groups were still alive after the fifth day. Surprisingly, while the survival rates of those silkworms in the control group that was injected with PBS were significantly different from the groups that were injected with *C. neoformans* strains, there was almost no difference among the latter (Table 1, Fig. 1). Further, among the treatment groups, there were significant differences in survival rates of silkworms injected with the wild type and complemented strain in the 6×10^8 CFU/ml and 2×10^8 CFU/ml concentrations. However, they were not different from the groups treated with *met5Δ* mutant in the same cell concentrations. This marginally weaker virulence of the complemented strain is likely caused by the reintroduced wild-type gene[17]. In this experiment, the mutant *met5Δ* strain unexpectedly showed similar virulence as the wild-type strain toward the silkworm. From these results, the effect of *MET5* gene on the virulence of *C. neoformans* could, therefore, not

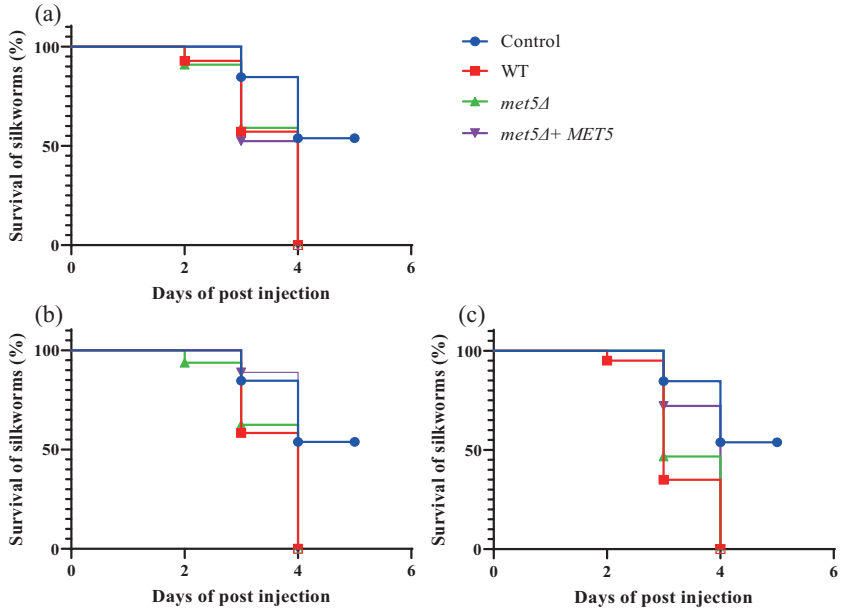

Fig. 1. Survival of silkworms injected with different cell concentrations of each *C. neoformans* strains.

a: 2×10^9 CFU/ml, b: 6×10^8 CFU/ml, c: 2×10^8 CFU/ml. Comparison of the silkworm killing abilities are summarized in Table 1.

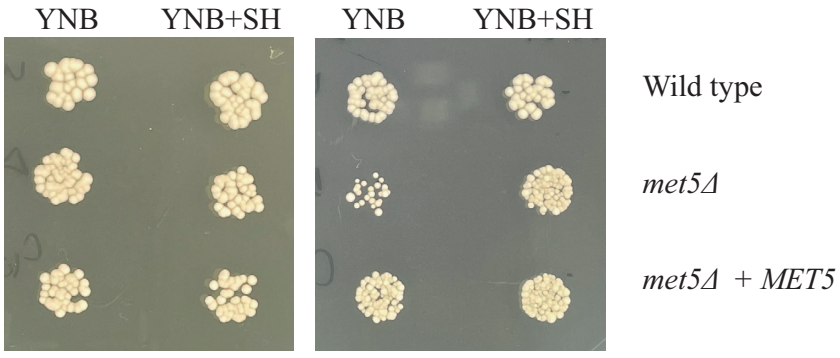

Fig. 2. Viability of yeast cells incubated in the YNB medium or the silkworm hemolymph (SH).

C. neoformans wild-type, *met5Δ*, and *met5Δ+ MET5* strains were incubated in YNB or YNB supplemented with SH; the 10^{-3} dilution of the incubations on the first (a) and third (b) day were spotted onto YPD plates to visualize the effect of SH on the viability of the yeast cells.

be elucidated using the silkworm model. In addition, we suspected that the silkworm might provide sufficient nutrition to enable the mutant strain to survive. This hypothesis was tested and confirmed in the later experiment.

Viability of *C. neoformans* strains after incubating with the silkworm hemolymph

To examine the effect of the silkworm hemolymph on the survival of *C. neoformans* mutant strains, we incubated each strain in a mixture including YNB medium and hemolymph extracted from the silkworm, and examined the viability of the cells on YPD media. All the strains except the *met5Δ* strain showed similar viability before and after the treatment with hemolymph. In particular, the *met5Δ* strain showed less viability after two days incubation in the medium with YNB only, which was restored by supplementing with the silkworm hemolymph (Fig. 2). These results supported our hypothesis that the silkworm hemolymph contain certain components that can help the *met5Δ* strain to survive despite an impaired sulfur assimilation pathway. From previous findings, the *met5Δ* mutant grew well in the presence of cystathionine, cysteine, and homocysteine, but poorly in the presence of methionine[10]. The available cysteine (up to 62 μg/ml) and cystathionine (up

to 310 μg/ml) in silkworm hemolymph[18] might be sufficient for the *met5Δ* mutant to remain viable. In contrast, the concentration of these sulfur-containing amino acids in mammalian animals appear to be considerably lower; cysteine concentration in mouse blood was reported to be ca. 240 ng/ml[19], while the availability of these amino acids in humans is even lower[20, 21]. Therefore, we believe that the deletion of *MET5* will render *C. neoformans* avirulent toward the mammalian animals, and that this should be further examined on mammalian infection models to fully understand its anti-cryptococcal potential.

Our study demonstrated a previously unreported disadvantage of the silkworm infection model. Even though the silkworm has multiple advantages as an infection model[12, 13, 16], it may not be suitable for some experiments due to the nutrients present in its body. This highlights the need to carefully consider how the characteristics of animal models may influence the results of virulence experiments.

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Conflicts of interest

Self-declared COI content: none.

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