Original Article

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Two-spotted cricket as an animal infection model of human pathogenic fungi

Yuto Kochi[1], Yasuhiko Matsumoto[2], Kazuhisa Sekimizu[2], Chikara Kaito[1,*]

[1] *Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan;*
[2] *Institute of Medical Mycology, Teikyo University, Tokyo, Japan.*

Summary **Invertebrate infection models that can be evaluated at human body temperature are limited. In this study, we utilized the two-spotted cricket, a heat-tolerant insect, as an animal infection model of human pathogenic fungi. Injection of human pathogenic fungi, including *Candida albicans*, *Candida glabrata*, and *Cryptococcus neoformans* killed crickets within 48 h at both 27˚C and 37˚C. The median lethal dose values (LD_{50} values) of *C. albicans* and *C. glabrata* against crickets were decreased at 37˚C compared to that at 27˚C, whereas the LD_{50} value of *C. neoformans* was not different between 27˚C and 37˚C. Heat-killed cells of the three different fungi also killed crickets, but the LD_{50} value of the heat-killed cells was higher than 5-fold that of live fungal cells in the respective species. *C. neoformans* gene-knockout strains of *cna1*, *gpa1*, and *pka1*, which are required for virulence in mammals, had greater LD_{50} values than the parent strain in crickets. These findings suggest that the two-spotted cricket is a valuable infection model of human pathogenic fungi that can be used to evaluate fungal virulence at variable temperatures, including 37˚C, and that the killing abilities of *C. albicans* and *C. glabrata* against animals are increased at 37˚C.**

Keywords: Cricket, animal infection model, temperature, virulence, human pathogenic fungi

1. Introduction

Human pathogenic fungi such as *Candida albicans*, *Candida glabrata*, and *Cryptococcus neoformans* cause superficial infections in the skin and oral cavity as well as deep infections in organs such as the lung and brain. Immunocompromised patients, such as those with AIDS and cancer, are especially susceptible to lethal fungal infection (*1-4*). Because fungi are eukaryotes, limited numbers of antifungal drugs are available and thus novel antifungal drugs are desired. To develop new antifungal drugs, it is important to understand the molecular mechanisms of fungal infectious processes using an animal infection model and to identify new drug targets. Many mammalian pathogens including fungi are assumed to detect an increase in the environmental temperature as information regarding the host environment (*5*). At 37°C, the human body temperature, *C. albicans* modulates its nucleosome structure with temperature-induced transcription factors to accomplish physiologic alterations that enhance virulence, such as hyphae formation (*6,7*). To clarify the significance of such temperature-dependent processes for fungal infectious processes, it is essential to utilize animal infection models at both low and high temperatures.

Animal models of infection that can withstand both low and high temperatures, however, are scarce. Mammals such as mice and rabbits have been used to evaluate fungal virulence properties in various organs, including the skin (*8*), lung (*9*), stomach (*10*), oral cavity (*11*), urethral tube (*12*), and vagina (*13*). Mammals are homeothermic animals, however, and cannot be used to evaluate fungal virulence at low temperatures. Many non-mammalian animals have been used as animal models of fungal infection to overcome the ethical and cost-related issues associated with mammalian model animals (*14,15*); a vertebrate model such as zebra fish (*16*), and invertebrate models such as nematode (*Caenorhabditis elegans*) (*17*), fruit fly (*Drosophila melanogaster*) (*18*), silkworm (*Bombyx mori*) (*19,20*), and the greater

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**Address correspondence to:*
Dr. Chikara Kaito, Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 3-1, 7-chome, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
E-mail: kaito@mol.f.u-tokyo.ac.jp

wax moth larva (*Galleria mellonella*) (*21,22*). The heterothermic characteristics of zebra fish were used to evaluate fungal virulence at a high temperature of 33°C (*23,24*). The applicability of this model at 37°C, however, is not known. The nematode *C. elegans* and the fruit fly *D. melanogaster* cannot survive at 37°C. Silkworms and the greater wax moth larva can be used as animal infection models at 37°C (*25-27*), but their infection sensitivities to fungi are drastically increased at 37°C compared to that at a lower temperature (*19,28-31*). The increased infection sensitivity of these insects at 37°C is considered to be due to damage to the immune system at a high temperature (*32-34*).

We focused on the two-spotted cricket, *Gryllus bimaculatus*, an Orthopteran insect that is distributed across tropical and subtropical regions in the world, to investigate the effects of temperature on the infectious processes of human pathogens. The two-spotted cricket develops from nymph to adult at a wide range of temperatures, from 19°C to 37°C (*35,36*). We previously reported that the two-spotted cricket is infected and killed by human pathogenic bacteria, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Listeria monocytogenes* (*37*). The infection sensitivity of the two-spotted cricket to *S. aureus* and *P. aeruginosa* does not differ between 27°C and 37°C, but the infection sensitivity to *L. monocytogenes* is higher at 37°C than at 27°C (*37*). Thus, the two-spotted cricket does not generally increase the infection sensitivity to pathogens at 37°C, and is an animal infection model that can be used to evaluate the effect of temperature on infectious processes without perturbing host system to identify the temperature-dependent virulence mechanisms of specific pathogens. In addition, the two-spotted cricket is available at a low price throughout the world, because crickets are cultured as food for amphibians and reptiles. The body size of the cricket is appropriate for injecting accurate amounts of samples and to quantitatively evaluate the virulence properties of pathogens by determining the median lethal dose (LD_{50}) value (*37*). In this study, we examined the virulence properties of human pathogenic fungi, including *C. albicans*, *C. glabrata*, and *C. neoformans*, using the cricket infection model at 27°C and 37°C. The findings revealed that the two-spotted cricket can be used as an animal infection model of human pathogenic fungi, and that *C. albicans* and *C. glabrata* exhibit increased killing abilities against crickets at a high temperature.

2. Materials and Methods

2.1. *Crickets*

Two-spotted crickets in the final nymph stage were purchased from Tsukiyono Farm (Tone-gun, Gunma, Japan) and raised to adults by feeding them water and food at 27°C, as previously reported (*38,39*). Briefly, 100 crickets were kept in a plastic cage (W320 × L170 × H210) with food 'Koorogi-food' (Tsukiyono-Farm), wet paper towel, and paper egg trays. Adult crickets within 1 week after eclosion were used for the infection experiments.

Table 1. Fungal strains used in this study

Strain	Genotypes and Characteristics	*Ref.*
Candida albicans		
ATCC10231	Serotype A	(*56*)
Candida glabrata		
CBS138	ATCC2001	(*57*)
Cryptococcus neoformans		
H99	Serotype A, clinical isolate	(*58*)
Δ*cna*	H99, *cna1*::*ade2*, *matα*	(*43*)
Δ*gpa1*	H99, *gpa1*:: *ade2*, *matα*	(*45*)
Δ*pka1*	H99, *pka1*:: *ade2*, *matα*	(*47*)

2.2. *Fungal strains and culture conditions*

Fungal strains, including *C. albicans*, *C. glabrata*, *C. neoformans*, and the gene-knockout strains of *C. neoformans*, which were stocked at -80˚C, were streaked on YPD agar plates and cultured overnight at 30°C. A single colony of each strain was inoculated into 30 ml of YPD liquid medium in a 225-mL conical tube (cat. no. 352075, BD Falcon, Bedford, MA) and aerobically cultured overnight by shaking at 150 rpm (BR-3000LF, TAITEC co., ltd., Koshigaya, Saitama, Japan) at 30°C. The details of the fungal strains used in this study are listed in Table 1.

2.3. *Infection experiments using crickets*

Overnight fungal cultures were centrifuged at 5,000 *g* for 6 min and the precipitated cells were suspended in saline. The fungal cell solution was serially diluted with saline. Crickets were injected with 50 μL of fungal solution *via* an intra-hemolymph route from the ventral abdominal region using a 1-mL syringe equipped with a 30 gauge-needle, as previously reported (37). The injected crickets (n = 5/group) were maintained in a disposable cylindrical dish (Φ129 × H97 mm, MINERON KASEI Co., Ltd., Higashi-Osaka, Osaka, Japan) with food and wet Kimwipes (NIPPON PAPER CRECIA Co., Ltd., Chiyoda-ku, Tokyo, Japan) in dark conditions at 27°C or 37°C. Cricket survival was monitored after the injection. The fungal solution used for the infection experiment was 10^5-fold diluted with saline, spread onto YPD agar plates, and incubated overnight at 30°C. The appearing colonies were counted and the number of live fungal cells injected into the crickets was calculated. The LD_{50} values of fungal strains against the crickets were determined by logistic regression from the dose-survival plots. All the survival data are listed in Table S1 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=16*).

2.4. *Preparation of heat-killed fungal cells*

Overnight fungal cultures were centrifuged at 5,000 *g* for 6 min, and the precipitated cells were suspended in 1 mL of saline and transferred to a 2-mL Eppendorf tube. A small part of the fungal solution was 10^5-fold diluted with saline, spread onto YPD agar plates, and incubated overnight at 30°C to measure the number of fungal cells. The fungal solution in a 2-mL Eppendorf tube was autoclaved at 121°C for 20 min, serially diluted with saline, and used for the infection experiments.

2.5. *Measurement of fungal doubling time*

Single colonies of *C. albicans*, *C. glabrata*, or *C. neoformans* were inoculated into 10 mL of YPD liquid medium in a 50-mL conical tube (Cat. No. 352070, BD-Falcon) and aerobically cultured overnight at 30°C by shaking at 150 rpm. A 100-µL aliquot of the overnight culture was inoculated into 10 mL of YPD liquid medium in a 50-mL conical tube and aerobically cultured by shaking at 150 rpm at 27°C or 37°C. The OD_{600} values were measured over time using a spectrophotometer (UV-1280, SHIMADZU Co., Kyoto, Japan). To measure the condensed cell culture, the culture was appropriately diluted with saline. The doubling time was calculated from the exponential growth phase by linear regression, as previously reported (*40,41*).

2.6. *Statistical analysis*

Cricket survival at different fungal doses were plotted on an X-Y graph and the dose-response survival curves were determined by logistic regression. To compare the two dose-survival curves, a likelihood ratio test was performed using R ver. 3.2.3 (*42*). The LD_{50} value and the standard error were determined using 'Mass' in R.

3. Results

3.1.*Cricket killing by human pathogenic fungi*

To determine whether human pathogenic fungi kill the two-spotted crickets, we injected *C. albicans*, *C. glabrata*, and *C. neoformans* into crickets *via* the intra-hemolymph route and maintained the crickets at 27°C or 37°C. At both temperatures, a high number of *C. albicans* cells killed crickets within 18 h after the injection, whereas a low number of *C. albicans* cells killed crickets 40 h after injection (Figure 1A). A similar tendency was

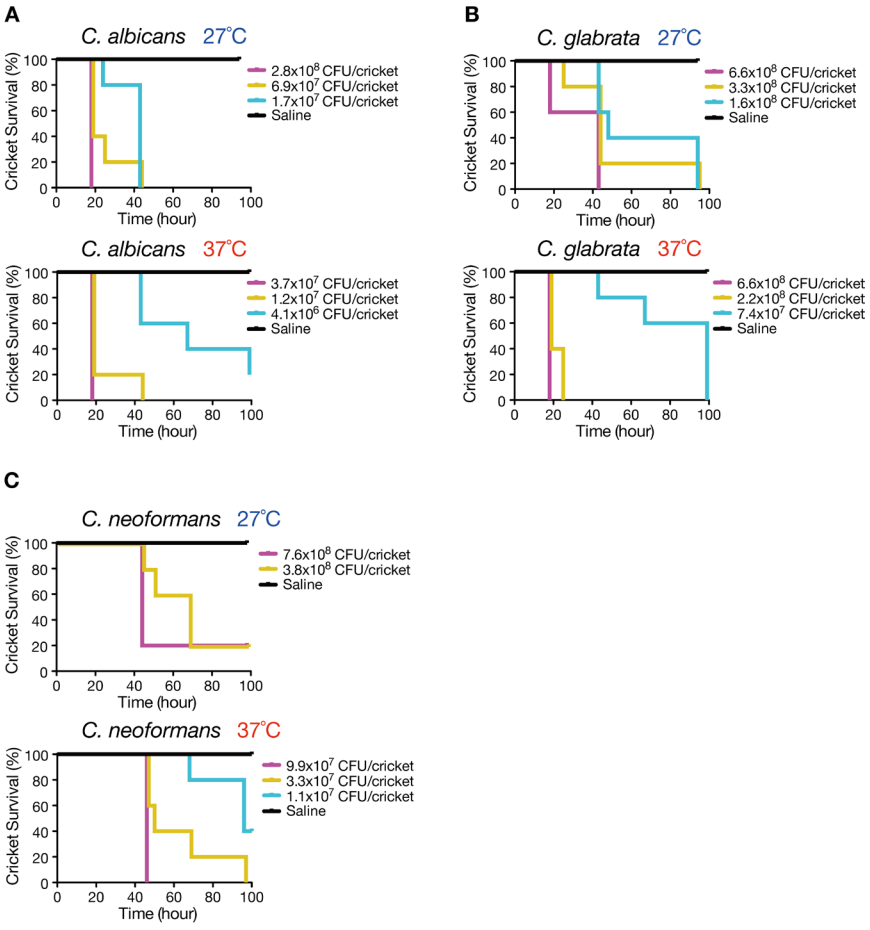

Figure 1. Killing of crickets after injecting human pathogenic fungi. Crickets (n = 5/group) were injected with saline or various doses of *C. albicans* **(A)**, *C. glabrata* **(B)**, or *C. neoformans* **(C)**, and were maintained at 27°C or 37°C. The time-course of cricket survival was monitored.

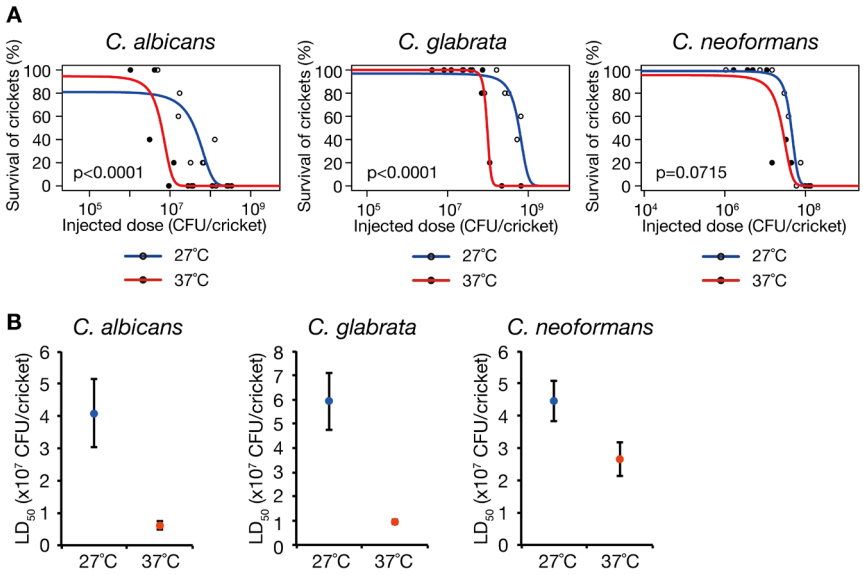

Figure 2. Effect of temperature on cricket sensitivity against fungal infection. (A) The dose-response survival curve of crickets injected with *C. albicans*, *C. glabrata*, or *C. neoformans* was examined at 27°C or 37°C. Serial dilutions of the fungal solution were injected into crickets (n = 5/dose) and survival was monitored at 24 h (*C. albicans* and *C. glabrata*) or 48 h (*C. neoformans*) after the injection. Results from independent experiments (*C. albicans* [27°C], three times; *C. albicans* [37°C], two times; *C. glabrata* [27°C], three times; *C. glabrata* [37°C], three times; *C. neoformans* [27°C], three times; *C. neoformans* [37°C], two times) were pooled and the survival curve was determined by logistic regression. The p-values determined by using likelihood ratio tests between the survival curves at 27°C and 37°C are presented in the graphs. All survival data are presented in Table S1 (Supporting Information) and no crickets injected with saline died in any of the experiments. **(B)** The LD_{50} values of *C. albicans*, *C. glabrata*, or *C. neoformans* at 27°C or 37°C were determined by logistic regression in **(A)**. Error bars indicate standard errors.

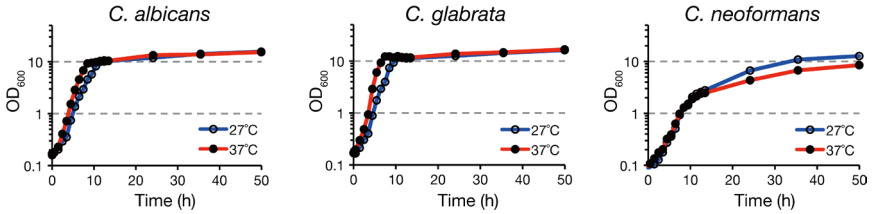

Figure 3. Effect of temperature on fungal growth curve. Overnight cultures of *C. albicans*, *C. glabrata*, or *C. neoformans* were inoculated into 100-fold amounts of fresh YPD medium and aerobically cultured at 27°C or 37°C. OD_{600} was measured during the time-course.

observed in the injection of *C. glabrata* cells (Figure 1B). *C. neoformans* killed crickets 43 h after injection (Figure 1C). In all experiments, injection of saline did not kill the crickets (Figures 1A-1C). These results suggest that *C. albicans*, *C. glabrata*, and *C. neoformans* kill crickets.

3.2. *Comparison of cricket killing ability by fungi between 27°C and 37°C*

Because the two-spotted cricket does not universally increase infection sensitivity to pathogens (*37*), the two-spotted cricket is useful for evaluating the temperature effect on the infection properties of pathogens. We examined whether *C. albicans*, *C. glabrata*, and *C. neoformans* increase their killing activities against crickets at a higher temperature. Crickets were injected with fungi, maintained at 27°C or 37°C, and survival was measured. The dose-response survival curve of *C. albicans* differed between 27°C and 37°C (Figure 2A), and the LD_{50} value at 37°C was less than one-fifth that at 27°C (Figure 2B). The dose-response survival curve of *C. glabrata* was different between 27°C and 37°C (Figure 2A), and the LD_{50} value at 37°C was less than one-fifth that at 27°C (Figure 2B). In contrast, the dose-response survival curve of *C. neoformans* did not differ between 27°C and 37°C (Figure 2A). These results suggest that *C. albicans* and *C. glabrata* have increased killing ability against crickets at 37°C compared to 27°C.

3.3. *Comparison of fungal growth between 27°C and 37°C*

We hypothesized that one reason for the increased virulence of *C. albicans* and *C. glabrata* at a high temperature is an increased growth rate at high temperature. To address this point, we measured the growth curves of *C. albicans*, *C. glabrata*, and *C. neoformans* at 27°C and 37°C, and determined the doubling times. All fungal strains showed logarithmic growth from 2 h to 6 h after inoculation (Figure 3). The

doubling times of *C. albicans* and *C. glabrata* were shorter at 37°C than at 27°C (Table 2). In contrast, the doubling time of *C. neoformans* was not shorter at 37°C than at 27°C (Table 2). These results suggest that the growth rates of *C. albicans* and *C. glabrata* increase at 37°C compared to at 27°C.

3.4. *Killing activity of heat-killed fungal cells against crickets*

To address whether the cricket killing ability by fungi is caused by live fungal cells, we examined the killing activities of heat-killed fungal cells against crickets. *C. albicans*, *C. glabrata*, and *C. neoformans* cells were autoclaved and injected into crickets. In all fungal species, the dose-response survival curve was different between the live fungal cells and the heat-killed fungal cells (Figure 4A). The LD_{50} value of heat-killed fungal cells was higher than 5-fold that of live fungal cells in each species (Figure 4B). These results suggest that live fungal cells have higher killing activity against crickets than dead fungal cells.

Table 2. Doubling times of fungal strains

Temperature	*C. albicans*	*C. glabrata*	*C. neoformans*
27°C	1.56 ± 0.09	1.34 ± 0.16	1.80 ± 0.04
37°C	1.05 ± 0.04	0.802 ± 0.020	2.16 ± 0.22
P value	0.0099	0.0259	0.0776

Doubling time (h) was calculated from fungal growth curves at 27°C or 37°C. Data are the means ± standard errors from three independent experiments. Student's *t*-test p values between 27°C and 37°C are presented.

3.5. *Killing activities of C. neoformans gene-knockout strains against crickets*

To determine the applicability of the cricket model for evaluation of fungal virulence factors, we examined whether the *C. neoformans* gene-knockout strains of *cna1*, *gpa1*, and *pka1*, which are virulence factors in mammals, exhibit decreased killing activities against crickets. *cna1* encodes a subunit of calcineurin, a protein phosphatase involved in the signaling pathway (*43,44*). *gpa1* and *pka1* are involved in capsule formation and melanin synthesis *via* a calcineurin-independent pathway (*45-47*). Crickets were injected with the gene-knockout strains and the dose-response survival curve at 37°C was determined. The survival curve differed between the parent strain and the respective gene-knockout strain (Figure 5A). The LD_{50} values of the *cna1*-, *gpa1*-, and *pka1*-knockout strains were higher than 2-fold that of the parent strains (Figure 5B). These results suggest that the cricket-fungus infection model is effective for evaluating fungal virulence factors.

4. Discussion

This study revealed that human pathogenic fungi,

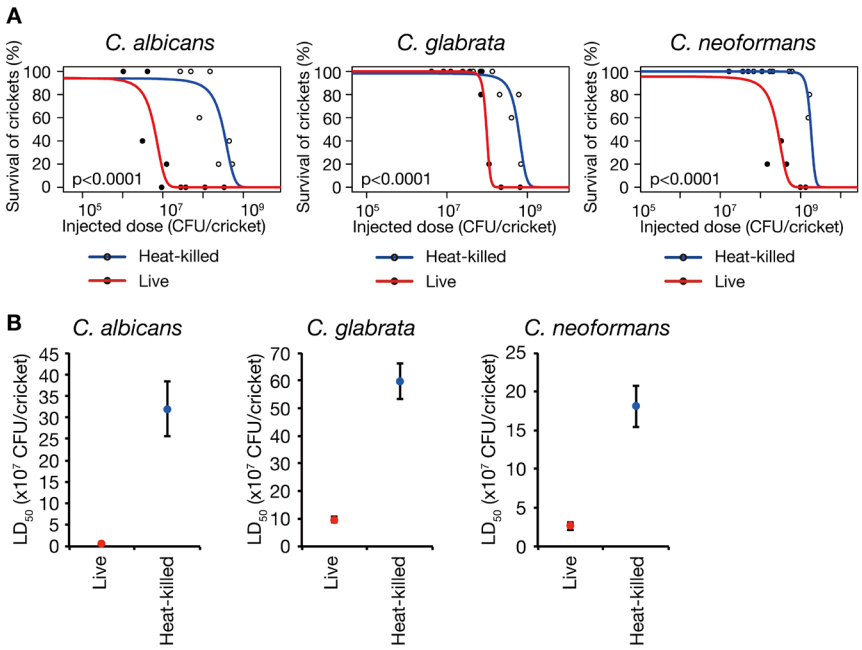

Figure 4. Killing effects of heat-killed fungal cells against crickets. (A) The dose-response survival curve of crickets injected with heat-killed cells of *C. albicans*, *C. glabrata*, or *C. neoformans* was examined at 37°C. Serial dilutions of heat-killed fungal solutions were injected into crickets (n = 5/dose) and survival was monitored at 24 h (*C. albicans* and *C. glabrata*) or 48 h (*C. neoformans*) after the injection. Results from three independent experiments were pooled and the survival curve was determined by logistic regression. Results of live fungal cells (closed symbols and red line) are the same as in Figure 2A. The p-values determined by using likelihood ratio tests between the survival curves of dead fungal cells and of live fungal cells are presented in the graphs. All survival data are presented in Table S1 (Supporting Information), and no crickets injected with saline died in any of the experiments. **(B)** The LD_{50} values of heat-killed fungal cells were determined by logistic regression in **(A)**. The LD_{50} values of live fungal cells were the same as those in Figure 2B. Error bars indicate standard errors.

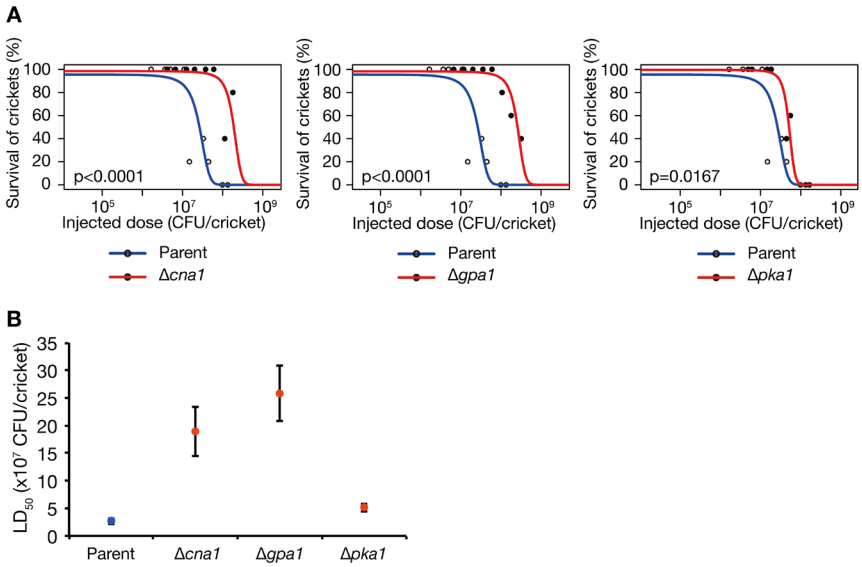

Figure 5. Evaluation of *C. neoformans* virulence factors in the cricket infection model. (A) The dose-response survival curves of crickets (n = 5/dose) injected with *C. neoformans* gene knockout strains of *cna1*, *gpa1*, and *pka1* were determined at 37°C. Survival was monitored at 48 h after fungal injection. Results from two independent experiments were pooled and the survival curve was determined by logistic regression. All survival data are presented in Table S1 (Supporting Information) and no crickets injected with saline died in any of the experiments. The p-values determined using likelihood ratio tests between the parent strain and the gene-knockout strain are presented in the graphs. The survival curve of crickets infected with the parent strain of *C. neoformans* was the same as in Figure 2A (*C. neoformans*, 37°C). **(B)** The LD_{50} values of *C. neoformans* gene knockout strains of *cna1*, *gpa1*, and *pka1* were determined by logistic regression in **(A)** and compared with the parent strain. The LD_{50} value of the parent strain was the same as that in Figure 2B (*C. neoformans*, 37°C). Error bars indicate standard errors.

including *C. albicans*, *C. glabrata*, and *C. neoformans*, kill the two-spotted cricket. The killing activity of the fungi was drastically decreased by heat inactivation of the fungal cells, indicating that live fungal cells contribute to kill crickets. Furthermore, we evaluated fungal virulence against crickets at both 27°C and 37°C, and revealed that the killing activities of *C. albicans* and *C. glabrata* were increased at 37°C compared to that at 27°C, but the killing activity of *C. neoformans* was not different between the two temperatures. This study also demonstrated that the *C. neoformans* gene-knockout strains of virulence factors attenuated the killing activities against crickets. Based on these results, we propose that the two-spotted cricket is a useful animal infection model of human pathogenic fungi, and is effective for clarifying the temperature-dependent virulence system of fungal pathogens.

To our knowledge, this study is the first to reveal that *C. albicans* and *C. glabrata* exhibit a temperature-dependent increase in killing activity against animals. This finding was obtained because we utilized the two-spotted cricket, a heterothermic animal, which can grow at both 27°C and 37°C. We found that the growth rate of these two fungi increases at 37°C, compared to that at 27°C, and assumed it to be a possible reason for the increased killing activities at 37°C. In addition to the growth rate, increasing the temperature leads to many physiologic alterations of fungi. *C. albicans* forms hyphae at high temperature, and a gene-knockout strain that is unable to form hyphae exhibits attenuated virulence (*48,49*). *C. glabrata* alters cell surface polysaccharides and shows different cell surface hydrophobicity at 37°C compared to that at a lower temperature (*50,51*). *C. neoformans* upregulates nucleotide metabolism and capsule formation at a high temperature, enabling growth at a high temperature (*52,53*). Because *C. neoformans* did not exhibit increased killing activity against crickets at 37°C, the physiologic alteration that is present in the two *Candida* species but is absent in *C. neoformans* may contribute to the temperature-dependent killing activity against crickets. These points should be investigated in future studies by evaluating the virulence properties of fungal genetic mutants for each physiologic process in the cricket infection model to clarify the temperature-dependent virulence system of these fungi.

Heat-killed fungal cells showed killing activity against crickets, although the killing activity of the heat-killed fungal cells was drastically lower than that of live fungal cells. It is plausible that live fungal cells proliferate in the cricket body and the proliferated fungal cells kill crickets in the same manner as the heat-killed cells. In silkworms, β-glucan, a component of the fungal cell wall, excessively activates the humoral immune system, which kills silkworms (*54,55*). To optimize the utility of the two-spotted cricket as an animal infection model of human pathogenic fungi, further studies are needed to investigate the immune response of crickets against fungal cells and to reveal the molecular mechanism underlying infection-induced death in crickets.

Acknowledgements

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