

Development of an Efficient Gene-targeting System for Elucidating Infection Mechanisms of the Fungal Pathogen *Trichosporon Asahii*

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Research Article

Keywords: *Trichosporon asahii*, silkworm, homologous recombination, Ku70, infection, calcineurin

Posted Date: June 22nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-636253/v1>

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Abstract

Trichosporon asahii is a pathogenic fungus that causes severe, deep-seated fungal infections in neutropenic patients. Elucidating the infection mechanisms of *T. asahii* based on genetic studies requires a specific gene-targeting system. Here, we established an efficient gene-targeting system in a highly pathogenic *T. asahii* strain identified using the silkworm infection model. By comparing the pathogenicity of *T. asahii* clinical isolates in a silkworm infection model, *T. asahii* MPU129 was identified as a highly pathogenic strain. Using an *Agrobacterium tumefaciens*-mediated gene transfer system, we obtained a *T. asahii* MPU129 mutant lacking the *ku70* gene, which encodes the Ku70 protein involved in the non-homologous end-joining repair of DNA double-strand breaks. The *ku70* gene-deficient mutant showed higher gene-targeting efficiency than the wild-type strain for constructing a mutant lacking the *cnb1* gene, which encodes the beta-subunit of calcineurin. The *cnb1* gene-deficient mutant showed reduced pathogenicity against silkworms compared with the parental strain. These results suggest that an efficient gene-targeting system in a highly pathogenic *T. asahii* strain is a useful tool for elucidating the molecular mechanisms of *T. asahii* infection.

Introduction

Trichosporon asahii is a basidiomycete yeast that is widely distributed in the environment and is often isolated from human blood, sputum, skin, feces, and urine¹⁻⁶. *T. asahii* causes severe, deep-seated fungal infections in neutropenic patients⁷⁻⁹. Deep mycoses caused by *T. asahii* has a 2-fold higher mortality rate than those caused by *Candida albicans* (80% vs 40%)¹⁰. Since *T. asahii* is resistant to echinocandin antifungals, patients treated with micafungin are susceptible to the development of severe infections¹¹. *T. asahii* strains resistant to amphotericin B and azole antifungals such as fluconazole have also been isolated from patients^{12,13}. *T. asahii* is therefore a highly problematic clinical pathogen⁹.

In general, mammals such as mice are used as experimental models in studies of infectious diseases¹⁴. The use of mammalian animals in infection experiments requires specialized experimental facilities, and the large number of animals required for these studies is a severe limitation due to ethical issues regarding animal welfare¹⁵. *T. asahii* infection experiments are not easy to perform in mice because immunosuppressive drugs must be administered¹⁶⁻¹⁸. To address these issues, we established a silkworm infection model for elucidating the mechanisms of *T. asahii* infection¹⁹. Compared with mammals such as mice, the use of invertebrate silkworms is advantageous because they are less costly to house and easier to rear in large numbers in simple facilities, and fewer ethical problems are associated with their use. Therefore, the use of silkworms as an experimental animal enhances the feasibility of performing large-scale, *in vivo* screening using a large number of individuals¹⁴.

Novel virulence genes in the pathogenic bacterium *Staphylococcus aureus* were identified using a silkworm infection model and a library of gene-deficient strains^{20,21}. Silkworm infection models have also been used to identify virulence genes of the pathogenic fungi *C. albicans* and *Candida glabrata*²²⁻²⁴. In

Cryptococcus neoformans, a basidiomycete yeast like *T. asahii*, a strain that is highly pathogenic to mice is also highly pathogenic to silkworms²⁵. Moreover, a *C. neoformans* strain lacking the gene encoding the calcineurin subunit, which contributes to the pathogenicity against mice, was less virulent against silkworms²⁵. Since the silkworm can be used to evaluate differences in the pathogenicity between strains of pathogenic fungi, the silkworm infection model with *T. asahii* may be useful for elucidating the infection mechanisms of *T. asahii* with the gene-deficient strains. We successfully established a *T. asahii* strain that expresses green fluorescent protein using an *Agrobacterium tumefaciens*-mediated gene transfer (ATMT) system¹⁹. A method for generating a gene-deficient strain of *T. asahii*, however, has not yet been established.

Homologous recombination (HR), a repair mechanism for DNA double-strand breaks (DSBs), is required to introduce mutations into a gene-targeting system using homologous DNA fragments^{26,27}. Another repair mechanism is the non-homologous end joining (NHEJ) of DSBs²⁶. These 2 main repair mechanisms affect gene-targeting efficiency by introducing homologous DNA fragments. NHEJ repair mediates the insertion of introduced homologous DNA fragments into genome sites that are different from the target region, thereby reducing the gene-targeting efficiency for generating a gene-deficient strain²⁶. Therefore, gene-targeting efficiency can be increased by inhibiting NHEJ repair^{26,28}. Ku70 and Ku80 proteins form heterodimers and are involved in the NHEJ repair for DSBs²⁹. In several fungi, deletion of the genes encoding these proteins led to the increase of gene-targeting efficiency for generating gene-deficient strains^{28,30-33}. In *C. neoformans*, gene-deficient strains could be generated in the *ku80* gene-deficient strain, but not in the wild-type strain, by electroporation, a gene transfer method³⁴. Therefore, strains with inhibited NHEJ repair due to disruption of the gene encoding Ku proteins are useful parental strains for promoting genetic studies.

In this study, we identified the *T. asahii* MPU129 strain, a clinical isolate that is highly pathogenic to silkworms, and generated a *T. asahii* MPU129 mutant deficient in the *ku70* gene. Gene-targeting efficiency to obtain the *ku70* gene-deficient strain was higher than that in the wild-type strain. Our findings suggest that a *T. asahii* strain showing high gene-targeting efficiency and the silkworm infection model are useful tools for studying infectious diseases as a preliminary step to conducting experiments in mice.

Results

Comparison of the pathogenicity of *T. asahii* strains using a silkworm infection model. Highly pathogenic strains are useful for understanding the molecular mechanisms of pathogens because several pathogenic strains obtain virulence genes by horizontal gene transfer and gene mutation³⁵⁻³⁹. First, we identified *T. asahii* strains that are highly pathogenic to silkworms. Using a silkworm infection model, we determined the median lethal dose (LD₅₀ values) on the basis of curves drawn by a simple logistic regression model (Fig. 1). The LD₅₀ values of the 17 clinical isolates were $9.3 \times 10^3 - 2.0 \times 10^6$ cells/larva and the LD₅₀ value of the MPU129 strain was the lowest, more than 10-fold lower than that of

the JCM2466 strain (Fig. 1h). The result suggests that the pathogenicity of the MPU129 strain against silkworms is highest among these *T. asahii* strains.

Generation of the *ku70* gene-deficient *T. asahii* MPU129 strain mutant. We next obtained a *ku70* gene-deficient mutant of the MPU129 strain using the ATMT system. The experimental scheme is shown in Fig. 2a. The targeting plasmid, pAg1-5'UTR (*ku70*)-*nptII*-3'UTR (*ku70*), contained the *nptII* gene that leads to resistance against G418, an aminoglycoside used as a selective agent for eukaryotic cells (Fig. 2b). Colonies on Sabouraud dextrose agar containing G418 were obtained using the ATMT system (Fig. 2c). In the genome of the 414th candidate colony, polymerase chain reaction (PCR) amplification revealed DNA fragments of the predicted size (Fig. 2d and e). The results suggest that the *ku70* gene-deficient *T. asahii* MPU129 strain mutant was obtained using the ATMT system.

Effect of *ku70* gene deficiency on growth and pathogenicity of the *T. asahii* MPU129 strain. We investigated whether the deficiency of *ku70* in the *T. asahii* MPU129 strain affected its growth on nutrient media and its pathogenicity in silkworms. The growth of the *ku70* gene-deficient mutant in RPMI-1640 or Sabouraud liquid medium was similar to that of wild-type at either 27°C or 37°C (Fig. 3a-d). Moreover, microscopic analysis did not reveal significant differences in the morphology (Fig. 3e). Furthermore, the time required for the *ku70* gene-deficient mutant to kill all the silkworms was similar to that of the wild-type strain (Fig. 3f-h). These results demonstrated that *ku70* gene deficiency in the *T. asahii* MPU129 strain did not significantly affect its growth on nutrient media or its pathogenicity to silkworms.

Increased gene-targeting efficiency in the *ku70* gene-deficient *T. asahii* mutant. We examined whether gene-targeting efficiency was increased in the *ku70* gene-deficient strain by determining the ratio of the strain lacking the *cnb1* gene, which encodes the β -subunit of calcineurin. Since gene-targeting efficiency in *ku80* gene-deficient strain of *C. neoformans* was tested by electroporation, a faster and simpler gene transfer method³⁴, we also used electroporation to investigate *T. asahii*. The experimental scheme is shown in Fig. 4a. A DNA fragment, 5'UTR (*cnb1*)-*NAT1*-3'UTR (*cnb1*), was introduced to delete the *cnb1* gene by electroporation (Fig. 4a). Nourseothricin-resistant strains were obtained, and each colony was confirmed by PCR to be deficient in the *cnb1* gene (Fig. 4b-e). Of the 21 nourseothricin-resistant colonies obtained by introducing the 5'UTR (*cnb1*)-*NAT1*-3'UTR (*cnb1*) into the *ku70* gene-deficient mutant, 4 were deficient for the *cnb1* gene (Table 2). On the other hand, none of the 120 nourseothricin-resistant colonies obtained by introducing the 5'UTR (*cnb1*)-*NAT1*-3'UTR (*cnb1*) into the wild-type was deficient for the *cnb1* gene (Table 2). These results suggest that the deficiency of the *ku70* gene in the MPU129 strain increases the gene-targeting efficiency for generating a gene-deficient mutant by electroporation.

Attenuated pathogenicity of the *cnb1* gene-deficient mutant against silkworms. In *C. neoformans*, the pathogenicity of the *cnb1* gene-deficient mutant against silkworms was reduced²⁵. We examined whether the *cnb1* gene-deficient mutant of *T. asahii* had reduced pathogenicity against silkworms. The survival time of silkworms injected with the *cnb1* gene-deficient mutant was longer than that of the parental strain (Fig. 5a). The LD₅₀ value of the *cnb1* gene-deficient mutant was 89-fold higher than that of the parent

strain (Fig. 5b). The result suggests that pathogenicity against silkworms was reduced by *cnb1* gene deficiency in *T. asahii*.

Discussion

In this study, we identified a *T. asahii* strain that is highly pathogenic against silkworms and established a platform for generating a gene-deficient mutant. The *cnb1* gene-deficient mutant obtained using the technique showed decreased pathogenicity against silkworms. To our knowledge, this is the first report of a method for obtaining a gene-deficient mutant of *T. asahii*. Our results suggest that the calcineurin pathway is involved in the pathogenicity of *T. asahii*.

Silkworms are suitable experimental animals for performing large-scale *in vivo* evaluations because they are relatively inexpensive and few ethical issues are associated with their use. Therefore, silkworm infection models are suitable for quantitative evaluation of the pathogenicity of microorganisms based on the calculation of LD₅₀ values. Using the silkworm infection model and a gene-deficient mutant library of *S. aureus*, we previously identified the virulence genes that contribute to pathogenicity against mice²⁰. The gene-targeting system in *T. asahii* developed in the present study will facilitate the construction of a gene-deficient mutant library of *T. asahii*. It is expected that a gene-deficient mutant library of *T. asahii* for application to silkworm infection models will help elucidate the molecular mechanisms of *T. asahii* infection. The contribution of candidate virulence genes to pathogenicity that is identified using the silkworm infection model should be confirmed by infection experiments in mice.

Gene-targeting efficiency by electroporation was higher in the *ku70* gene-deficient mutant, while growth on nutrient media and pathogenicity to silkworms remained unaltered. Therefore, the *ku70* gene-deficient mutant is useful as a parental strain for elucidating the infection mechanism of *T. asahii* based on genetic studies. In the pathogenic fungus *Aspergillus fumigatus*, the pathogenicity of the *ku80* gene-deficient mutant did not differ from that of wild-type *Aspergillus fumigatus* against mice⁴⁰. We considered that NHEJ repair of DSBs might not be greatly involved in the pathogenicity of *T. asahii* or *A. fumigatus*. Although electroporation is a faster and simpler gene transfer method than the ATMT system for obtaining gene-deficient mutants, homologous recombination by electroporation occurs at low frequency^{34,41}. When using electroporation, no *cnb1* gene-deficient mutants were obtained in the wild-type, but a 19% ratio of mutations was obtained in the *ku70* gene-deficient mutant. The result suggests that NHEJ of DSBs occurs at a high frequency in *T. asahii*. Therefore, we reasoned that the generation of target gene-deficient mutants by electroporation requires the *ku70* gene-deficient mutant that lacks NHEJ repair activity. Construction of a gene-deficient mutant library of *T. asahii* using the *ku70* gene-deficient mutant as a parent strain is thus planned for future studies.

Although we tried to obtain a *cnb1* gene-deficient mutant using the *ku70* gene-deficient mutant as the parent strain with the ATMT system, we did not obtain a drug-resistant candidate. Optimization of the ATMT system using the *ku70* gene-deficient mutant is needed to obtain target gene-deficient mutants.

In *C. neoformans*, the calcineurin pathway is involved in capsule production and melanin synthesis, which are responsible for evading host immunity⁴². A calcineurin-deficient strain of *C. neoformans* showed decreased pathogenicity against mice and silkworms^{25,43,44}. The present study also showed that the calcineurin pathway is involved in the pathogenicity of *T. asahii* against silkworms. Further studies are needed to investigate the generation of a revertant strain obtained by reintroducing the *cnb1* gene into the *cnb1* gene-deficient mutant and to perform a detailed functional analysis using the *cnb1* gene-deficient mutant and its revertant strain.

In conclusion, we established a simple method for generating a gene-deficient *T. asahii* strain that is highly pathogenic against silkworms. The *ku70* gene-deficient *T. asahii* MPU129 strain mutant is useful as a parental strain for genetic studies and an important tool for studying infectious diseases of *T. asahii*.

Methods

Reagents. Kanamycin, cefotaxime, and chloramphenicol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Nourseothricin and G418 were purchased from Jena Bioscience (Dortmund, Germany) and Enzo Life Science, Inc. (Farmingdale, NY, USA), respectively.

Culture of *T. asahii*. The *T. asahii* strains (MPU105, MPU107, MPU108, MPU113, MPU120, MPU129, MPU131, MPU133, MPU147, MPU153, MPU159, MPU160, MPU161, MPU162, MPU330, MPU331, and MPU344) used in this study were stocked in the previously reported MPU library⁴⁵. *T. asahii* strains were grown on Sabouraud dextrose agar (1% hipolypepton [Nihon Pharmaceutical Co., Ltd., Tokyo, Japan], 4% dextrose and 1.5% agar [both from FUJIFILM Wako Pure Chemical Industries, Osaka, Japan]) and incubated at 27°C for 2 days.

For growth on liquid medium, RPMI-1640 (RPMI medium 1640 [Life Technologies Ltd., Paisley, UK] containing 165 mM 3-(*N*-morpholino) propanesulfonic acid [Dojindo Laboratories, Kumamoto, Japan], pH 7.0) and Sabouraud liquid medium (1% hipolypeptone, 4% dextrose) were used in this study. Suspensions of wild-type *T. asahii* (MPU129 strain) and *ku70* gene-deficient *T. asahii* mutants were prepared with RPMI-1640 or Sabouraud medium and adjusted to 0.01-0.02 on absorbance at 630 nm. The *T. asahii* suspensions were incubated at 27°C or 37°C and absorbance at 630 nm was measured using a microplate reader (iMark™ microplate reader; Bio-Rad Laboratories Inc., Hercules, CA, USA). After incubation for 2 days, the *T. asahii* cells were observed with a light microscope (CH30; Olympus, Tokyo, Japan).

Silkworm infection experiments. Silkworm infection experiments were performed according to a previous report¹⁹. Eggs of silkworms were purchased from Ehime-Sanshu Co., Ltd. (Ehime, Japan), disinfected, and hatched at 25–27°C. The silkworms were fed an artificial diet, Silkmate 2S, containing antibiotics purchased from Ehime-Sanshu Co., Ltd.. Fifth instar larvae were used in the infection

experiments. Silkworm fifth instar larvae were fed the artificial diet (Silkmate 2S; Ehime-Sanshu Co., Ltd.) overnight. *T. asahii* grown on Sabouraud agar plates was suspended in physiologic saline solution (0.9% w/v NaCl) and filtered through a 40- μ m cell strainer (Corning Inc., Corning, NY, USA). A 50- μ l suspension of *T. asahii* cells was injected into the silkworm hemolymph using a 1-ml tuberculin syringe (Terumo Medical Corporation, Tokyo, Japan). Silkworms injected with *T. asahii* cells were placed in an incubator and their survival was monitored.

LD₅₀ measurement. The dose of *T. asahii* required to kill half of the silkworms (LD₅₀) was determined according to the previous report¹⁹. *T. asahii* strains (1×10^2 to 2×10^7 cells) were injected into the silkworm hemolymph. Survival of the silkworms at 48 h was monitored. The LD₅₀ was determined from the combined data of 2-3 independent experiments by simple logistic regression model using Prism 9.1.2 (GraphPad Software, LLC, San Diego, CA, USA, <https://www.graphpad.com/scientific-software/prism/>).

Construction of gene-deficient *T. asahii* mutants. The plasmid for gene-deficient *T. asahii* strains was constructed according to a previous report³⁰. To generate the *ku70* gene-deficient strain, the 5'-UTR of the *ku70* gene, neomycin phosphotransferase gene (*nptII*) cassette, and 3'-UTR of the *ku70* gene were introduced into a pAg1 vector⁴⁶. To generate the *cnb1* gene-deficient strain, the 5'-UTR and 3'-UTR of the *cnb1* gene were introduced into a pAg1-NAT1 vector⁴⁶. Cloning was performed by the infusion method according to the general method (In-Fusion HD Cloning Kit, Takara, Shiga, Japan). The primers used for PCR amplification of each DNA region are shown in Table 1. The pAg1-5'UTR(*ku70*)-*nptII*-3'UTR(*ku70*) was introduced into the *T. asahii* MPU129 strain using the *A. tumefaciens*-mediated transformation method described previously¹⁹. The pAg1-5'UTR(*ku70*)-*nptII*-3'UTR(*ku70*) was introduced into the *A. tumefaciens* EHA105 strain by electroporation and transformants were grown on 2 \times YT agar (1.6% tryptone and 1% yeast extract [both from Becton, Dickinson, and Company, NJ, USA], 0.5% NaCl, and 1.5% agar) containing chloramphenicol (25 μ g/ml) and kanamycin (50 μ g/ml). The transformant was co-cultured with the *T. asahii* MPU129 strain at 27°C for 2 days. The candidates were isolated as colonies grown on Sabouraud dextrose agar containing G418 (50 μ g/ml) and cefotaxime (100 μ g/ml). Introduction of the mutation into the genome of the candidate strains was confirmed by PCR using the primers shown in Table 1.

To prepare competent cells for electroporation, *T. asahii* MPU129 strain was spread on a Sabouraud dextrose agar plate and cultured at 27°C for 3 days. *T. asahii* cells on the agar were suspended by physiologic saline solution (2 ml), and the suspension was transferred to a 1.5-ml tube. The fungal cells were collected by centrifugation at 8000 rpm for 3 min (TOMY-MX100, TOMY Digital Biology Co. Ltd, Tokyo, Japan) and suspended by adding 1 ml of ice-cold water and centrifuged at 8000 rpm for 3 minutes. This washing process was repeated 4 times. The washed cells were suspended by adding 1 ml of 1.2 M sorbitol solution and centrifuged at 8000 rpm for 3 min. The obtained fungal cells were suspended with 0.2 ml of 1.2 M sorbitol solution as competent cells. The PCR-amplified 5'-UTR (*cnb1*) - NAT1-3'-UTR (*cnb1*) fragment (180 ng/2 μ l) was added to the *T. asahii* competent cells (40 μ l) and placed on ice for 15 minutes. The suspension was added to a 0.2-cm gap cuvette (Bio-Rad Laboratories, Inc.)

and electroporated (Time constant protocol: 1800 V, 5 ms) using a Gene Pulser Xcell (Bio-Rad Laboratories, Inc.). The cells were suspended by adding 500 µl YPD containing 0.6 M sorbitol and incubated at 27°C for 3 h. After incubation, the cells were collected by centrifugation at 10,000 rpm for 5 min and suspended in 100 µl of physiologic saline solution and applied to Sabouraud dextrose agar containing nourseothricin (300 µg/ml). The cells were incubated at 27°C for 3 days and the growing colonies were isolated as *cnb1* gene-deficient strain candidates. Introduction of the mutation into the genome of the candidate strains was confirmed by PCR using the primers shown in Table 1.

Statistical analysis. The significance of differences between groups in silkworm infection experiments was calculated by the log-rank test based on the curves by the Kaplan-Meier method using Prism 9.1.2.

Declarations

Data availability. The datasets generated during the current study are available from the corresponding author on reasonable request.

Acknowledgements

We thank Tae Nagamachi, Asami Yoshikawa, Yu Sugiyama, Eri Sato, and Asuka Toshima (Meiji Pharmaceutical University) for their technical assistance rearing the silkworms. This study was supported by JSPS KAKENHI grant number JP20K07022 (Scientific Research (C) to Y.M.).

Author contributions

Study conception and design: Y.M. Acquisition of data: Y.M., T.N., A.Y., H.Y., Y.Y., T.Y.; Analysis and interpretation of data: Y.M. Drafting of manuscript: Y.M. Critical revision: Y.M., T.N., A.Y., H.Y., Y.Y., T.Y., T.S. All authors have read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

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Tables

Due to technical limitations, table 1-2 is only available as a download in the Supplemental Files section.

Figures

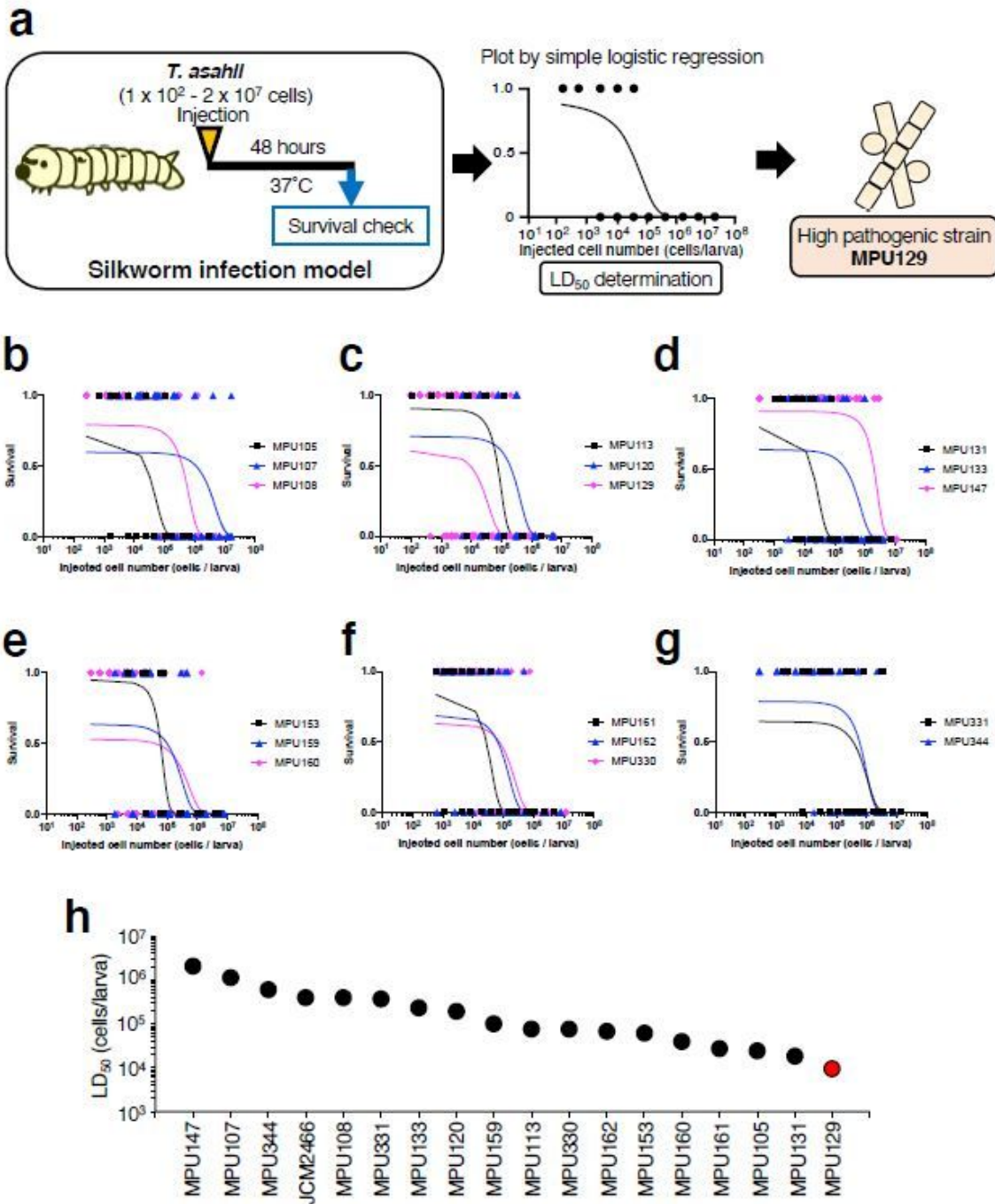


Figure 1

Figure 1

Comparison of pathogenicity of *T. asahii* strains against silkworms. (a) Experimental scheme for selecting high pathogenic strain MPU129 using the silkworm infection model. (b-g) The number of surviving silkworms was determined 48 h after administration of the fungal cells (1×10^2 to 2×10^7 cells) into the hemolymph of silkworms. Survived and dead silkworms are indicated as 1 and 0, respectively. The curves were drawn from the combined data of 2-6 independent experiments by a simple logistic

regression model. (h) LD50 values of *T. asahii* strains. Data for JCMM2466 are cited from Matsumoto et al.19.

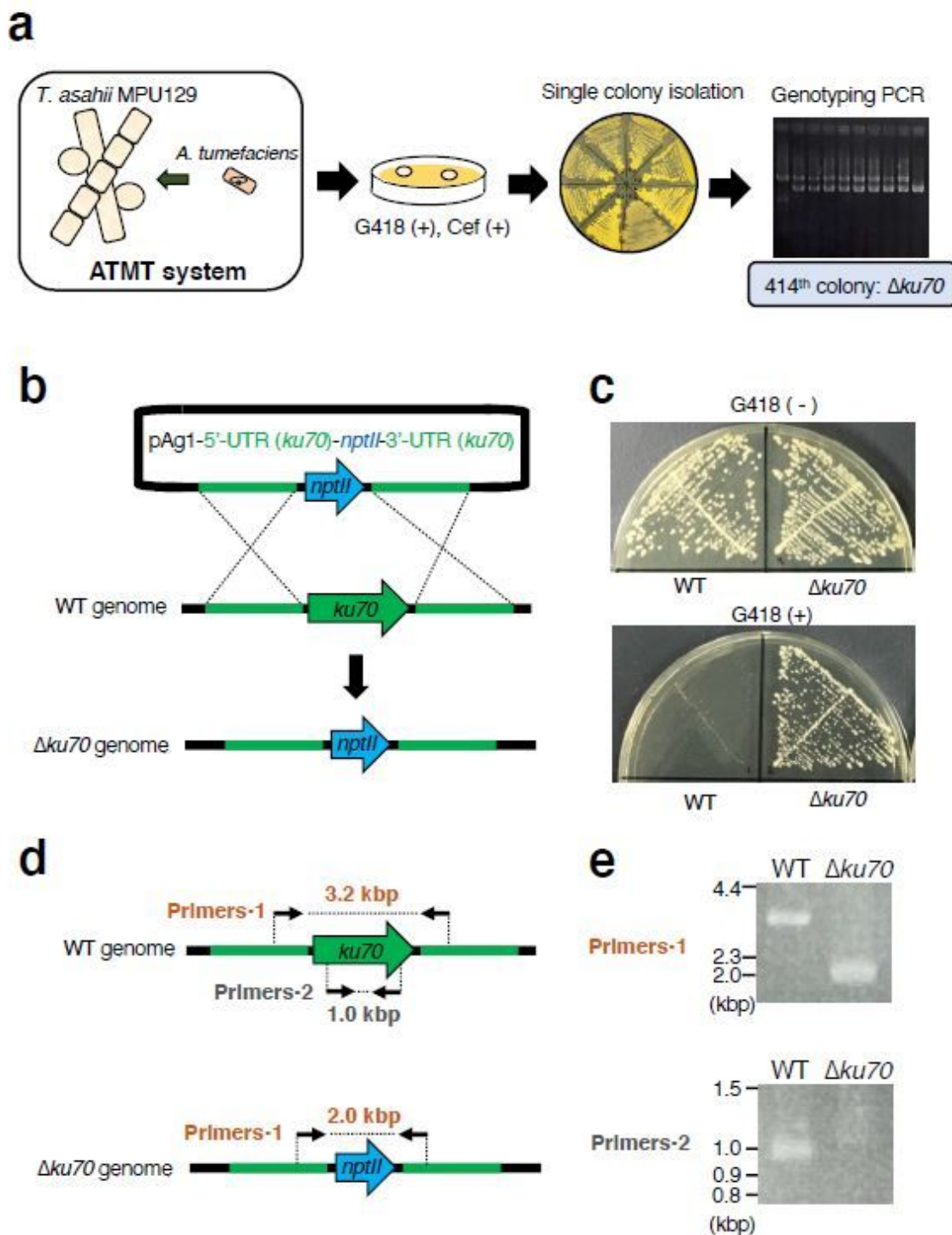


Figure 2

Construction of the *ku70* gene-deficient *T. asahii* MPU129 strain mutant by the ATMT system. (a) Experimental scheme for obtaining a *ku70* gene-deficient mutant candidate using the ATMT system. (b) Structure of the plasmid used to construct the *ku70* gene-deficient mutant and the predicted genome of

the *ku70* gene-deficient mutant. (c) The wild-type (MPU129) and *ku70* gene-deficient candidate were spread on Sabouraud agar medium with or without G418 (100 $\mu\text{g}/\text{ml}$) and incubated at 27°C for 2 days. (d) Location of the primers for confirming the genome structure of the *ku70* gene-deficient candidate by PCR. (e) Confirmation of the *ku70* gene-deficiency of the *ku70* gene-deficient candidate by PCR.

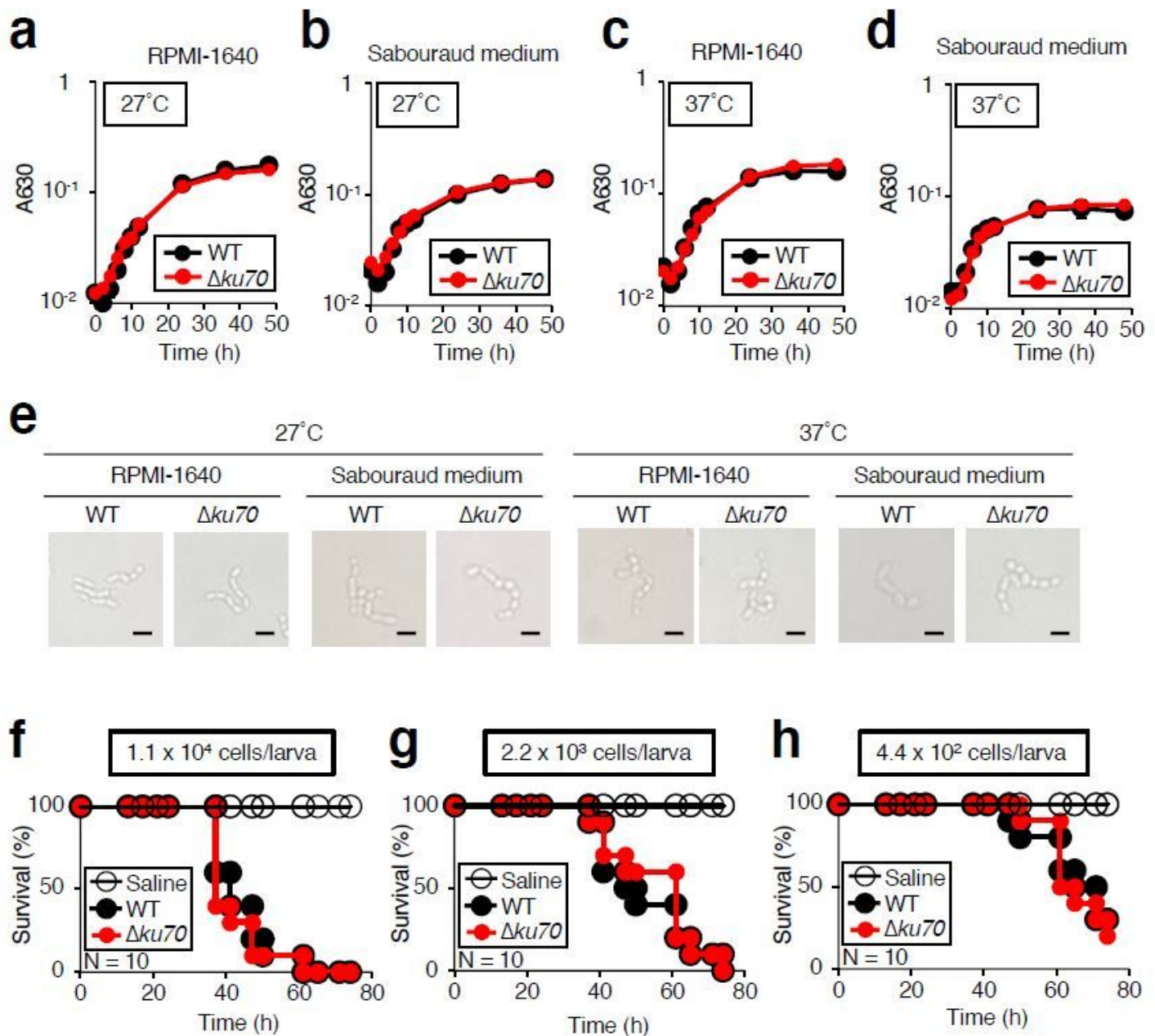


Figure 3

Effects of *ku70* gene-deficiency in the *T. asahii* MPU129 mutant on growth, morphology, and pathogenicity against silkworms. (a-d) The wild-type (MPU129) and *ku70* gene-deficient mutant were inoculated on RPMI-1640 medium and Sabouraud medium and incubated at 27°C or 37°C. Absorbance 630 nm of the culture was monitored. (e) The wild-type (MPU129) and *ku70* gene-deficient mutant were incubated at 2 days after inoculation and observed with a microscope. (f-h) Saline, *T. asahii* MPU129

(WT), or the *ku70* gene-deficient mutant ($\Delta ku70$) [1.1×10^4 cells (f), 2.2×10^3 cells (g), 4.4×10^2 cells (h)] were injected into the silkworm hemolymph and incubated at 37°C. The survival of the silkworms was monitored. $n = 10/\text{group}$.

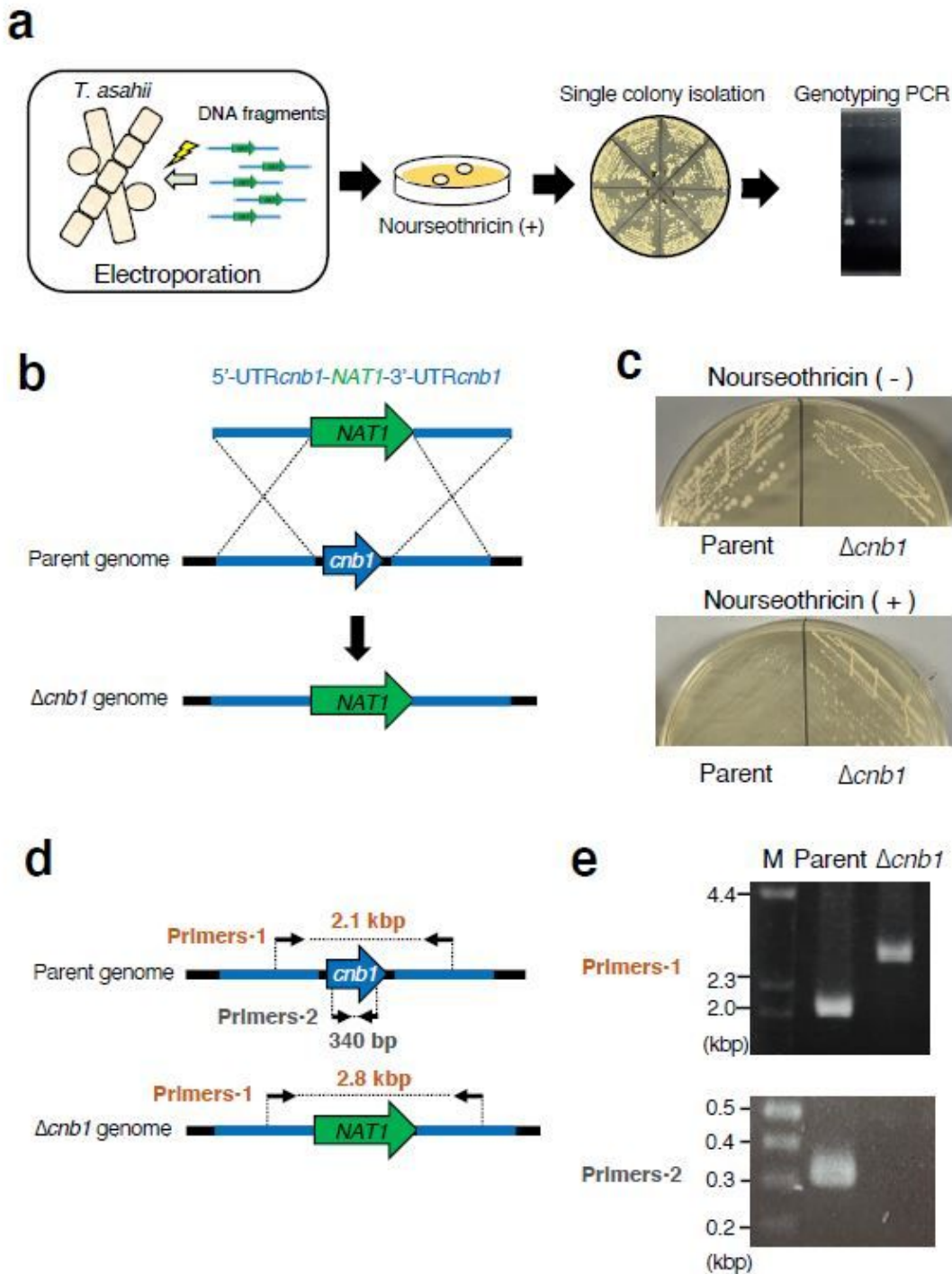


Figure 4

Construction of *cnb1* gene-deficient mutant in the *ku70* gene-deficient *T. asahii* MPU129 mutant by electroporation. (a) Experimental scheme for obtaining a *cnb1* gene-deficient mutant candidate by

electroporation. (b) Structure of the DNA fragment for construction of the *cnb1* gene-deficient mutant and the predicted genome of the *cnb1* gene-deficient mutant. (c) The parent strain (MPU129 $\Delta ku70$) and *cnb1* gene-deficient candidate were spread on Sabouraud agar medium with or without nourseothricin (100 $\mu\text{g/ml}$) and incubated at 27°C for 2 days. (d) Location of the primers for confirming the genome structure of the *cnb1* gene-deficient candidate by PCR. (e) Confirmation of the *cnb1* gene-deficiency of the *cnb1* gene-deficient candidate by PCR.

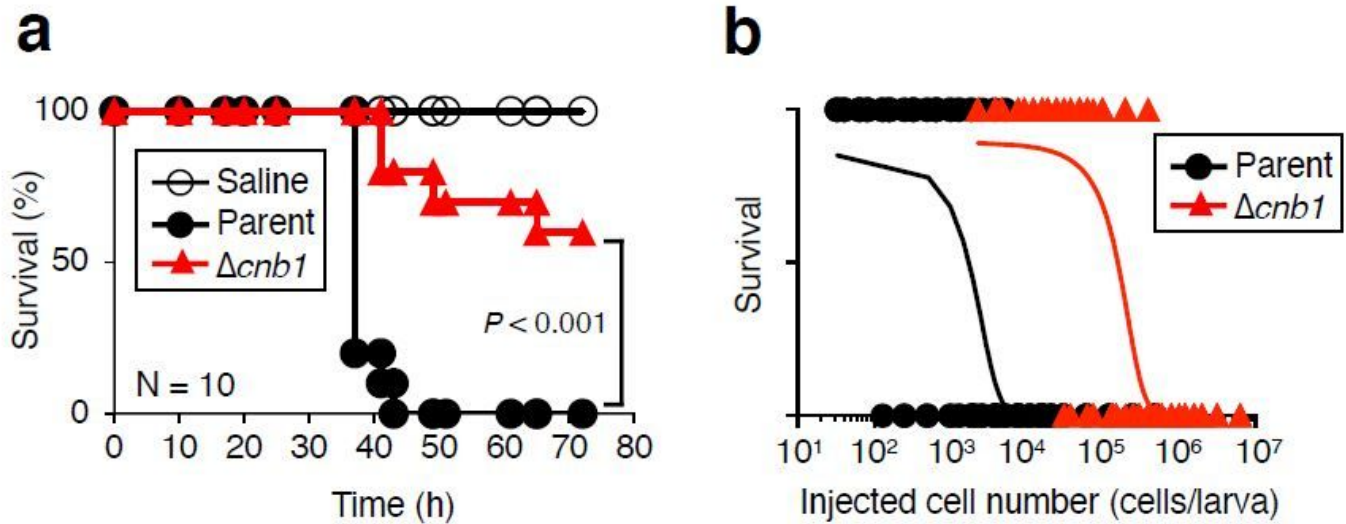


Figure 5

Attenuated pathogenicity of *T. asahii* against silkworms by *cnb1* gene-deficiency. (a) Saline, *T. asahii* MPU129 $\Delta ku70$ (parent strain) (7.1×10^4 cells), or the *cnb1* gene-deficient mutant ($\Delta cnb1$) (1.3×10^5 cells) were injected into the silkworm hemolymph and incubated at 37°C. The survival of the silkworms was monitored. The significance of differences between groups in silkworm infection experiments was calculated by the log-rank test based on the curves by the Kaplan-Meier method. $n = 10/\text{group}$. (b) The number of surviving silkworms was determined at 48 h after administration of the fungal cells (3.3×10^2 to 6.2×10^6 cells) into the hemolymph of silkworms. Survived and dead silkworms were indicated as 1 and 0, respectively. The curves were drawn from combined data of 3 independent experiments by simple logistic regression model.

Supplementary Files

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