Silkworm as an experimental animal for research on fungal infections

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Abstract
Silkworm, Bombyx mori, has various advantages as an experimental animal, such as the low cost for rearing and fewer ethical problems. Models utilizing silkworms of infection with pathogenic bacteria have been established for identification of genes encoding virulence factors by large-scale in vivo screening. In this review, we describe recent progress in the study of silkworm infection models for elucidating the mechanisms of fungi infection. Silkworm infection models have been established for Candida albicans, Candida tropicalis, Candida glabrata and Cryptococcus neoformans, which are yeast type fungi, and Aspergillus fumigatus, Arthroderma vanbreuseghemii, Arthroderma benhamiae, Microsporum canis, Trichophyton rubrum, and Rhizopus oryzae, which are filamentous fungi. Novel genes encoding virulence factors in C. albicans and C. glabrata have been identified by using the silkworm infection models. We here outline the benefits of using silkworm infection models and a strategy for identifying the genes responsible for pathogenicity of microorganisms such as fungi. © 2019 The Authors. Microbiology and Immunology Published by The Societies and John Wiley & Sons Australia, Ltd.

KEYWORDS
human pathogenic fungus, infectious disease, silkworm, virulence factor

1 | INTRODUCTION

1.1 | Animal models for understanding infection systems of pathogens
Pathogenic microorganisms infect humans and cause various infectious diseases. Understanding the pathogenic mechanisms of infectious diseases caused by pathogenic microorganisms is necessary for establishing therapeutic and preventive methods. To achieve this, basic research using animal models mimicking human infectious diseases is indispensable.

Models involving infection of various mammals by pathogenic microorganisms have been proposed [1–3]. However, use of mammalian animals has problems in terms of both cost and ethical considerations. Therefore, studies of
screening for identifying virulence factors of pathogenic microorganisms using mammals are not easy, because many animals are required. To overcome these problems, infection models using invertebrates such as fruit flies, nematodes, and greater wax moths have been proposed [4–7]. Invertebrate animals generally have the following benefits compared with mammals: (i) lower cost of breeding the animals, (ii) larger numbers of individuals can be reared in a small space, (iii) fewer ethical problems concerning killing the animals, and (iv) fewer samples needed because of the smaller body sizes (Table 1) [8].

Silkworms have been proposed as an experimental model animal for pathogenic microorganisms that infect humans [9–12]. Various strains of silkworm and rearing methods have been established in the long history of sericulture. Therefore, researchers can easily rear a large number of silkworms in a small space (Table 1). Body temperatures of silkworm can be easily controlled by changing the rearing temperature, whereas this is difficult with mammals. Because silkworms are much larger than fruit flies and nematodes, it is easy to perform experiments requiring injections, for which tuberculin syringes can be used (Figure 1). Researchers can inject accurate volumes of pathogen culture and solutions of drugs into silkworms. Furthermore, in silkworm experiments, researchers can distinguish between intra-hemolymph and intra-midgut injections (Figure 1). The former corresponds with intravenous injection in humans and the latter with oral administration. In larva of Galleria mellonella, an invertebrate, approximately 10 μl of sample solution can be injected into hemolymph with using a syringe (Table 1). However, methods for distinguishing between intra-hemolymph and intra-midgut injections have not yet been established for these larvae. Given that G. mellonella larvae are smaller than silkworm larvae, accurate injection into the intra-midgut may be more difficult than with silkworms. Using intra-midgut injections, silkworms can be used to investigate the pathogenicity of bacteria that infect the intestinal tract. Utilizing the various advantages of the silkworm as an experimental animal, researchers can study molecular mechanisms of infection by pathogenic microorganisms in humans.

### 1.2 Identification of pathogenic genes of bacteria using a silkworm infection model

Silkworm infection models have been established for human pathogenic bacteria such as Staphylococcus aureus [9], Streptococcus pyogenes [10], Pseudomonas aeruginosa [9], pathogenic Escherichia coli [13], Listeria monocytogenes [14], Serratia marcescens [15], and Vibrio cholerae [9]. Silkworms are also killed by injection of extracellular toxins, such as α-toxin and β-toxin of S. aureus, exotoxin A of P. aeruginosa, diptheria toxin, and hemolysin of B. cereus [16,17]. These results suggest that the virulence of various pathogenic bacteria infecting humans can be evaluated using silkworm infection models.

Injection of Porphyromonas gingivalis cells causes silkworm death and such death is not prevented by administration of antibiotics [18]. Moreover, silkworms die as a result of excessive activation of the innate immune system induced by P. gingivalis peptidoglycans [18]. Silkworms recognize not only peptidoglycan and lipopolysaccharide, which are constituents of bacteria, but also β-glucan, which is a cell wall component of fungi and activates innate immune systems [19–22]. Thus, these studies suggest that death resulting from excessive activation of the innate immune system by bacterial and fungal infection can be evaluated using a silkworm model.

| Animals | Cost of rearing | Space for rearing | Permission from the Ethics Committee | Requirement for biosafety measures | Quantitative injection of samples with a syringe | Reference |
|---------|----------------|------------------|-------------------------------------|----------------------------------|-----------------------------------------------|-----------|
| Silkworm (larva) [Bombyx mori] | Low | Small | Not necessary | Low | Easy | [53] |
| Fruit fly (adult) [Drosophila melanogaster] | Low | Small | Not necessary | High | Difficult | [75] |
| Nematode [Caenorhabditis elegans] | Low | Small | Not necessary | Low | Difficult | [76] |
| Greater wax moth (larva) [Galleria mellonella] | Low | Small | Not necessary | Low | Easy | [77] |
| Mouse [Mus musculus] | High | Large | Necessary | High | Easy | [78–80] |

This table is a modified version from Ishii et al. [12], with permission.
The silkworm infection model is applicable to a variety of pathogenic microorganisms. The strategy for searching for pathogenic genes of microorganisms is shown in Figure 2. The steps include: (i) preparation of gene-disrupted mutants of the pathogenic microorganism; (ii) screening for mutants with less ability to kill silkworms; (iii) confirming their low killing ability against mice; and (iv) genetic and biochemical analysis of the function of the protein encoded by the identified pathogenic gene. We have succeeded in identifying several pathogenic genes of some bacteria, including S. aureus, by this strategy [10,23–26]. We constructed a gene-deficient mutant library of S. aureus and then screened avirulent mutants that showed low pathogenicity against silkworms. Novel genes, cvfA, cvfB, and cvfC, have been identified by screening using a silkworm infection model with S. aureus [10]. Mutants of these genes also showed reduced ability to kill silkworms [36]. In addition, deficiency of the ptc1 gene, which has not been reported to be associated with pathogenicity of C. albicans, causes decreased ability to kill silkworms [36]. The pathogenicity

2 | SILKWORM INFECTION MODELS WITH PATHOGENIC FUNGI

Studies using silkworm infection models with individual pathogenic fungi are described below.

2.1 | Candida albicans

C. albicans, a resident fungus on the human body surface and in the gastrointestinal tract and vaginal mucosa, causes candidiasis in patients with reduced immunity [27,28]. C. albicans adheres to tissues and forms hyphae, causing tissue destruction and inflammation [29]. Moreover, C. albicans regulates hyphal formation by signal transduction via a protein kinase pathway [30–34]. CMP1 (CNA1), a component of calcineurin complex, which is a serine/threonine protein kinase, is necessary for the pathogenicity of C. albicans mice [30,31,34]. Protein kinases SIT4 and YVH1 are also required for infection against mice [32]. Therefore, these protein kinase pathways are thought to play an important role in regulation of infection against mammals.

Silkworms reportedly die when incubated at 27°C after injection of C. albicans cells into their hemolymph [35]. Administration of antifungal drugs to silkworms infected with C. albicans has a therapeutic effect. Thus, killing of silkworms seems to require proliferation of C. albicans within them.

Hanaoka and colleagues constructed gene-deficient mutants of 21 protein kinases of C. albicans and examined their pathogenicity against silkworms [36]. The cmp1, sit4, and yvh1 genes are required for infection of C. albicans against mice [32,33,37]. Mutants deficient in these genes also showed reduced ability to kill silkworms [36]. In addition, deficiency of the ptc1 gene, which has not been reported to be associated with pathogenicity of C. albicans, causes decreased ability to kill silkworms [36]. The pathogenicity

FIGURE 1 Injection of solution into silkworm. (a) A fifth instar silkworm fed with an artificial diet for one day. (b) Red ink is injected into the silkworm’s hemolymph. (c) Red ink has diffused into the silkworm’s hemolymph and its legs are stained red (intra-hemolymph injection). (d) When red ink has been injected into the silkworm’s intestinal tract, it stays in the intestinal tract and the silkworm’s legs do not stain red (intra-midgut injection).
of a ptc1 gene-deficient mutant of *C. albicans* has also been shown to be decreased in mice [36]. This study suggests that novel virulence genes of *C. albicans* can be identified by using a silkworm infection model. Thus, the silkworm infection model is a useful *in vivo* means of identifying novel virulence factors and exploring the infectious system of *C. albicans*.

2.2 | *Candida tropicalis*

*C. tropicalis* infects patients with neutropenia is frequently isolated from patients with leukemia [38–40]. In patients with neutropenia, *C. tropicalis* is considered more likely to form disseminated lesions than *C. albicans*. Therefore, *C. tropicalis* and *C. albicans* may have different regulatory mechanisms for pathogenicity; however, the molecular mechanisms remain uncertain.

When silkworms are injected with *C. tropicalis* and incubated at 27°C, they die; however their death under these conditions can be prevented by administration of antifungal drugs [35]. Therefore, the silkworm infection model is useful for evaluating the pathogenicity of *C. tropicalis*. Now that a technique for constructing gene-deficient mutants of *C. tropicalis* has been established [41], a gene-deficient mutant is expected to be used for further studies.

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**FIGURE 2** Identification of virulence factors of pathogens using a silkworm infection model. (a) Method for evaluating avirulent mutants from a gene-deficient mutant library. The wild type strain of a pathogen or gene-deficient mutants are injected into silkworms and the number that survive measured. (b) Strategies for clarifying the infectious systems of pathogenic microorganisms. A gene-deficient mutant library of a pathogenic microorganism is prepared and new genes necessary for pathogenicity to silkworms identified. Furthermore, the pathogenicity of the identified mutants against mice is confirmed. Genetic analyses of the identified pathogenic genes and biochemical analysis of the proteins (gene products) are carried out.
library can be prepared using the technique. It is that new virulence genes of *C. tropicalis* will be identified by screening avirulent mutants from a gene-deficient mutant library using a silkworm infection model.

### 2.3 Candida glabrata

*C. glabrata* is an opportunistic fungus that is resident in the human intestinal tract and infects individuals with diabetes [42]. *C. glabrata* is rarely isolated alone and is often isolated with other *Candida* species such as *C. albicans* [43]. Moreover, *C. glabrata* is resistant to azoles, antifungal drugs; thus, relapse of infections with this organism is a problem clinically [44]. Because *C. glabrata* has low infectivity for mice, establishment of an experimental animal system has been difficult.

Silkworms infected with *C. glabrata* do not die within 4 days when incubated at 27°C. To establish a silkworm infection model with *C. glabrata*, we constructed an experimental system for infection by this organism using diabetic silkworms. Feeding a high glucose diet induces diabetes in silkworms [45–47]. When diabetic silkworms injected with *C. glabrata* are incubated at 37°C, they die within 3 days [48].

Ueno and colleagues have constructed a gene-deficient mutant library of *C. glabrata* and searched for genes necessary for infection caused by *C. glabrata* using a diabetic silkworm infection model [48]. Screening for avirulent mutants in this model resulted in identification of lactate dehydrogenase Cyb2 as a virulence-related factor in *C. glabrata* [48]. A mutant of the gene encoding the lactate dehydrogenase Cyb2 of *C. glabrata* had decreased ability to kill the diabetic silkworms [48]. Furthermore, in an experimental system of intestinal colonization using diabetic mice, the cyb2 gene-deficient mutant of *C. glabrata* was found to have decreased ability to adapt in the intestinal tract [48]. The CYB2 gene in *S. cerevisiae* is regulated at the transcriptional stage by the Hap family transcription factor, which is activated by recognizing glucose deprivation [49]. Ueno and colleagues consider that cyb2 gene expression is controlled by Hap family transcription factors in *C. glabrata* and investigated this using gene-deficient mutants of the transcription factors. The expression of the cyb2 gene was decreased in deficient mutants of *hap2* and *hap5* genes of *C. glabrata* [48]. However, the deficient mutant of the *hap1* gene in *C. glabrata* was not found to have decreased expression of the cyb2 gene [48]. Strains of *C. glabrata* deficient in the *hap2* and the *hap5* genes have reduced ability to kill diabetic silkworms, whereas the *hap1* gene-deficient mutant has not been found to have decreased killing ability [48]. These results suggest that *C. glabrata* adapts to environments of diabetic hosts by promoting expression of Cyb2 via transcription factors Hap2 and Hap5. Thus, research using the gene-deficient mutant library of *C. glabrata* and a silkworm infection model has revealed a novel mechanism that is necessary for infection of *C. glabrata*.

### 2.4 Cryptococcus neoformans

*C. neoformans* causes cryptococcosis, a fatal fungal disease [50], and is frequently detected in patients with reduced immunity. Patients with AIDS in Africa south of the Sahara Desert have died of infection with *C. neoformans* [51]. *C. neoformans* meningitis, which has a high mortality rate, is particularly problematic in areas where AIDS is prevalent [52].

Although silkworms infected with *C. neoformans* do not die after incubation at 27°C for 4 days, they die within 3 days when incubated at 37°C [53]. *C. neoformans* strains of Serotype A have a higher infectivity in mammals than those of Serotype D [54]. In infection experiments using silkworms, *C. neoformans* strains of Serotype A had a greater ability to kill silkworms than those of Serotype D [53], suggesting that a silkworm infection model is useful for distinguishing between weakly pathogenic and highly pathogenic strains of *C. neoformans*. *C. neoformans* cells translocate to the brain by evading host immunity as a result of capsular formation and melanin production [55]. In *C. neoformans*, capsule formation and melanin production are regulated by GPA1, an α-subunit of G protein, PKA1, a cyclic AMP-dependent protein kinase, and CNA1, a catalytic subunit of calcineurin [56–58]. Thus, the pathogenicity of *C. neoformans* is controlled by various signaling pathways, including the GPA-PKA and calcineurin pathways [59]. Deficient mutants of *gpa1*, *pkal* and *cna1* genes, which are necessary for the pathogenicity of *C. neoformans* against mammals, are less able to kill silkworms than the parent strain [53]. On the basis of these results, it is expected that silkworm infection models will be used to clarify the mechanism of pathogenicity of *C. neoformans* via the GPA-PKA and calcineurin pathways.

### 2.5 Aspergillus fumigatus

*A. fumigatus*, a type of environmental filamentous fungus that is widely present in nature, causes opportunistic infections [60]. *A. fumigatus* has low infectivity against healthy individuals, but causes pulmonary infections in humans with reduced immunity [61]. Given that the number of patients with invasive aspergillosis caused by *A. fumigatus* is increasing and this disease is fatal unless treatment is started early, this pathogen is a clinical problem [62–64].

When silkworms are injected with *A. fumigatus* and incubated at 27°C, they die [65]. Amphotericin B and voriconazole, anti-fungal drugs that are used to treat aspergillosis show therapeutic effects against silkworms infected with *A. fumigatus* [65]. Using a silkworm infection
model, Nakamura and colleagues succeeded in identifying a novel antifungal agent, ASP2397, for treating infections caused by A. fumigatus [65]. Whether it will be possible to identify pathogenic genes of A. fumigatus by using a silkworm infection model is yet to be determined.

2.6 | Arthroderma vanbreuseghemii, Arthroderma benhamiae, Microsporum canis and Trichophyton rubrum

A. vanbreuseghemii, A. benhamiae, M. canis and T. rubrum are all fungi that can cause dermatophytoses [66]. Given that a quarter of the world’s population contracts superficial cutaneous fungal infections caused by these dermatophytes, they are a problem worldwide [67]. Dermatophytes grow as hyphae, forming filamentous structures that are referred to as a mycelium. The hyphae can damage host tissues and cause inflammation, thus causing superficial mycoses [68]. Yamada and colleagues succeeded in establishing a genetic method for constructing recombinant strains of the dermatophyte A. vanbreuseghemii [69]. Therefore, it is considered that it will be possible to evaluate pathogenic factors of dermatophytes by studying their gene-deficient mutants in an infected animal model. A skin infection model using guinea pigs has been

**FIGURE 3** Visualization of infection in organs of silkworm using a dermatophyte expressing eGFP. (a) Method for evaluation of hyphal growth of dermatophytes in organs of silkworm by fluorescent imaging. (b) Fluorescence microscope images of midgut of silkworm infected with A. vanbreuseghemii expressing eGFP. Figure 3b was reproduced from Ishii et al., (8) with permission.
established; however, this model is costly and associated with ethical problems. Therefore, it has been difficult to conduct large-scale in vivo screening to isolate avirulent mutants from a gene-deficient mutant library.

A silkworm infection model for dermatophytes has been established [8]. After injection of conidia of *A. vanbreuseghemii*, silkworms die within 100 hours of incubation at 30°C [8]. Moreover, germination of conidia of *A. vanbreuseghemii* in a liquid medium is strongly pathogenic to silkworms. Furthermore, microscopic analysis of a strain of *A. vanbreuseghemii* expressing eGFP revealed that this fungus forms hyphae in the organs of silkworms (Figure 3). Terbinafine is an antifungal agent that inhibits hyphal formation by dermatophytes. Administration of terbinafine to silkworms injected with *A. vanbreuseghemii* suppresses hyphal formation in their organs and the infected silkworms administrated with terbinafine live longer than those that do not receive terbinafine [8]. These results suggest that hyphal formation of *A. vanbreuseghemii* plays an important role in its pathogenicity to silkworms. Therefore, it is expected that the pathogenic factors related to hyphal formation by *A. vanbreuseghemii* will be identified by using a silkworm infection model.

Because a single hypha may comprise numerous cells, quantification of hyphal formation in animals by a colony counting method is difficult [70]. To overcome this problem, a fluorescence imaging system has been developed for quantitatively evaluating dermatophyte growth in vivo. Hyphal formation can be evaluated on the basis of detection of the fluorescence of eGFP-expressing dermatophytes in silkworms [8]. Thus, a silkworm infection model with dermatophytes expressing eGFP is useful for evaluating hyphal formation in vivo.

In addition, other dermatophytes such as *T. rubrum*, *A. benhamiae* and *M. canis* kill silkworms under the same experimental conditions as *A. vanbreuseghemii* [8]. In particular, silkworms are a useful animal for investigating infection with *T. rubrum*, which is the most frequent cause of dermatophytosis in humans [71]. Thus far, silkworm models can only determine the pathogenicity of dermatophytes by monitoring the death of individual animals. Research on identification of pathogenic factors using a gene-deficient mutant library of dermatophytes and silkworm infection models is expected to progress.

### 2.7 *Rhizopus oryzae*

*Rhizopus oryzae*, which infects from the nasal cavities of severely immunocompromised patients, causes deep mycosis [72]. There are many clinical reports but few basic studies on the pathogenic factors of *R. oryzae*.

Tominaga and colleagues found that silkworms die when incubated at 27°C after being injected with *R. oryzae* [73]. Furthermore, administration of amphotericin B increases the survival time of these silkworms [73]. A silkworm infection model with *R. oryzae* is expected to contribute to the understanding of zygomycosis.

### 3 SILKWORM DEATH CAUSED BY FUNGAL INFECTION

The fact that antifungal drugs can be successfully used to treat silkworms in silkworm infection models with pathogenic fungi indicates that growth of fungi contributes to the silkworms’ death. Calcineurin is involved in the hyphal formation that enables *C. albicans* to kill silkworms; tissue damage and inflammation caused by hyphal formation may cause silkworm death. In comparison, in *C. neoformans*, calcineurin contributes to cell wall synthesis at 37°C [74]. Cell wall components of fungi activate silkworms’ immune systems [19]. The cell wall components of *C. neoformans*, which grows in the bodies of silkworms, may induce excessive immunity that causes silkworm death. Elucidation of the molecular mechanisms of silkworm death caused by fungal infection is an important subject for future study.

**TABLE 2** Assay conditions in silkworm infection models with pathogenic fungi

| Species                  | Incubation temperature | Diabetic state | Reference |
|--------------------------|------------------------|----------------|-----------|
| *Candida albicans*       | 27°C                   | –              | [35]      |
| *Candida tropicalis*     | 27°C                   | –              | [35]      |
| *Candida glabrata*       | 37°C                   | +              | [48]      |
| *Cryptococcus neoformans*| 37°C                   | –              | [53]      |
| *Aspergillus fumigatus*  | 27°C                   | –              | [65]      |
| *Arthroderma vanbreuseghemii* | 30°C             | –              | [8]       |
| *Arthroderma benhamiae*  | 30°C                   | –              | [8]       |
| *Microsporum canis*      | 30°C                   | –              | [8]       |
| *Trichophyton rubrum*    | 30°C                   | –              | [8]       |
| *Rhizopus oryzae*        | 27°C                   | –              | [73]      |
4 | CONCLUSIONS

In this review, we have stated that silkworm infection models for several pathogenic fungi have been established and that genes necessary for their pathogenicity can be identified by screening of gene-deficient mutant libraries. Being relatively cheap and lacking ethical problems, silkworms are suitable for large-scale in vivo screening. In silkworm infection models, it is easy to quantitatively measure pathogenicity of fungi on the basis of determination of the lethal dose for 50% of these animals. Moreover, it is possible to establish an infectious system for investigating weakly pathogenic fungi, which are difficult to investigate in mammals, by changing the incubation temperature (Table 2). Silkworm infection models will contribute to establishing novel preventive and therapeutic strategies by elucidating the infectious systems of pathogenic fungi.

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