

Usefulness of silkworm as a model animal for understanding the molecular mechanisms of fungal pathogenicity

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Summary *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, and *Cryptococcus neoformans* are causative pathogens of opportunistic diseases in immunocompromised human patients. Silkworms are killed by injection of these pathogenic fungi into their hemolymph. In this paper, we describe recent results by our laboratory and other researchers using gene-deficient strains of these pathogenic fungi. The silkworm is considered to be a useful model animal for understanding the pathogenicity of these fungi. Silkworms are also beneficial for evaluating therapeutically active anti-fungal reagents.

Keywords: Fungi, silkworm, infectious disease

1. Introduction

Pathogenic fungi cause serious infectious diseases, such as pneumonia, in humans. Older persons, HIV-infected patients, and patients undergoing treatment with immunosuppressive therapies are particularly susceptible to fungal infections. Pathogenic fungi have various virulence factors that are required for their survival in host environments. The expression of virulence factors in fungi seems to be regulated in response to the host environments. Understanding the molecular mechanisms of pathogenicity by fungi will help to establish effective therapeutic strategies for fungal infection.

In general, animal models mimicking human infectious diseases are used to identify virulence factors in pathogens. Mice are widely used as a model animal for fungal infections (1). The use of large numbers of mammalian animals, however, is costly and associated with ethical issues regarding animal welfare. To address these problems, we propose the use of silkworms as an animal model to study human pathogens (2-4). The cost to purchase and rear silkworms is much lower than that of mice, and the use of silkworms avoids the ethical problems of killing mammals. We previously reported silkworm-infection model of *Staphylococcus*

aureus, which causes opportunistic diseases in humans (5-9). We recently demonstrated that the silkworm *S. aureus* infection model was useful for discovering novel antibiotics effective in a mouse model (5,6). Moreover, disruption mutants of *S. aureus* with attenuated killing abilities in silkworms exhibited less pathogenicity than wild-type strains in mice (7-9). Silkworms are killed by injection of *Candida albicans* (10,11), *Candida tropicalis* (10), *Candida glabrata* (12), or *Cryptococcus neoformans* (13). Therefore, the silkworm seems to be a suitable animal model for identifying genes of bacteria and fungi responsible for the expression of pathogenicity. In this mini review, we describe recent findings in silkworm infection models of each pathogenic fungus.

2. Silkworm-fungal infection models

2.1. *Candida albicans*

C. albicans is frequently isolated from patients with fungal infection. We previously reported that injection of *C. albicans* kills silkworms and that administration of anti-fungal drugs has therapeutic effects (10). The regulatory mechanisms of the pathogenesis of *C. albicans* mediated by protein kinases in *C. albicans* have been reported (14,15). Important roles for type 2B serine/threonine protein kinases, called the calcineurin complex CMP1 (also known as CNA1), in the pathogenicity of *C. albicans* have been demonstrated (15). Protein kinase SIT4 and YVH1 are also involved in the pathogenicity (16,17). Hanaoka *et al.* screened

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virulence factors among protein kinases in *C. albicans* using the silkworm infection model (11). They injected 21 gene-disrupted mutants of genes encoding protein kinases into the hemolymph of silkworms, and found four gene mutants (*cmp1*, *sit4*, *yvh1*, and *ptc1*) with attenuated killing ability in silkworms (11). Among these genes, the pathogenicity of the *ptc1* gene had not been previously reported. Thus, this gene was first identified as a virulence gene of fungi in a study using silkworms. The *ptc1* gene-disrupted mutant exhibited low hyphae formation activity and low protease activity. Moreover, the mutant also exhibited attenuated pathogenicity in mice (11). The other three genes were previously shown to be associated with pathogenicity by altered expression of virulence genes in a mouse infection model (16-18). These findings suggest that the silkworm-*C. albicans* infection model is useful for identifying the virulence factors of *C. albicans*.

2.2. *Candida tropicalis*

C. tropicalis is a pathogenic fungus that is frequently isolated from human patients. We demonstrated that antifungal reagents have therapeutic effects in a silkworm infection model with *C. tropicalis* (10). *C. tropicalis* is a fungus closely related to *C. albicans*. Thus, future experiments with *C. tropicalis* gene-disrupted mutants using the silkworm fungal infection model may allow us to identify the virulence genes of the fungus.

2.3. *Candida grabrata*

C. grabrata resides in the human intestinal tract. This fungus causes opportunistic infections in patients with the metabolic diseases, such as diabetes (19). Ueno *et al.* screened *C. grabrata* mutants with low pathogenicity in hyperglycemic silkworms, created by feeding a high glucose diet (20). They demonstrated that the *cyb2* gene of *C. grabrata* is required for the pathogenicity

of *C. grabrata* against hyperglycemic silkworms (12). In addition, a *hap2* gene mutant and *hap5* gene mutant in which the RNA level of the *cyb2* gene is decreased, exhibit less virulence in the hyperglycemic silkworm. The *cyb2* gene encodes a protein with 65% homology to lactate dehydrogenase in *Saccharomyces cerevisiae* (12). A *C. grabrata cyb2* deficiency mutant exhibits decreased colonization ability in the gastrointestinal tract based on a mouse model of diabetes (12). These findings indicate the usefulness of the silkworm infection model with *C. grabrata* for searching for virulence factors of *C. grabrata*.

2.4. *Cryptococcus neoformans*

C. neoformans is a causal microbial of severe fungal infections, such as pneumonia and encephalitis. Antifungal agents have therapeutic effects in silkworms infected with *C. neoformans* (13). *C. neoformans* has a characteristic capsular structure on the cell surface. The capsular structure is suggested to be required for the pathogenicity of *C. neoformans*. *C. neoformans* has at least two serotypes, serotype A and serotype D. Serotype A of the fungus has higher capsule-forming ability and higher pathogenicity in mammals than the serotype D (21). As in mammalian infection experiments, the *C. neoformans* serotype A exhibits higher pathogenicity in the silkworm infection model than *C. neoformans* serotype D (13). Furthermore, mutants of the *can*, *gpa1*, and *pkal* genes, which are required for *C. neoformans* pathogenicity in mammals, also exhibit lower virulence in silkworms. The protein encoded the *cna* gene is suggested to contribute to the pathogenicity via calcineurin signaling (22).

The *gpa1* gene encodes a G-protein α -subunit that contributes to the capsular formation (23). Protein kinase A acts downstream of Gpa1 and contributes to capsule formation (24). We also demonstrated that the pathogenicity of *C. neoformans* is significantly altered

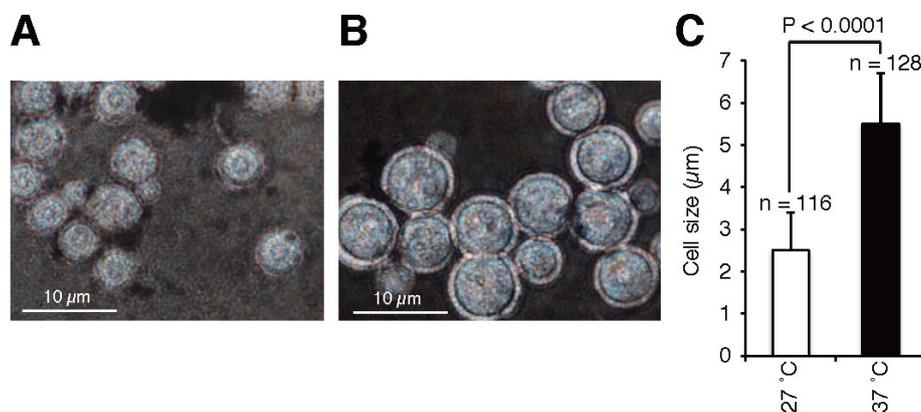


Figure 1. Effect of temperature on the capsular formation of *C. neoformans* in the hemolymph of live silkworms. Cells of *C. neoformans* were injected into the silkworm hemolymph. Twenty-four hours after injection, *C. neoformans* cells were harvested from the silkworm hemolymph, stained with Indian ink, and observed under a microscope. (A) Cells of *C. neoformans* in the hemolymph of silkworms reared at 27°C. (B) Cells of *C. neoformans* in the hemolymph of silkworms reared at 37°C. (C) Mean diameters of *C. neoformans* cells at 27°C and 37°C. Figures were taken from Matsumoto *et al.* (13).

Table 1. Virulence genes tested in the silkworm-fungal infection-model

Species	Genes	Conditions	Ref.
<i>Candida albicans</i>	<i>cmp1</i>	27°C	(11)
	<i>syf4</i>	27°C	(11)
	<i>yvh1</i>	27°C	(11)
	<i>PTC1</i>	27°C	(11)
<i>Candida grabrata</i>	<i>cyb2</i>	37°C, Diabetic	(12)
	<i>hap2</i>	37°C, Diabetic	(12)
	<i>hap5</i>	37°C, Diabetic	(12)
<i>Cryptococcus neoformans</i>	<i>gpa1</i>	37°C	(13)
	<i>pka1</i>	37°C	(13)
	<i>cna1</i>	37°C	(13)

by temperature; the pathogenicity of the fungi against silkworms is much stronger at 37°C than at 27°C. The capsule size and cell size of *C. neoformans* at 37°C in the silkworm hemolymph is significantly greater than that at 27°C condition (Figure 1). This finding suggests that capsule formation is required for the pathogenicity of *C. neoformans* in both silkworms and mammals. Taken together, these findings suggest that the silkworm is a suitable animal model for evaluation of the pathogenicity of *C. neoformans*.

3. Conclusions

The silkworm fungal infection model was used to evaluate the pathogenicities of four different species of fungi, *C. albicans*, *C. tropicalis*, *C. grabrata*, and *C. neoformans*. Among these pathogenic fungi, gene-disrupted mutants of pathogenic genes in *C. albicans*, *C. grabrata*, and *C. neoformans* exhibited less pathogenicity in silkworms (Table 1). In particular, strains deficient in genes encoding the intracellular signaling proteins such as protein kinases and G proteins had decreased killing ability in silkworms. In *C. albicans* and *C. grabrata*, pathogenic genes identified by screening in silkworm infection models are required for pathogenicity in mammals. Therefore, we suggest that the silkworm fungal infection model is useful for identifying the genes necessary for fungal pathogenicity in mammals.

Fungal infections, which cause opportunistic diseases, are anticipated to become a serious problem in future along with advances in medical care and increasing number of aged people in our society. Identification of virulence factors in fungi using silkworm infection models will help to elucidate the molecular mechanisms of fungal pathogenesis and facilitate the development of strategies for preventing and treating fungal infections.

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