An arthropods' role in the life cycle of *Francisella tularensis*

(節足動物が野兎病菌の生活環に果たす役割)

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CONTENTS

PREFACE

Arthropods, the most successful animal group in the world include a large number of species (Ødegaard, 2000) and are distributed widely regardless of environmental condition (Chown et al., 2007). Arthropods harbor numerous pathogens as vectors. Vector-borne diseases continued to be important topics in the history, e.g. malaria, pestis and dengue fever (Jamrozik et al., 2015; Achtman, 2016; Patterson et al., 2016). In addition, vector-borne diseases are highly sensitive to environmental changes, because land-use changes and adaptation to climate change are likely to affect the geographical distribution of vectors and their host (Medone et al., 2015). The risk of vector-borne disease emergence has increased markedly according to environmental changes such as global warming and/or modernization (Medlock and Leach, 2015). Therefore, control of vectors and vector-borne pathogens is top priority in public health.

Francisella tularensis is one of the causative agents of zoonotic vector-borne diseases, tularemia which is highly pathogenic in humans and animals (WHO, 2007; Decors et al., 2011). *F. tularensis* establishes its life cycle between wild mammals and blood-sucking

arthropods in environment (Carvalhoa et al., 2014). Transmission of *F. tularensis* occurs through arthropod vectors, inhalation of infectious drops or aerosolized pathogens from infected animals, or through ingestion of contaminated food or water (Ellis et al., 2002). It is thought that interactions between *F. tularensis* and arthropods play important roles in maintenance of life cycle in environment (Petersen et al., 2009). However, the life cycle of *F. tularensis* in nature is not fully understood. Especially, primary vector species are still unclear in Japan.

For preventing infection with vector-borne pathogens, it is necessary to clarify interactions between pathogens and host arthropods in the environment. I examined the life cycle of *F. tularensis* from following two different aspects:

1. epidemiological surveillance using a large number of ticks in Japan and an experimental infection using the tick species maintained in our laboratory (chapter 1)
2. establishment of a novel *F. tularensis* infection model using silkworms and investigation of the innate immune response to *F. tularensis* infection (chapter 2)

The main goal of this study is to reveal the reservoir of *F. tularensis* in the environment, and subsequently to elucidate interaction between *F. tularensis* and host arthropods through newly developed infection model.

CHAPTER 1

Detection of *Francisella tularensis* and analysis of bacterial growth in ticks in Japan

INTRODUCTION

Francisella tularensis is a gram-negative, facultative, intracellular bacterium that causes tularemia in animals and humans. It is transmitted to humans mainly by handling infected animals, arthropod bites, ingestion of contaminated water or food, and inhalation of infectious aerosols (Ellis et al., 2002). *F. tularensis* can infect a wide range of vertebrates and invertebrates. Its maintenance in nature is primarily associated with rodents and lagomorphs, although amoebae are a potential reservoir (Oyston et al., 2004; Santic et al., 2006). Vector-borne transmission of tularemia to mammalian hosts has an important role in the pathogenesis of the disease (Petersen et al., 2009). Several environmental arthropod vectors have been reported to harbor *F. tularensis*, including fleas, lice, midges, bedbugs, mosquitoes, and ticks (Guryčová, 1998; Petersen et al., 2009). Ticks are considered to be significant vectors and potential reservoirs capable of maintaining the organism for long periods of time in the environment, as well as transmitting *F. tularensis* to animals and humans via tick bites (Výrosteková, 1994; Petersen et al., 2009).

Many reports have described tularemia surveillance in North America and European countries (Goethert and Telford, 2009; Gehringer et al., 2013), but ecological studies of *F. tularensis* have been less thorough in Asian countries. In Japan, Japanese black bears are considered a candidate sentinel species for tularemia (Hotta et al., 2012). In addition, seropositive Japanese raccoon dogs were detected by serosurveillance of tularemia among various species of wild animals in Japan (Sharma et al., 2014). However, *F. tularensis* had not been detected in ticks in Japan since 1991 (Fujita et al., 2008). In the present study, to investigate the life cycle of *F. tularensis* in the environment, I tried to detect *F. tularensis* DNA using a large number of tick specimens collected in Japan. I also constructed an experimental infection model using ticks and I assessed bacterial growth in ticks to identify important factors that affect its growth in this organism.

MATERIALS AND METHODS

Bacteria

F. tularensis subsp. *holarctica* LVS was obtained from the Pathogenic Microorganism Genetic Resource Stock Center, Gifu University. *F. tularensis* LVS was cultured aerobically at 37°C in brain heart infusion broth (BD, Franklin Lakes, NJ, USA) supplemented with cysteine (BHIc; Mc Gann et al., 2010) on BHIc agar.

Tick collection and DNA extraction

Ticks were collected by flagging over vegetation or mammals, including *Procyon lotor*, *Nyctereutes procyonoides*, *Cervus nippon centralis*, *C. nippon yesoensis*, *Lepus brachyurus*, *Meles anakuma*, *Mustela itatsi*, *Sus scrofa*, *Vulpes vulpes japonica*, *Muntiacus reevesi*, *Ursus thibetanus*, *Paguma larvata*, and *Martes melampus melampus* in Japan between 1998 and 2015 (Table 1). After morphological identification, DNA was extracted from whole or half bodies, as described previously (Takano et al., 2014). Briefly, tick tissues were lysed in 50 µl of 25-mM sodium hydroxide (NaOH) for 10 min at 95°C. After adding 4 µL of Tris-HCl (1 M, pH 7.5) for neutralization, the lysate

was centrifuged at 4°C, and the resulting supernatant was used as template DNA for PCR. The DNA template was stored at −30°C until use.

Detection of *Francisella* DNA in ticks

The genera *Francisella* DNA was detected in ticks using TaqMan real-time PCR by targeting the *23kDa* gene (Versage et al., 2003). The sequences of the primers and probes are shown in Table 2. The 23kDaP fluorogenic probe was synthesized with a 6-carboxy-fluorescein reporter molecule attached to the 5' end and a Black Hole Quencher (Applied Biosystems, CA, USA) attached to the 3' end.

Real-time PCR was performed with a StepOne Real-Time PCR system (Applied Biosystems) using a Premix Ex Taq PCR kit (Probe qPCR) (TaKaRa Bio Inc., Shiga, Japan). Real-time PCR was performed in a 12.5 μL final volume, which contained 6.25 μL of Premix Ex Taq (2×), 1.0 μL of DNA template, 1.0 μL of 3.75 pmol/μL F primers, 1.0 μl of 11.25 pmol/μL R primers, 0.3 μL of 250 nmol/L TaqMan probe, 0.25 μL of ROX reference dye (50×), and 2.7 μL of distilled water. All of the reactions were repeated in triplicate with the following cycling parameters: 95°C for 20 s followed by

45 cycles at 95°C for 1 s and 60°C for 20 s.

I performed nested PCR for real-time PCR positive samples using a KOD-Plus-Neo high fidelity DNA polymerase kit (Toyobo Co. Ltd, Osaka, Japan) to detect a fragment of the *fopA* gene from the genus *Francisella* (Fulop et al., 1996). The primers used are shown in Table 2. Each PCR reaction comprised 2.0 μL of 10× KOD-Plus-Neo buffer, 2.0 μL of dNTPs (2 mmol/L), 1.2 μL of 25 mM$MgSO_4$, 1.0 μL of each primer (10 μg/μL), 1.0 μl of DNA (100 ng/μL), 0.4 μL (1 U/μL) of KOD-Plus-Neo DNA polymerase, and 11.4 μL of distilled water. The PCR reaction conditions were: 95°C for 3 s and 35 cycles (first) or 25 cycles (nested) at 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min.

The nested PCR amplicons were purified using NucleoSpin Gel and a PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). DNA was subjected to direct nucleotide sequencing with a Big Dye Terminator v3.1 Cycle Sequencing Kit and an ABI3031 Genetic Analyzer (Thermo Fisher Scientific Inc., MA, USA).

Phylogenetic analysis

Sequence data were aligned using MEGA6.06 software (http://www.megasoftware.net; Tamura et al., 2013) and compared with representative *fopA* gene sequences from GenBank. After obtaining the alignment, a phylogenetic tree was constructed by the algorithms implemented in the CLUSTALW (version 2.1; DDBJ, Japan) using the neighbor-joining method (Saitou and Nei, 1987) according to the bootstrap probability values based on 1,000 replicates (Felsenstein et al., 1985).

Isolation of the *hlyD* mutant

The *F. tularensis hlyD* mutant was generated by group II intron insertion using the TargeTron gene knockout system (Sigma-Aldrich, MO, USA) modified for *Francisella* species (Rodriguez et al., 2008), as described previously (Uda et al., 2014).

Microinjection of *Francisella tularensis* into ticks

I. persulcatus was maintained by feeding on female ICR mice (Kyudo, Kumamoto,

Japan) at Yamaguchi University. Mice were kept in a temperature-controlled room with a constant supply of food and water, and all the protocols for animal experiments have been approved in the Animal Research Committee of Yamaguchi University (Permit Number: 211 and 256). Animal studies were performed in compliance with the Yamaguchi University Animal Care and Use guidelines. The mice were sacrificed by cardiac puncture under isoflurane anesthesia and overdose of isoflurane, and all efforts were made to minimize suffering by using isoflurane anesthesia. All of the ticks subjected to microinjection were used 2 weeks after molting to nymphs.

F. tularensis LVS was grown in BHIc broth at 37°C for 18 h and washed twice with Hanks' balanced salt solution (HBSS; Sigma-Aldrich). After determining a suitable dilution, bacterial cells suspended in HBSS were injected (0.2-0.5 μL/tick) into *I. persulcatus* via the anus (Kariu et al., 2011). Control ticks were injected with an equal volume of 75°C/30-min heat-killed *F. tularensis* LVS. Groups of four ticks were used per assay. Equal volumes (0.5 μL/tick) of heat-killed *F. tularensis* LVS or *F. tularensis* LVS suspension were injected into three groups of ticks. *F. tularensis* LVS DNA was

extracted from ticks as described above and the amount of *F. tularensis* LVS DNA in ticks was quantified by real-time PCR using a standard containing a known bacterial concentration based on the colony count.

Statistical tests

Significant differences were determined using the Student's *t*-test. Significant differences were accepted at $P < 0.05$ and they are indicated by asterisks in the figures. Data were expressed as means based on four samples from three identical experiments and the error was represented as the standard error of the mean (SEM, n = 12).

RESULTS

Detection of *Francisella* DNA in ticks

The prevalence of *Francisella* in tick species is shown in Table 3. Among 4527 tick specimens, 2093 were *Ixodes ovatus*, 2107 were *I. persulcatus*, 73 were *I. monospinosus*, 48 were *I. pavlovskyi*, six were *I. tanuki*, 138 were *Haemaphysalis flava*, 21 were *H. megaspinosa*, 20 were *H. longicornis*, 14 were *H. kitaokai*, two were *H. hystricis*, one was *H. formosensis*, three were *Amblyomma testudinarium*, and one was *Dermacentor taiwanensis*. A DNA fragment of a *Francisella* gene was detected by real-time polymerase chain reaction (PCR) in 45 *I. ovatus* (2.15%), 14 *I. persulcatus* (0.66%), six *I. monospinosus* (8.22%), and one *H. flava* sample(s) (0.72%). The DNA samples with positive results by real-time PCR were then tested by nested PCR. Finally, *Francisella* DNA was detected by nested PCR in 42 and five samples of *I. ovatus* and *I. persulcatus*, respectively, which were positive according to real-time PCR (Table 3, Fig.2). The *I. monospinosus* and *H. flava* DNA with positive results by real-time PCR were negative according to nested PCR (Table 3).

Phylogenetic analysis

The nested PCR amplicon from each *I. ovatus* and *I. persulcatus* sample was purified and analyzed randomly to obtain nucleotide sequences. The two sequences (331 bp) obtained were exactly the same. The consensus sequence was compared with representative sequences and phylogenetic analysis was performed. Neighbor-joining phylogenetic trees showed that the *Francisella* strains detected in *I. ovatus* and *I. persulcatus* clustered with *F. tularensis* subsp. *holarctica* LVS, FSC022 and *F. tularensis* subsp. *novicida* U112 (Fig. 1). The phylogenetic analysis demonstrated that the *F. tularensis* strains detected in ticks in this study were closely related to strains distributed throughout Eurasia (Wang et al., 2011).

Microinjection of *Francisella tularensis* into *I. persulcatus*

The presence of *F. tularensis* in ticks in Japan was demonstrated by this study, so I next investigated bacterial growth in ticks using *I. persulcatus* nymphs, which I maintained in our laboratory. The copy number of *F. tularensis* was measured using DNA isolated from ticks, which I microinjected with live or heat-killed *F. tularensis*. *F. tularensis*

DNA decreased significantly in ticks injected with heat-killed bacteria, but *F. tularensis* DNA was detected until 21 days after injection when live bacteria were injected (Fig. 3). This is the first report of the experimental maintenance of *F. tularensis* in *Ixodes* ticks for at least 3 weeks.

Bacterial growth of the *hlyD* mutant in *I. persulcatus*

To investigate the bacterial factors associated with bacterial growth in ticks, a *hlyD* mutant was constructed in *F. tularensis* LVS and microinjected into ticks. The *F. tularensis hlyD* gene shares homology with the *Escherichia coli hlyD* gene and it is highly expressed at 26°C in *F. tularensis* LVS (Horzempa et al., 2008). In *E. coli*, HlyD is known to be a hemolysin translocator protein in the type I secretion system (Koronakis and Hughes, 1993; Low et al., 2010). The bacterial gene quantity is shown in Fig. 4A and the relative gene quantity relative to day 0 is shown in Fig. 4B. Both the parental strain and *hlyD* mutant were kept in ticks, but the relative gene quantity decreased in the *hlyD* mutant at 14 days after injection. There was no significant difference in the relative gene quantity at 21 days after injection in the two strains.

DISCUSSION

It is thought that interactions between *F. tularensis* and arthropods play important roles in maintenance of life cycle in environment (Petersen et al., 2009). Although *F. tularensis* DNA had not been detected in ticks in Japan since 1991, surveys of *F. tularensis* among wild mammals in Japan have been reported (Hotta et al., 2012; Sharma et al., 2014; Hotta et al., 2015). These reports suggest that *F. tularensis* is still present in Japan. In the present study, the ticks with positive results by real-time and nested PCR were considered to be positive for *F. tularensis*. According to my large-scale surveillance study of ticks, *F. tularensis* DNA were detected from 2.00% (42/2093) of *I. ovatus* and 0.23% (5/2107) of *I. persulcatus* (Table 3). In addition, all of the positive ticks were collected by flagging over vegetation but not from mammals, indicating that *F. tularensis* was maintained in ticks after blood-sucking followed by molting. In previous study, almost all of clinical cases of tularemia in Japan were limited in northern Japan (Ohara et al., 1999). I detected *F. tularensis* DNA from1.27% (11/866) of the ticks collected in non-endemic areas (Fig. 2). These results reveal that the ticks harboring *F. tularensis* are widely spread in Japan including southern part, and

I. ovatus and *I. persulcatus* play an important role in the *Francisella* life cycle.

In the phylogenetic analysis, *F. tularensis* strains detected in this study were closely related to strains distributed throughout Eurasia (Fig. 1; Wang et al., 2010). In several samples, *Francisella* DNA was detected by real-time PCR, but not by nested PCR (Table 2). Recently, *Francisella*-like endosymbionts (FLEs) have been reported in ticks (Sun et al., 2000; Dergousoff et al., 2012; Michelet et al., 2013). Since the primer pairs used in real-time PCR targeted a wide range of members of the genera *Francisella*, these results suggests that real-time PCR might detect symbionts similar to FLEs.

To assess the property of *I. persulcatus* for harboring *F. tularensis*, I next established a novel infection model using *I. persulcatus* nymphs with micro injection method. The copy number of live *F. tularensis* DNA in *I. persulcatus* were retained for 21 days, whereas heat-killed *F. tularensis* DNA were significantly decreased (Fig. 3), suggesting that *F. tularensis* maintained their numbers by some biological activity in *I. persulcatus*. These results are consistent with previous report that *F. tularensis* LVS colonized

capillary-fed *Dermacentor variabilis* (Mani et al., 2012). These findings strongly substantiated that *I. persulcatus* are important carrier of *F. tularensis* in Japan.

Although many bacterial factors associated with bacterial growth in mammals were reported (Carvalhoa et al., 2014), there are few information about bacterial factors associated with bacterial growth in vector arthropod. I hypothesized that genes which is highly expressed at 26°C in *F. tularensis* LVS (Horzempa et al., 2008) were involved in bacterial maintenance in host arthropods. In this study, I constructed a *hlyD* mutant in *F. tularensis* LVS and microinjected it into *I. persulcatus*. Both the parental strain and *hlyD* mutant were kept in ticks (Fig. 4A), but the relative gene quantity was decreased in the *hlyD* mutant (Fig. 4B), suggesting that HlyD contribute to adaptation or survival in ticks. The *F. tularensis hlyD* gene shares homology with the *Escherichia coli hlyD*, a configuration factor of Hly operon (Schmidt et al., 1995). Hly operon consists of the part of type I secretion system (*hlyB* and *hlyD*) and hemolysin (*hlyA*) which is known to be a major virulence factor in *E. coli* (Schmidt et al., 1995; Thanabalu et al., 1998). *F. novicida* and *F. novicida*-like strains have hemolytic activities and they encode proteins

related to RTX toxin as HlyA homologs (Lai et al., 2003; Siddaramappa et al., 2011). The function and structure of hemolysin in *F. tularensis* is still undefined, but hemolysin-like proteins and/or hemolysin-associated factors in this species may contribute to the bacterial infection with ticks.

Ticks possess an innate immune system to combat infection (Zivkovic et al., 2010; Aung et al., 2012), but *F. tularensis* speculates to escape from the innate immune system and survive as an endosymbiont in these organisms. Previously, it was reported that *F. novicida* can replicate in a mosquito cell line (Read et al., 2008). Obvious bacterial growth in ticks was not observed in the present study, but *F. tularensis* may have the ability to escape the innate immune system and establish symbiosis with ticks (see chapter 2). Disrupting the *Francisella* lifecycle is thought to be alternative method to control *Francisella* infection in human. In this study, I identified HlyD as an important factor for symbiosis. Therefore, HlyD may be a possible target for *Francisella* infection. Thus, my experimental tick-infection model will be useful for analyzing the symbiosis between *F. tularensis* and ticks

TABLES AND FIGURES

Table 1. Tick collection sites and sources in this study.

Sampling method	Total	Endemic area	Non-endemic area
Flagging	4373	3582	791
Mammals from			
Procyon lotor	40	8	32
Nyctereutes procyonoides	34	15	19
Cervus nippon yesoensis /centralis	29	23	6
Lepus brachyurus	15	14	1
Meles anakuma	4	2	2
Mustela itatsi	3	3	0
Sus scrofa	6	1	5
Vulpes vulpes japonica	8	2	6
Muntiacus reevesi	7	7	0
Ursus thibetanus	4	0	4
Paguma larvata	1	1	0
Martes melampus melampus	3	3	0
Total	4527	3661	866

Table 2. The primer and probe list used in this study.

Gene target	Primer or probe	Sequence	Product size
Real-time PCR			
23kDa	23kDaF	5'-TGAGATGATAACAAGACAACAGGTAACA-3'	84 bp
	23kDaR	5'-GGATGAGATCCTATACATGCAGTAGG-3'	
	23kDaP	5'-TCAGTTCTCACATGAATGGTCTCGCCA-3'	
Nested PCR			
fopA	First PCR		
	FNA8L	5'-CGAGGAGTCTCAATGTACTAAGGTTTGCCC-3'	900 bp
	FNB2L	5'-CACCATTATCCTGGATATTACCAGTGTCAT-3'	
	Second PCR		
	FNA7L	5'-CTTGAGTCTTATATGTTTCGGCATGTGAATAG-3'	409 bp
	FNB1L	5'-CCAACTAATTGGTTGTACTGTACAGCGAAG-3'	

Table 3. Prevalence of *Francisella* in tick species.

Species		Positive (%)		
		Real-time PCR	Nested PCR	Total
Ixodes	*I. ovatus*	45/2093 (2.15)	42/45 (93.3)	42/2093 (2.00)
	I. persulcatus	14/2107 (0.66)	5/14 (35.7)	5/2107 (0.23)
	I. monospinosus	6/73 (8.22)	0/6	0/73
	I. pavlovskyi	0/48		0/48
	I. tanuki	0/6		0/6
Haemaphysalis	*H. flava*	1/138 (0.72)	0/1	0/138
	H. megaspinosa	0/21		0/21
	H. longicornis	0/20		0/20
	H. kitaokai	0/14		0/14
	H. hystricis	0/2		0/2
	H. formosensis	0/1		0/1
Amblyomma testudinarium		0/3		0/3
Dermacentor taiwanensis		0/1		0/1
Total		66/4527 (1.46)	47/66 (71.2)	47/4527 (1.04)

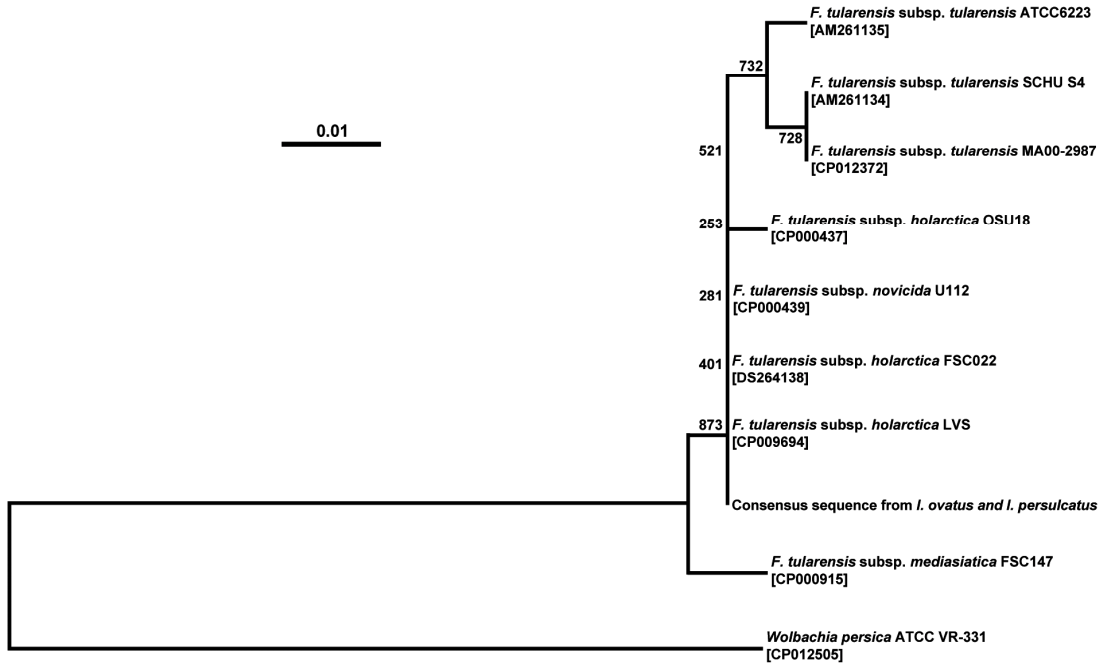

Figure 1. Phylogenetic tree of *fopA* gene in representative *Francisella* strains.

Gene accession numbers are shown in brackets. The numbers on the branches refer to the bootstrap probability values based on 1000 replicates. The scale bar indicates the number of nucleotide substitutions per site.

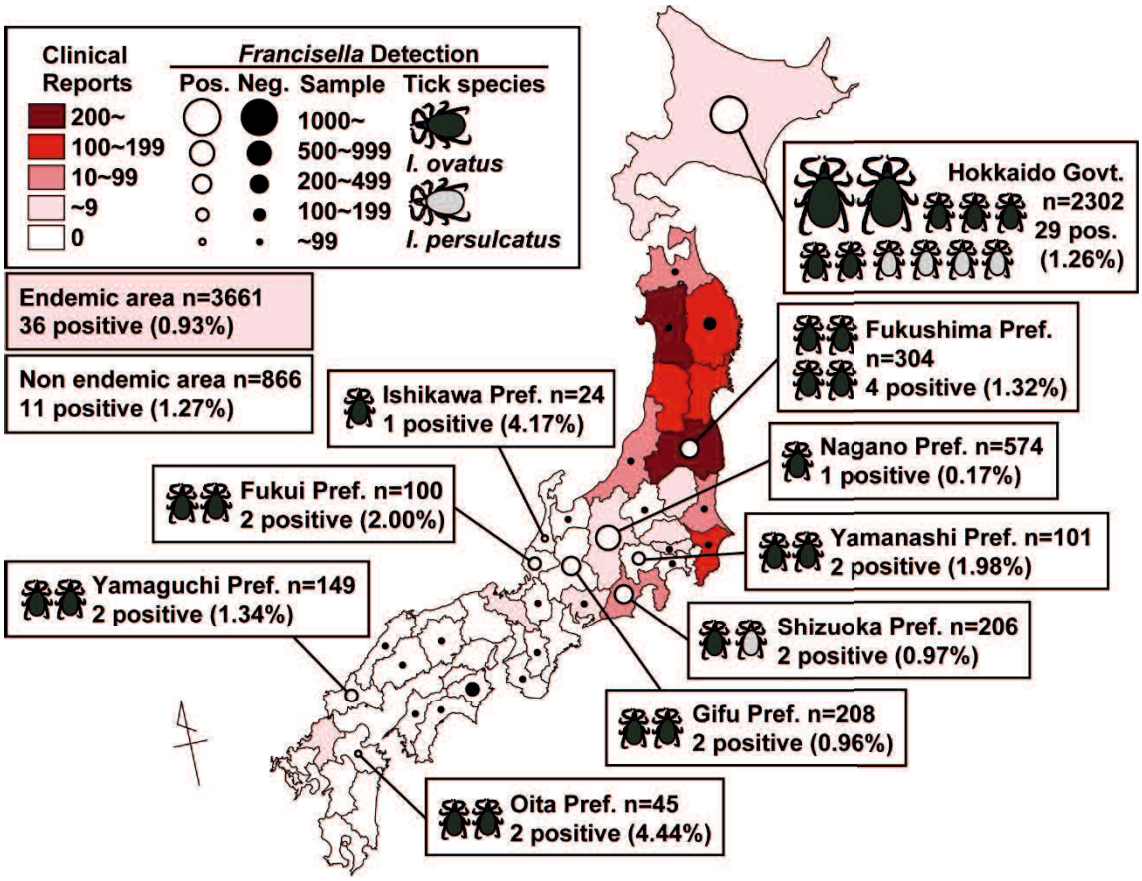

Figure 2. Distribution of ticks containing *Francisella* DNA.

Tularemia case numbers in each area (Ohara et al., 1996) are shown in the color chart. The total of endemic area was calculated using the data from the area reported one or more clinical case in humans. Sample numbers (n) are indicated by circle size. Open and filled circles denote *Francisella* DNA positive area or negative area, respectively. The species and numbers of *Francisella* DNA positive ticks were represented by the color and size of tick drawings. Black drawings indicate *I. ovatus* and white drawings is *I. persulcatus*. Large drawings indicate 10 ticks, and small one is 1 tick.

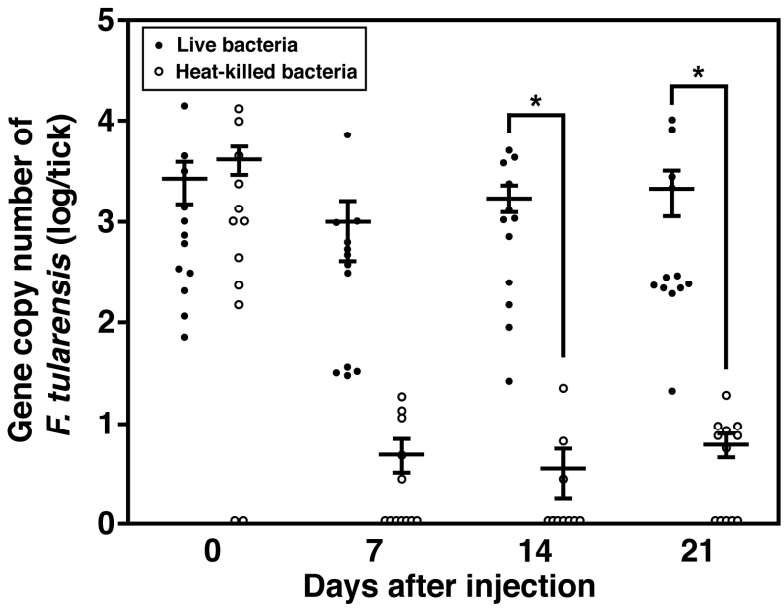

Figure 3. Bacterial quantity based on the *23kDa* gene copy number in ticks.

Open or filled circles denote live or heat-killed *Francisella tularensis* LVS-injected nymphs, respectively. The calculated mean for each time point is represented by the horizontal line. The error bars represent the standard error of the mean (n = 12). Significant differences were accepted at $P < 0.05$ and they are indicated by asterisks.

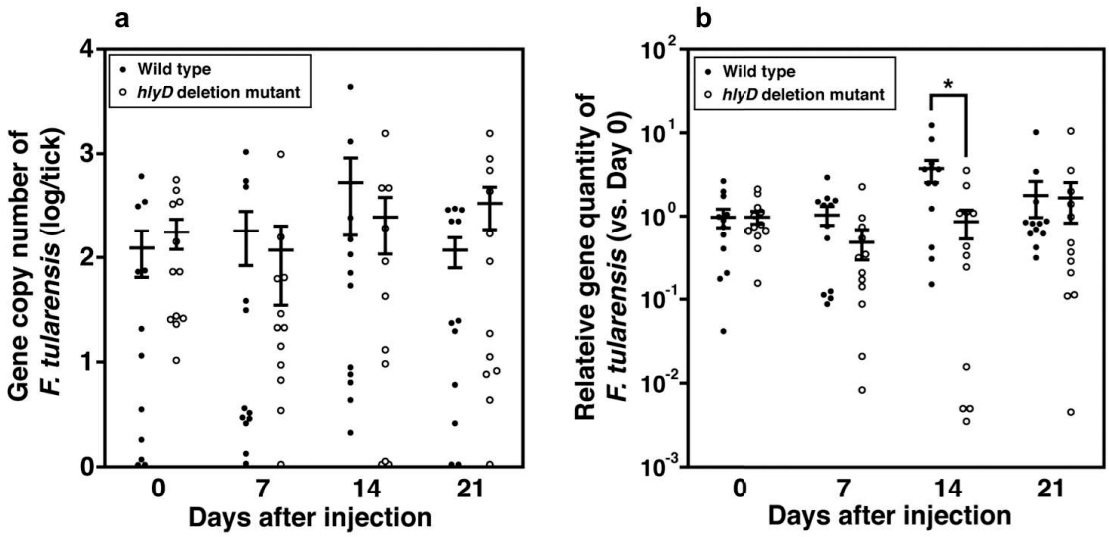

Figure 4. Bacterial quantity based on the *23kDa* gene copy number in ticks.

(**a**) Gene copy number in ticks and (**b**) the ratio of each time point relative to day 0. Open or filled circles denote live or heat-killed *Francisella tularensis* LVS-injected nymphs, respectively. The calculated mean for each time point is represented by the horizontal line. The error bars represent the standard error of the mean (n = 12). Significant differences were accepted at $P < 0.05$ and they are indicated by asterisks.

CHAPTER 2

Symbiosis with *Francisella tularensis* provides resistance to pathogens in the silkworm

INTRODUCTION

Francisella tularensis is a facultative intracellular bacterium and the etiological agent of tularemia, which is highly pathogenic in humans and animals (WHO, 2007; Decors et al., 2011). Tularemia can be induced by various transmission routes, such as insect bites, inhalation of contaminated aerosols, and ingestion of contaminated food or water (Ellis et al., 2002). *F. tularensis* has a wide range of animal hosts, such as rabbits, hares, voles, and other rodents, but its life cycle in nature is not fully understood. In addition to mammals, it has also been isolated from environmental water, mud, ticks, and mosquito larvae collected in endemic areas (Petersen et al., 2009; Carvalhoa et al., 2014). Therefore, it is thought that may the vector-borne transmission of *F. tularensis* to mammalian hosts is an important route in development of tularemia. Indeed, interactions between *F. tularensis* and arthropods play major roles in the ecology of this bacterium and its maintenance in the environment (Petersen et al., 2009). Deer flies, horse flies, ticks, and mosquitoes are common arthropod vectors for its transmission between mammals (Carvalhoa et al., 2014), but symbiosis between the bacterium and arthropods is still unclear.

Eukaryotes have evolved and diversified in the context of persistent colonization by non-pathogenic microorganisms (Douglas, 2014). The benefit of symbiosis can be attributed to two types of interaction. The first interaction is symbiosis as a source of novel capabilities, which is based on metabolic or other traits possessed by the microbial partner but not the eukaryotic host (Douglas, 2014). By exploiting these capabilities, eukaryotes have repeatedly derived enhanced nutrition (Wilson et al., 2010; Russell et al., 2013), defenses against natural enemies (Piel, 2002; Degnan and Moran, 2008), or other selectively important characteristics (Shin et al., 2011). The second interaction is the symbiotic basis of health, which comprises improved vigor and fitness gained by eukaryote hosts via the microbial modulation of multiple traits, including growth rate (Broderick and Lemaitre, 2012), immune function (Login et al., 2011; Eleftherianos et al., 2013), nutrient allocation (Newell and Douglas, 2014), and behavior (Sharon et al., 2010).

Symbiosis between *F. tularensis* and insects is completely uncharacterized. Thus,

here I established a novel symbiosis model for the bacterium in the silkworm *Bombyx mori*. *B. mori* larvae have been employed as infection models for a large variety of agents, including bacteria, viruses, and fungi (Jiang et al., 2016; Kaito, 2016; Uchida et al., 2016). Silkworms can provide a useful model for analyzing innate immunity because insects and mammals share common innate immune mechanisms. Therefore, silkworms provide a useful model for studying host–pathogen interactions in the presence of innate immunity. Compared with mammals, these non-mammalian models have logistical, budgetary, and ethical advantages. Thus, large numbers of larvae can be simply obtained at a low cost to allow large-scale screening, which would not be possible in mammals. My results demonstrate that silkworms acquire host resistance to pathogenic bacteria via their symbiosis with *F. tularensis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Francisella tularensis subsp. *holarctica* LVS, *Escherichia coli* JM109, *E. coli* JM109 pAcGFP (Clontech, Mountain View, CA, USA), and *Staphylococcus aureus* ATCC923 were used in this study. Bacterial strains were maintained as frozen glycerol stocks. *F. tularensis* subsp. *holarctica* LVS was obtained from the Pathogenic Microorganism Genetic Resource Stock Center, Gifu University and was cultured aerobically at 37°C in brain heart infusion broth (BD, Franklin Lakes, NJ) supplemented with cysteine (BHIc; Mc Gann et al., 2010) or Brucella broth (BD) containing 1.5% agar (Wako, Osaka, Japan). *E. coli* JM109, and *S. aureus* ATCC923 were cultured in Luria–Bertani (LB) broth (Nacalai Tesque, Kyoto, Japan) or LB broth containing 1.5% agar. Ampicillin (100 μg/mL) and chloramphenicol (10 μg/mL) were used as necessary.

Establishment of a GFP-expressing *F. tularensis* strain

A GFP-expressing plasmid, pNVU-GFP, was constructed from a pNVU1-expressing plasmid (Uda et al., 2014). The tetracycline resistance gene was removed from pNVU1

by treating the plasmid with *Sma*I. The GFP gene containing an SD sequence was amplified from pGreenTIR (Miller and Lindow, 1997) using the primer pairs pNVU-GFP-F (5′-GAAATTATTGATCCCTGATTAACTTTATAAGGAGGAA-3′) and–R (5′-CTTGGTCTGACACCCCTATTTGTATAGTTCATCCATG-3′), and inserted into *Sma*I-digested pNVU1. pNVU-GFP was transformed and replicated in *E. coli* DH5α and purified using a Plasmid Midi Kit (Qiagen, Hilden, Germany). LVS was transformed with pNVU-GFP by electroporation. The transformed LVS was cultured in BHIc for 3 h and then selected on BHIc agar plates containing 5 μg/mL chloramphenicol.

Silkworms

Fourth instar *B. mori* larvae (Hu/Yo × Tukuba/Ne) were obtained from Ehime-Sanshu (Ehime, Japan). The larvae were raised by feeding them with Silkmate 2M (Nosan Corporation, Kanagawa, Japan) at room temperature (25°C).

Infection using silkworm larva

Day 2 fifth instar larva was inoculated in the hemocoel with 50 μL of bacterial solution containing 1 × 10^8 CFU/mL in PBS using a 1-mL syringe equipped with a 30-gauge needle (Terumo Inc., Tokyo, Japan). After inoculation, the silkworms were incubated at room temperature with food. To obtain bacterial counts (as CFU/mL), the infected silkworm larvae were weighed and placed in disposable 15-mL centrifuge tubes, before homogenizing with a Biomasher SP (Funakoshi Co., Ltd, Tokyo, Japan) and suspending in 3 mL of PBS. The suspension was subsequently centrifuged at 300 × *g* for 30 s and solid tissues were separated from the concentrated suspension. Using appropriate dilutions, the suspension samples were spread onto agar plates and the numbers of colonies were counted. To calculate the counts (CFUs), the summed volumes of the hemolymph and tissues were estimated together (1 g = 1 mL).

In vivo melanization analysis

Day 2 fifth instar larva was inoculated with 50 μL of bacterial solution containing 2 × 10^8 CFU/mL in PBS. Control groups were injected with PBS or an equal volume of 75°C/30-min heat-killed *F. tularensis*. After 1 h and 18 h, the hemolymph was collected

from the caudal horn and placed in a pre-chilled 1.5-mL tube on ice to prevent further melanization because of exposure to the air. The hemolymph samples were centrifuged at 6000 × *g* for 5 min at 4°C to remove the hemolymph cells. The optical density (λ = 405 nm) of each supernatant fraction was measured using a spectrometer immediately after centrifugation.

In vivo nodule formation analysis

Melanized nodules precipitated around the dorsal vessel were observed, as described previously (Suzuki et al., 2011). Photographs were taken from the seventh to ninth segments under the same conditions using a stereoscopic microscope. To quantify the formation of nodules, each photograph was imported into Image J 1.42l software (NIH, USA; http://rsbweb.nih.gov/ij/), and the total amount of pixels in each sample was calculated with the ImageJ area measurement tool. The relative melanized area was calculated as the ratio of each group relative to that of the control.

Fluorescence microscopy

GFP-expressing bacteria were used to inoculate fifth instar day 2 larvae, which were then incubated at room temperature with food. At 1, 24, 72, and 120 h post-inoculation, hemolymph was collected from the caudal horn and added to a 24-well tissue culture plate, before diluting up to 500 μL with IPL-41 Insect Medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum. The plates were then centrifuged for 5 min at 900 × *g* and incubated for 15 min at room temperature. After washing twice with PBS, the samples were fixed with 4% paraformaldehyde (Wako, Osaka, Japan) in PBS for 15 min at room temperature. Subsequently, the samples were washed twice with PBS. Fluorescent images were obtained using a FluoView FV100 confocal laser scanning microscope (Olympus).

Pre-inoculation of silkworms

I injected 50 μL of PBS, 1×10^8 CFU/mL live *F. tularensis*, or the same amount of 75°C/30-min heat-killed *F. tularensis* into each silkworm during pre-inoculation. After incubation for 72 h at room temperature with food, the silkworms were inoculated with 50 μL of PBS suspension containing 2×10^8 or 1×10^7 CFU/mL *S. aureus* to obtain the

survival curve or internal CFU measurements, respectively. For the *in vivo* melanization and nodule formation analyses, each silkworm was injected with 50 μL of 1×10^8 CFU/mL *S. aureus* suspension or PBS as a control to determine the effects of pre-inoculation.

RNA isolation and qPCR analysis of Antimicrobial peptides (AMPs)

To analyze the expression of AMP genes, I collected the fat bodies from silkworms dissected at 72 h after pre-inoculation. The total RNA was isolated from the fat body using NucleoSpin RNA (Macherey-Nagel, Düren, Germany). The RNA was quantified by absorption at 260 nm using a NanoDrop 2000 (Thermo Fisher Scientific Inc., MA, USA). Reverse transcription was conducted using ReverTra Ace qPCR RT Master Mix (Toyobo Co. Ltd, Osaka, Japan), and cDNA samples were stored at –30°C prior to use. Next, qPCR was performed with the StepOne Real-Time PCR system (Applied Biosystems, CA, USA) using KOD SYBR qPCR Mix (Toyobo). The primer sets used for gene quantification (*Actin A3* ,*Cecropin B*, *Lebocin*, *Attacin* and *Moricin*) were described previously (Lee et al., 2007). The *Actin A3* amplicon was used as an internal

control to normalize all of the data. The relative expression levels of the AMPs were calculated using the relative quantification method (ΔΔCt).

Immunoblotting

Day 2 fifth instar larva was inoculated with 50 μL of PBS, live or 75°C/30-min heat-killed *F. tularensis* solution containing 2×10^8 CFU/mL in PBS. After 1 h, 24 h, 48 h and 72 h, the hemolymph was collected from the caudal horn and centrifuge (6000 × *g*, 4 °C) for 10 min to isolate hemolymph plasma. The proteins in 1.5 μL of hemolymph plasma were separated by SDS-PAGE with 4-12% Bis-Tris Gel (Thermo Fisher Scientific Inc.), and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% nonfat dry milk in Tris-buffered saline (TBS) at room temperature for 2 h, the membranes were incubated overnight with anti-cecropin B antibody (1:1000; Abcam plc, Cambridge, UK) at 4 °C. After washing with TBS containing 0.02% (v/v) Tween 20, the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (0.01 μg/mL) at room temperature and immunoreactions were visualized using the enhanced

chemiluminescence detection system (GE Healthcare Life Science, Little Chalfont, UK).

Statistical analysis

Statistical analyses were performed using one-way ANOVA with the post hoc Tukey–Kramer test. Statistically significant differences between groups were accepted at $P < 0.05$ or $P < 0.01$. The survival curves were estimated with the Kaplan-Meier method and the log-rank test was used to determine significant differences between the live and heat-killed *F. tularensis* pre-inoculated groups ($P < 0.05$).

RESULTS

Silkworm as a novel host model for *F. tularensis* symbiosis

F. tularensis is often found in arthropods such as ticks, but symbiosis between the bacteria and arthropods remains unclear. Thus, we established a novel host model for *F. tularensis* subsp. *holarctica* LVS symbiosis in silkworms. Silkworms were infected with *F. tularensis*, *Escherichia coli,* or *Staphylococcus aureus* via injection into the hemocoel. Silkworms infected with *F. tularensis* and *E. coli* were alive at 7 days after infection (Fig. 1a and b). No significant differences were observed in the body weight of the silkworms infected with *F. tularensis* and *E. coli* compared with uninfected silkworm (data not shown). By contrast, silkworms infected with *S. aureus* were dead within one day of infection (Fig. 1a and b). I also evaluated the growth of the bacteria in silkworms. I found that the bacterial numbers of *F. tularensis* did not show acute fluctuation for 6 days after infection (Fig. 1c). The bacterial number of *E. coli* decreased each day after infection (Fig. 1c). *S. aureus* exhibited slight growth in silkworms one day after infection but further data could not be obtained because of the decreased growth and death of the silkworms caused by the bacterial infection (Fig. 1c). Green

fluorescent protein (GFP)-expressing *F. tularensis* were observed in the hemolymph isolated from silkworms until 5 days after infection (Fig. 1d). By contrast, GFP-expressing *E. coli* were not observed in the hemolymph at 5 days after infection (Fig. 1d). These results suggest that *F. tularensis* establish a symbiosis with the silkworms.

F. tularensis blocks the silkworm immune response trigger

In silkworms, melanization and nodulation are known to be the most common immune responses to bacterial infection (Tanaka and Yamakawa, 2011). To analyze the melanization and nodulation responses in the silkworms after bacterial infection, I collected hemolymph from the silkworms after bacterial infection and determined the optical density of the samples. Live *F. tularensis* did not induce melanization at 1 and 18 h after infection, whereas heat-killed *F. tularensis* significantly induced melanization at 18 h after inoculation (Fig. 2a and 2b). *E. coli* and *S. aureus* induced melanization after bacterial infection strongly (Fig. 2a and 2b). To analyze nodulation, the dorsal vessel was observed by microscopy in silkworms after bacterial infection. *E. coli* and *S.*

aureus strongly induced nodulation immediately after bacterial infection (Fig. 2c and 2d). Live *F. tularensis* did not induce nodulation at 1 and 18 h after infection, whereas heat-killed *F. tularensis* significantly induced nodulation at 18 h after inoculation (Fig. 2c and 2d). These results suggest that immune responses of silkworm against *F. tularensis* are inhibited during the early stage of the infection, and some activities of *F. tularensis* are involved in the inhibition.

Silkworm acquires host resistance to *S. aureus* infection after pre-inoculation with *F. tularensis*

Melanization and nodulation were not induced by *F. tularensis* infection, so I investigated whether the melanization and nodulation responses induced by *S. aureus* were inhibited by pre-inoculation with *F. tularensis*. Silkworms were inoculated with live or heat-killed *F. tularensis* and incubated for 72 h at room temperature. After 72 h incubation, melanization and nodulation induced by heat-killed *F. tularensis* calmed down. The silkworms were then inoculated with PBS or *S. aureus*, and I measured the melanization and nodulation responses (Fig. 3a). The results showed that

pre-inoculation with live *F. tularensis* inhibited the melanization and nodulation responses induced by *S. aureus* infection, whereas the heat-killed bacteria were not effective at immune inhibition (Fig. 3b-e).

I hypothesized that pre-inoculation with live *F. tularensis* may have affected the immune response in silkworms, so I investigated the survival rate of silkworms after bacterial infection. I found that silkworms pre-inoculated with live *F. tularensis* survived *S. aureus* infection and they exhibited significant host resistance to bacterial infection compared with those pre-inoculated with heat-killed *F. tularensis* (Fig. 4a). Pre-inoculation with live *F. tularensis* significantly inhibited the growth of *S. aureus* in silkworms at 24 h after infection compared to PBS inoculated control (Fig. 4b). By contrast, heat-killed *F. tularensis* did not affect the survival rate of silkworms or the growth of *S. aureus* in the silkworms (Fig. 4a and 4b).

AMPs are well-known immune factors that combat pathogens in arthropods (Yi et al., 2014). To investigate whether AMPs contribute to the host resistance caused by

pre-inoculation with live *F. tularensis*, I analyzed the expression of genes for typical AMPs, i.e., cecropin B, lebocin, attacin, and moricin, at 72 h after inoculation with live or heat-killed *F. tularensis*. I found that the expression levels of these AMP genes were significantly induced by pre-inoculation with live *F. tularensis*, whereas the heat-killed bacteria had no effect (Fig. 4c). I also confirmed the time-course expression of cecropin B by immunoblotting. (Fig. 4d). Live *F. tularensis* induced cecropin B expression and the induction was sustained at 72 h post infection. In contrast, the expression induced by heat-killed *F. tularensis* was reduced at 48 h and disappeared at 72 h post infection.

DISCUSSION

Arthropods are involved in the life cycle of *F. tularensis* (Carvalhoa et al., 2014). Therefore, the development of arthropod host models is useful for studying the mechanisms related to *F. tularensis* infection and symbiosis. In this study, I established a novel, symbiotic host model for *F. tularensis* using silkworms. The ecology of *F. tularensis* and the natural reservoirs of the bacterium in the environment are not fully understood. The wax moth (*Gallaria mellonella*) has been used as a mammalian infection model for *F. tularensis* (Aperis et al., 2007), but symbiosis between *F. tularensis* and insects is still unclear. However, I observed a symbiosis between *F. tularensis* and silkworms in the present study; therefore, some types of insect may be candidates as natural reservoirs. Since the silkmoths that hatched from *F. tularensis*-infected larva still retained the *F. tularensis* bacteria (data not shown), it is possible that the animals were infected with *F. tularensis* by eating insects and/or larvae containing the bacteria.

Insects only possess innate immunity (Babayan and Schneider, 2012); therefore,

insects are generally used as models to study the basis of innate immunity. Melanization and nodulation are known to be the first defensive responses to bacterial invaders in arthropods (Cerenius and Söderhäll, 2004). Melanin can seal off foreign organisms in the hemocoel and starve them of nutrients (Chen and Chen, 1995; Tang, 2009). Melanin synthesis also results in the production of reactive oxygen and nitrogen intermediates, which are toxic to some pathogens (Kan et al., 2008). Nodule formation is a rapid response that removes microorganisms from the hemocoel. Granulocytes release sticky material after bacterial infection, and the hemocytes and bacterial cells clump together, thereby resulting in the formation of nodules (Suzuki et al., 2011), which comprise aggregations of hemocytes and microorganisms that are subsequently subjected to melanization (Arai et al., 2016). By contrast, AMPs might work during a later stage of infection because their production and concentration in the hemolymph both increase after bacterial infection (Imamura et al., 1999; Zhang et al., 2015). In this study, I demonstrated that live *F. tularensis* inhibited melanization and nodulation, but not heat-killed *F. tularensis*, suggesting that some biological activities of *F. tularensis* may inhibit the immune responses. *F. tularensis* posses type VI secretion system which is

important for intracellular growth in host cells. This secretion system contribute to control immune system in silkworm. Indeed, the type VI secretion system is reported to be involved in intracellular growth in a mosquito cell line (Read et al, 2008). Thus, symbiosis between *F. tularensis* and silkworms may be established by inhibiting the silkworm immune responses during the early stage of infection. The over-activation of immune reactions can damage the host animal itself (Ha et al., 2005). *S. aureus* has a very rapid growth rate and it causes high mortality in silkworms (Kaito et al., 2002). However, although *F. tularensis* remained at similar bacterial numbers to *S. aureus* in silkworms, they never died. Thus, the over-activated immune reactions caused by *S. aureus* infection may lead to silkworm death in the early stage of infection.

A key insect-based immunological study discovered Toll in *Drosophila*, which led to the identification of mammalian Toll-like receptors (Tzou et al., 2002; Kurata, 2014). Insect Toll functions as a receptor for an endogenous ligand, which relays signals to transcription factors that produce AMPs (Cherry and Silverman, 2006). The silkworm has 14 Toll isotypes, some of which are expressed several hours after bacterial infection

(Cheng et al., 2008; Tanaka et al., 2008). These receptors mediate the induced immune responses that are known as pathogen-associated molecular patterns, such as those to lipopolysaccharide and peptidoglycan (Hoffmann et al., 1996; Ohta et al., 2006). I found that *F. tularensis* inhibited the silkworm cellular immune responses in the early stage of infection, but the production of AMPs was continued until the later stage of infection. *F. tularensis* is also sensitive to some AMPs (Vonkavaara et al., 2013), but the bacterium may escape from the effects of AMPs by endosymbiosis within host cells. Therefore, silkworms engaged in symbiosis with *F. tularensis* exhibited resistance to *S. aureus* infection.

F. tularensis can resist degradation in the phagosome and replicate inside mammalian macrophages (Fortier et al., 1995). I did not demonstrate the phagocytosis activity of hemocytes directly in this study, but intracellular bacteria were clearly observed in the hemocytes at 5 days after infection. Hemocytes may be one of the targets for bacterial invasion and *F. tularensis* may control the immune response in the silkworm to take advantage of suitable conditions for symbiosis. The intracellular signaling pathway

related to the expression of AMPs in silkworms is still unclear. However, Ishii et al. (2010) showed that the phosphorylation of p38 MAPK protein conferred protection against *S. aureus* infections in silkworms by up-regulating the expression of AMP genes, but it did not affect the melanization activity. In *Drosophila*, the activation of p38 induces host protection from various bacterial pathogens and fungi because of the up-regulated expression of genes for stress response factors and specific AMPs (cecropin B and attacin; Chen et al., 2010). Thus, *F. tularensis* may induce specific signaling pathways that are affected by intracellular bacteria. Therefore, these signaling pathways may be a possible target for disrupting the lifecycle of *Francisella* in the environment.

A mutualistic relationship is considered to provide benefits to both symbionts. Various insects possess intracellular bacteria within specialized cells known as bacteriocytes, the sole function of which appears to be the housing and maintenance of bacteria (Douglas, 1989). Insect immune effectors have been implicated in the regulation of the bacteria found in the bacteriocytes of the weevil *Sitophilus*. *Sitophilus*

bacteriocytes express a cationic AMP, coleoptericin A, at high levels, and this AMP plays an important role in controlling the maintenance of the symbionts (Login et al., 2011). *Wolbachia pipientis* is an obligate intracellular bacterium and a common endosymbiont of insects (Zientz et al., 2004). *Drosophila melanogaster* flies infected with *W. pipientis* are less susceptible to the induction of mortality by a range of RNA viruses (Hedges et al., 2008). *Wolbachia* also inhibits the ability of a range of pathogens, such as *Plasmodium*, dengue virus, and Chikungunya virus, to infect *Aedes aegypti* (Moreira et al., 2008). The depressed vector competence of *Wolbachia*-infected mosquitoes may be caused by an enhanced immune function, including the induction of AMPs, melanization, and reactive oxygen species (Zug and Hammerstein, 2015). I showed that silkworms in symbiosis with *F. tularensis* were protected from death caused by *S. aureus* infections. Thus, symbiosis with *F. tularensis* may provide fitness benefits for insects, and the human pathogen *F. tularensis* may have an important role in protecting natural reservoirs, such as arthropods, from pathogenic invaders. *F. tularensis* has been isolated from deer flies, horse flies, ticks, and mosquitoes (Petersen et al., 2009). *Francisella*-like endosymbionts have also been reported in various tick

species (Sun et al., 2000; Dergousoff and Chilton, 2012; Michelet et al., 2013). In this study, my results suggest that *F. tularensis* can infect and survive in endosymbiosis with silkworms; therefore, many other insect species may also be vectors of tularemia. Thus, *Francisella* may be distributed in more arthropod species than considered at present.

FIGURES

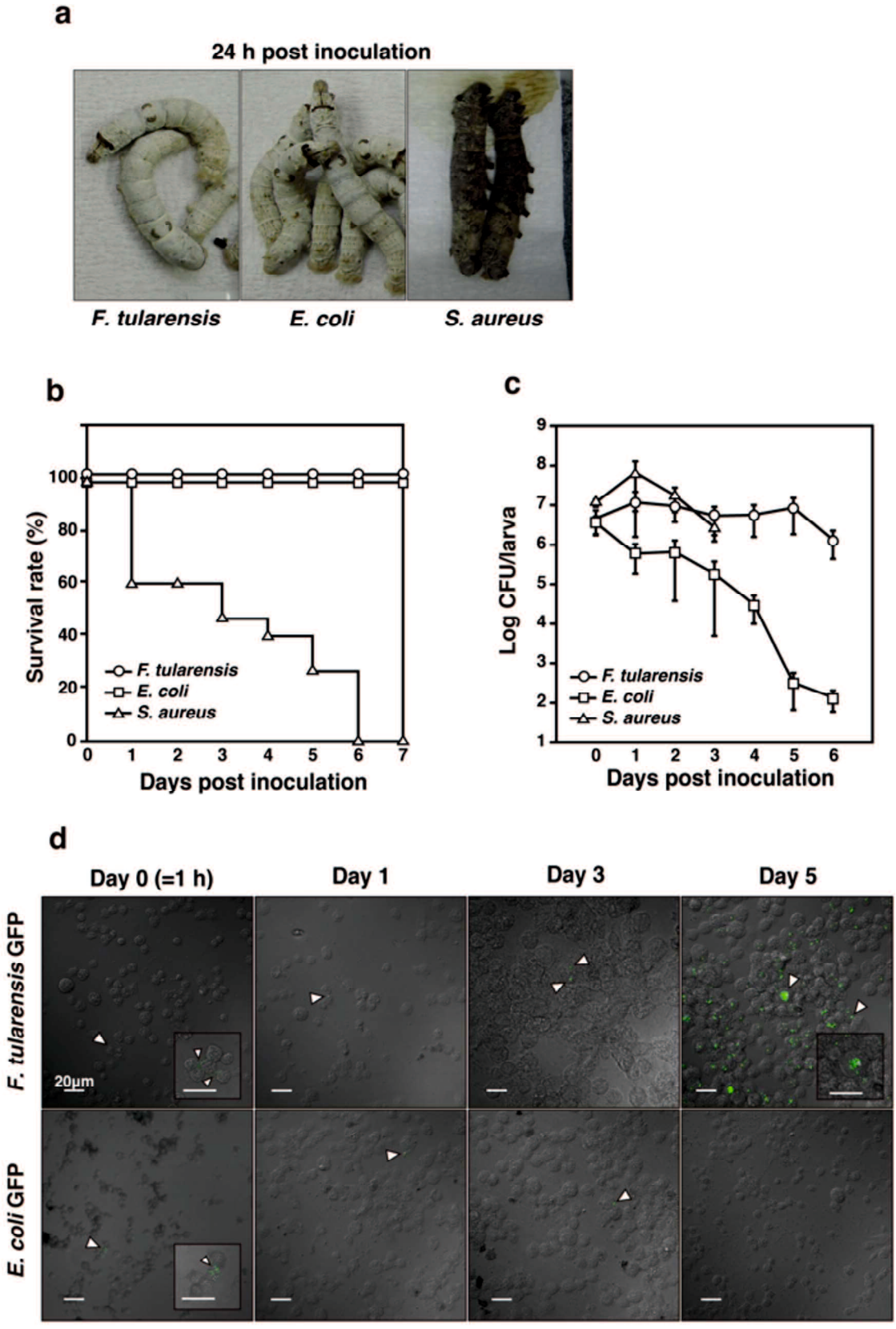
a
24 h post inoculation
F. tularensis
E. coli
S. aureus
b
Survival rate (%)
Days post inoculation
F. tularensis
E. coli
S. aureus
c
Log CFU/larva
Days post inoculation
F. tularensis
E. coli
S. aureus
d
Day 0 (=1 h)
Day 1
Day 3
Day 5
F. tularensis GFP
E. coli GFP
20μm

Figure 1. Symbiosis between *F. tularensis* and silkworms.

(**a**) Fifth instar larva were infected with *F. tularensis*, *E. coli*, or *S. aureus.* Larval conditions at 24 h post infection were shown. (**b**) Each silkworm group (n = 15) was infected with *F. tularensis*, *E. coli* and *S. aureus*, then survival rate was calculated at indicated day point. (**c**) Silkworms were infected with *F. tularensis*, *E. coli* and *S. aureus*. Bacterial numbers in silkworms were counted at indicated day points. The data represent the averages from triplicate samples based on three identical experiments and the error bars denote the standard error of the mean (n = 9). (**d**) Silkworms were infected with GFP-expressing *F. tularensis* or *E. coli* (arrowheads). Hemolymph cells were at indicated day points and observed by confocal laser scanning microscopy. Close-up images are shown in the box frame. Scale bar represents 20 μm.

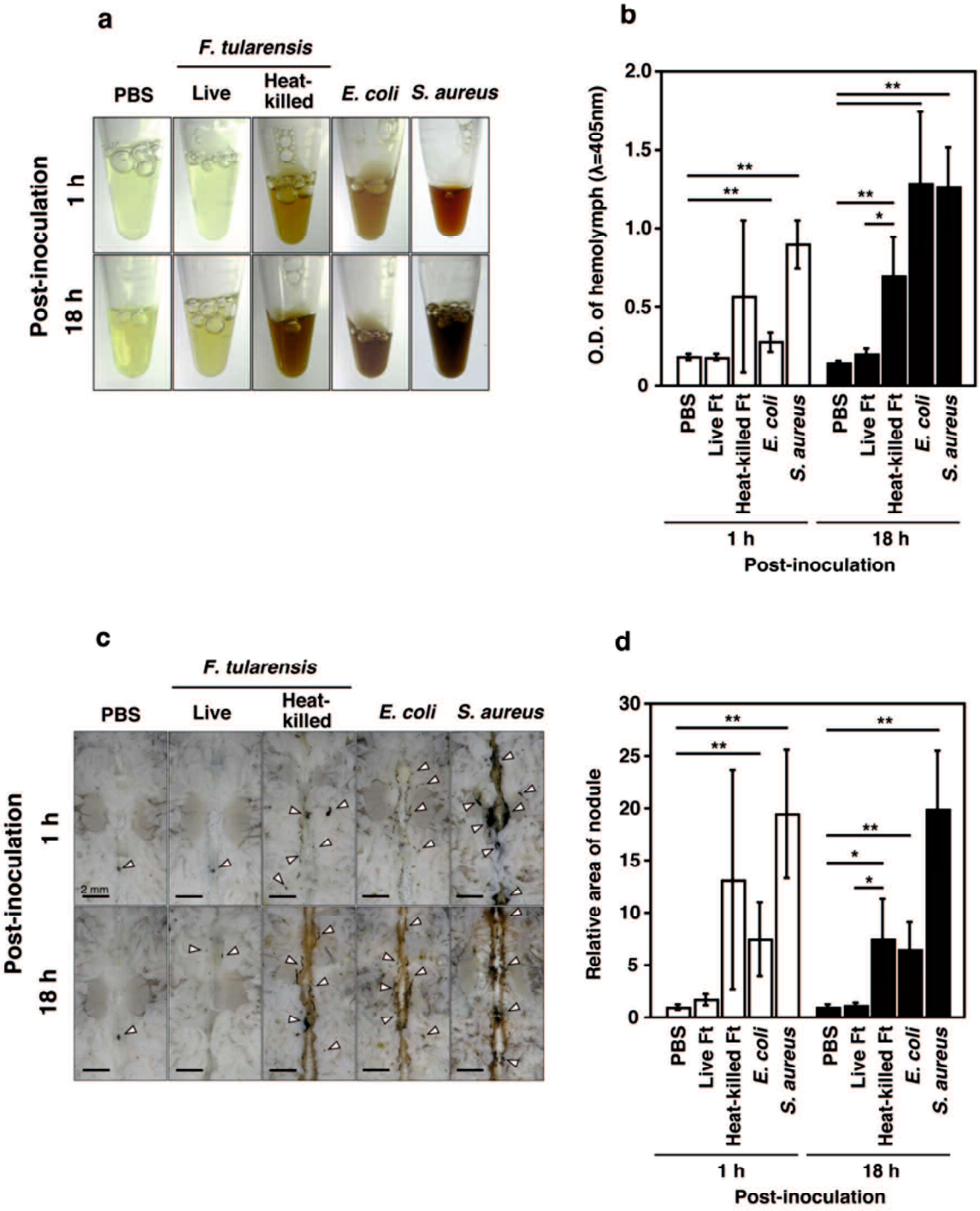

Figure 2. *F. tularensis* inhibits the immune response by silkworms.

(**a**) Hemolymph at 1 and 18 h post-inoculation was collected from fifth instar larva

inoculated with PBS, live *F. tularensis*, heat-killed *F. tularensis*, *E. coli*, and *S. aureus*. The condition of melanization was decided by color. (**b**) Silkworms were infected with indicated bacteria, and hemolymph was collected at 1 and 18 h post-inoculation. The optical density (λ = 405 nm) of the hemolymph was measured using a spectrometer immediately after centrifugation to remove hemolymph cells. (**c**) Silkworms were infected with indicated bacteria, and nodule formation around dorsal vessel was observed. Arrowheads indicate nodule formation induced by infected bacteria. (**d**) Silkworms were infected with indicated bacteria, and the total area of nodule formation was calculated using the area measurement tool. The relative melanized area was shown compared to PBS-inoculated control group. (**b** and **d**) The data represent the averages from triplicate samples based on three identical experiments, and the error bars denote the standard deviations. Significant differences were accepted at $P < 0.05$ or $P < 0.01$, and they are indicated by asterisks (*) or double asterisks (**), respectively.

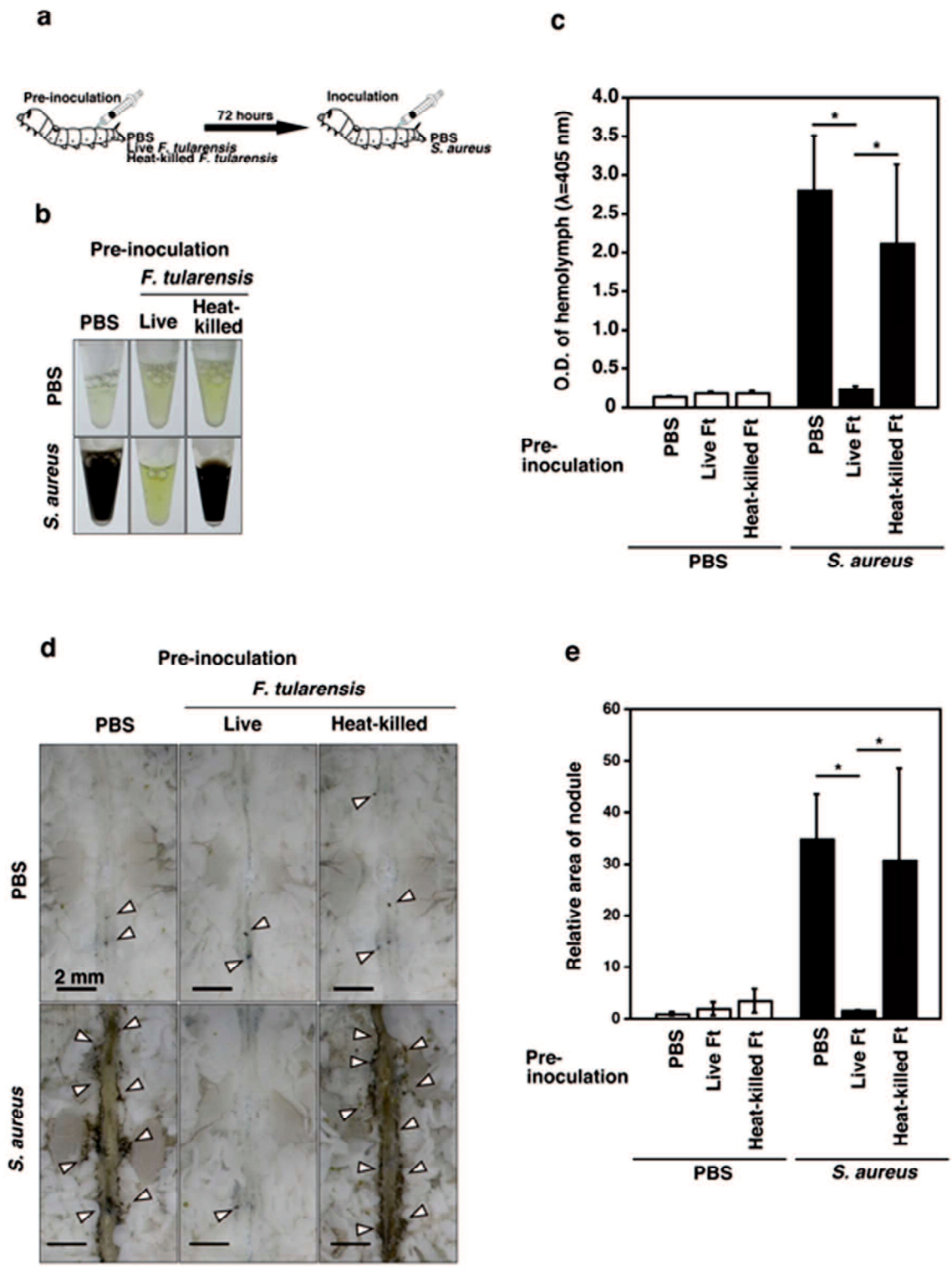

Figure 3. The silkworm immune response induced by *S. aureus* is inhibited by pre-inoculation with *F. tularensis*.

(**a**) The experimental scheme is shown. Silkworms were pre-inoculated with indicated bacteria. After 72 h incubation, silkworms were inoculated with PBS or *S. aureus*, and melanization and nodulation were observed. (**b**) Hemolymph was collected from silkworms at 1 h post-inoculation with PBS or *S. aureus*, and the condition of melanization was decided by color. (**c**) The optical density (λ = 405 nm) of the hemolymph collected at 1 h post second inoculation was measured using a spectrometer immediately after centrifugation to remove hemolymph cells. (**d**) Nodule formation around dorsal vessel at 1 h post second inoculation was observed. Arrowheads indicate nodule formation. (**e**) The total area of nodule formation was calculated using the area measurement tool. The relative melanized area of nodule formation was shown compared to PBS-inoculated control group. (**c** and **e**) The data are presented as averages from triplicate samples based on three identical experiments, and the error bars denote the standard deviations. Significant differences were accepted at $P < 0.05$, and they are indicated by asterisks (*).

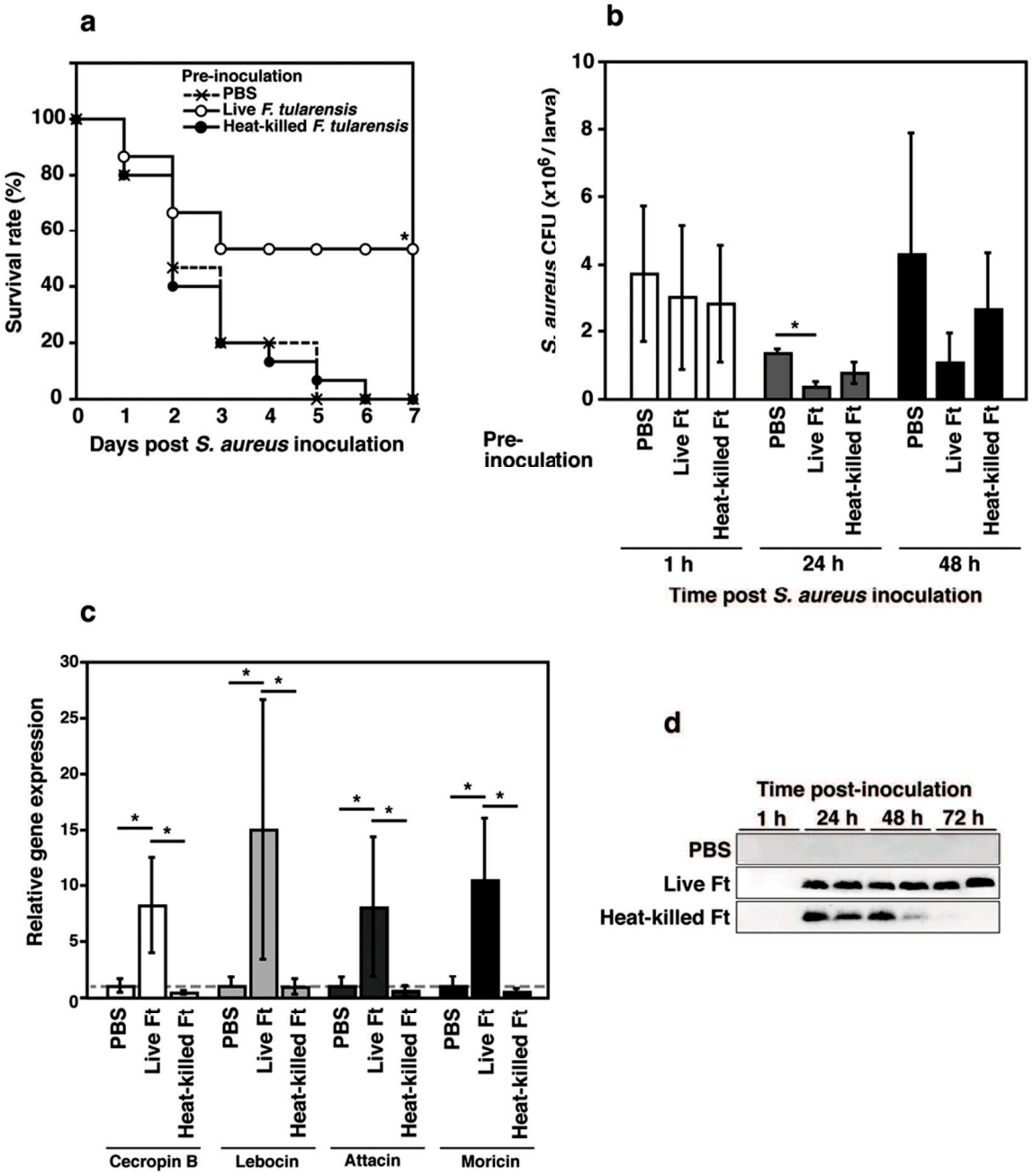

Figure 4. Silkworms acquire host resistance to *S. aureus* infection after pre-inoculation with *F. tularensis.*

(**a**) Each silkworm group pre-inoculated with PBS, live *F. tularensis*, or heat-killed *F. tularensis* (n = 15) were inoculated with *S. aureus*, then survival rate was calculated at

indicated day point. Significant differences between live and heat-killed *F. tularensis* pre-inoculated groups were accepted at $P < 0.05$ and indicated by asterisk (*). (**b**) Silkworms were pre-inoculated with PBS, live *F. tularensis*, or heat-killed *F. tularensis*. Silkworms were then infected with *S. aureus*. Bacterial numbers of *S. aureus* was counted at 1 h, 24 h, and 48 h post second inoculation. (**c**) RNA samples were collected from the fat bodies of silkworms inoculated with PBS, live *F. tularensis*, or heat-killed *F. tularensis*. The expression levels of AMP genes were determined by real-time PCR. The relative expression levels are presented compared to PBS inoculated group. (**d**) Hemolymph plasma samples from silkworms pre-inoculated with PBS, live *F. tularensis*, or heat-killed *F. tularensis* were collected at 1 h, 24 h, 48 h and 72 h post inoculation. Cecropin B expression levels were analyzed by immunoblotting. (**b** and **c**) The data represent the averages from triplicate samples based on three identical experiments and the error bars denote the standard error of the mean (n = 9). Significant differences were accepted at $P < 0.05$ and they are indicated by asterisks (*).

CONCLUSION

Tularemia has studied since 1911 mainly as bioterror agent (Dennis et al., 2001), and the numbers of reports about pathogenicity to human and epidemiological surveillance are still increasing. However, the life cycle of *F. tularensis* in nature is not fully understood. To control *F. tularensis* infection, it is necessary to clarify interactions between bacteria and host arthropod in environment. I focused on ticks which are candidate reservoir of *F. tularensis* and silkworm which is an alternative arthropod infection model.

In chapter 1, I demonstrate that *I. ovatus* and *I. persulcatus* play an important role in establishment of *F. tularensis* life cycle in environment, and symbionts similar to FLEs may exist in Japan. In addition, experimental infection model substantiated the property of *I. persulcatus* for harboring *F. tularensis*, and revealed a bacterial factor involved in internal growth. These findings are important to clarify interactions between ticks and *F. tularensis*. To clarify the relationship between *F. tularensis* and host arthropods, further study was needed. However, it was difficult to assess tick responses to *F. tularensis*

infection because of its body size and long life cycle period.

In chapter 2, I established alternative *F. tularensis* infection model using silkworm. This model demonstrated that *F. tularensis* control immune response to survive in silkworm. On the other hand, *F. tularensis* infection stimulates silkworm resistance against other pathogenic bacteria through AMPs induction. This phenomenon seems to be similar to symbiotic relationships between arthropods and their endosymbiosis, such as *Wolbachia* (Moreira et al., 2009; Zug and Hammerstein, 2015). These results led the conclusion that *F. tularensis* establishes symbiosis in silkworm.

Melanization, nodulation and production of AMPs demonstrated in this study are widely common immune system against microorganisms in invertebrates, including ticks and mosquitos (Cerenius and Söderhäll, 2004; Meekins et al., 2016). In addition, growth kinetics of *F tularensis* in *I. persulcatus* (Chapter 1 Fig. 3) were similar to that in silkworms (Chapter 2 Fig. 1c). On the other hand, FLEs in ticks have been reported all over the world (Sun et al., 2000; Dergousoff and Chilton, 2012; Michelet et al.,

2013), and Gerhart et al. (2016) showed that FLEs might evolved from pathogenic *Francisella* in mammals (Gerhart et al., 2016). Taken together, These data suggest that *F. tularensis* establish symbiotic relationship with vector arthropods via control of immune responses.

Recently, *Wolbachia* came into the global spotlight because the mosquito infected with *Wolbachia* could control a range of pathogens in environment, such as *Plasmodium*, dengue virus, and Chikungunya virus (Moreira et al., 2009). Here, I showed that induction of AMPs play an important role to establish symbiotic relationships. The spectrums of AMPs are quite wide, including various bacteria, virus and fungi (Hoffmann et al., 1996; Chiou et al., 2002; Cheng et al., 2008; Lu et al., 2016). Thus, *F. tularensis* may affect to other vector-borne microorganisms as a symbiont. My findings in this study will contribute to control not only *F. tularensns* infection but also other vector-borne pathogens in environment through further research.

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