Analysis of molecular mechanism of *Francisella* pathogenesis using mouse and silkworm infection model

(マウスおよびカイコモデルを用いた野兎病菌の病原性分子メカニズムの解析)

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PREFACE

Tularemia is a zoonotic disease caused by a Gram-negative facultative intracellular bacterium capable of producing disease in a diverse range of hosts; at least 190 different species of mammals, 23 birds, 3 amphibians and 88 invertebrates are reported as being susceptible to *Francisella tularensis* (Mörner *et al*. 2008). *Francisella* transmission occurs through direct contact of sick animal and ingestion or inhalation of contaminated food and water (Carvalho *et al.* 2014); besides, a wide range of arthropods act as a potential vectors for disease transmission (Carvalho *et al.* 2014). Due to its aerosol transmission to human with as few as 10 cells, CDC declares it as Tier 1 threat agent (Saslaw *et al.* 1961; Dennis *et al.* 2001). In spite of its sporadic outbreak throughout the Northern Hemisphere, potentiality of acting as a biological weapon *Francisella* is still catching a great attention towards scientist.

After entry, *F. tularensis* can invade and replicate both in phagocytic and non phagocytic cells (Meibom and Charbit 2010). For successful parasitism *Francisella* must replicate within macrophage and capable of escaping from phagocytic vacuole to cytosol. After initial multiplication, *Francisella* spreads into distant body parts and

produces different clinical form of disease. So, important aspect of *Francisella* virulence is its ability to proliferate within macrophage. Macrophage creates oxidative stress to kill the *Francisella,* but it possesses several antioxidant enzymes that can directly counteract the harsh intracellular environments, a number of them have already been described (Lindgren *et al.* 2007; Melillo *et al.* 2009; Dieppedale *et al.* 2011; Binesse *et al.* 2015). Not only this, *Francisella* has some oxidation repairing enzyme in its chromosome that repairs the damaged oxidized molecule to its free form. But the oxidative repairing mechanism of *Francisella* has not been studied yet.

Nobody will disagree of using mammalian model as a prior option for better understanding of bacterial pathogenecity. Though established mouse infection model is available for *Francisella* study, researchers are turning their interest to insect host system as- (i) it allows high- throughput research with low economic budget, (ii) no raise of ethical issues by the animal welfare association, (iii) laboratories where mammalian host rearing facilities are unavailable, and also (iv) it provides valuable information about environmental persistence and transmission. Among different non mammalian infection models, considering cheap and easy rearing facilities at room

temperature within minimal setup and administration facilities of target amount of bacterial number, silkworm (*Bombyx mori*) infection model are getting popular now a days.

Taken together, these two observations, for understanding molecular pathogenesis of *Francisella*, I set up my experimental objective in this study under two different aspects-

1. Analysis of methionine sulfoxide reductases of *Francisella tularensis* in oxidative stress condition using mouse infection model.

2. Establishment of a novel *Francisella novicida* infection model in silkworm.

CHAPTER 1

Contribution of methionine sulfoxide reductase B (MsrB) to *Francisella tularensis* infection in mice

INTRODUCTION

Francisella tularensis is a gram-negative facultative intracellular bacterium that causes tularemia in humans. It can serve as a biological weapon (Dennis *et al.* 2001). Only 10 cells of *Francisella* can cause disease in humans (Saslaw *et al.* 1961). *F. tularensis* can infect in a wide range of mammals, but it is mainly found in small rodents, hares, and rabbits (Carvalho *et al.* 2014). The organism is transmitted by ticks, mosquitoes, direct contact of sick animals, contaminated food and water, or aerosols (Carvalho *et al.* 2014). Depending upon the route of entry, the bacterium causes ulceroglandular, glandular, oropharyngeal, oculoglandular, typhoid, or pneumonic forms of tularemia (Evans *et al*. 1985; Carvalho *et al.* 2014). The disease is primarily distributed in the Northern Hemisphere. The genus *Francisella* contains two species, namely *F. tularensis* and *F. philomiragia*. *F. tularensis* has four subspecies, namely subsp. *tularensis* (type A), *holarctica* (type B), *mediasiatica*, and *novicida* (Sjödin *et al.* 2012). Among these four subspecies, the first two are responsible for human infection (Sandstrom *et al.* 1992). After infection, *Francisella* can survive and replicate within a variety of mammalian cell types including phagocytic and non-phagocytic cells of various species (Meibom

and Charbit 2010). Within macrophages, *Francisella* faces a number of host defenses including degradative enzymes, acidic pH, and oxidative stresses (Lenco *et al.* 2005; Bakshi *et al.* 2006; Clemens and Horwitz 2007; Guina *et al.* 2007; McCaffrey *et al.* 2010; Dieppedale *et al.* 2011). As a result of host-pathogen interactions, macrophages produce reactive oxygen species (ROS), which alter bacterial macromolecules (e.g., proteins, DNA, lipids) and ultimately induce bacterial death. *Francisella* detoxifies ROS using various enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase, organic hydroperoxides, alkyl hydroperoxide reductase, and MoxR ATPase (Lindgren *et al.* 2007; Melillo *et al.* 2009; Dieppedale *et al.* 2011; Binesse *et al.* 2015).

Methionine is one of the most vulnerable amino acids under oxidative stress. Upon oxidation, it is converted into methionine sulfoxide (MetO), which cannot perform normal cellular functions. Oxidation of methionine results in two diastereomeric forms of MetO: methionine-(S)-sulfoxide (Met-S-SO), and methionine-(R)-sulfoxide (Met-R-SO). The two methionine sulfoxide reductases encoded by msrA and msrB catalyze the reduction of Met-S-SO and Met-R-SO, respectively (Weissbach *et al.* 2002). Msr is different from conventional antioxidants such as SOD or alkyl hydroperoxide reductase.

The latter can directly detoxify ROS, whereas Msr can convert the oxidized protein into its reduced state. The performance of MsrA and MsrB has been studied in many pathogenic bacteria. In prior research, the lack of MsrA induced susceptibility to oxidative stress in *Salmonella typhimurium* (Denkel *et al.* 2011), *Staphylococcus aureus* (Singh *et al.* 2001, 2015), *Mycobacterium smegmatis* (Douglas *et al.* 2004), and *Escherichia coli* (Moskovitz *et al.* 1995). Both MsrA and MsrB combat oxidative stress in *Campylobacter jejuni* (Atack and Kelly 2008), *M. tuberculosis* (Lee *et al.* 2009), and *Enterococcus faecalis* (Zhao *et al.* 2010). Among these organisms, *S. typhimurium*, *C. jejuni*, and *Mycobacterium* sp. are considered facultative intracellular pathogens capable of replicating inside host macrophages. However, the role of MsrA and MsrB in the pathogenesis of *F. tularensis* has not been studied yet. This study described the contribution of MsrA and MsrB to the intracellular growth of *F. tularensis* and its response to oxidative stress. The results indicated the importance of the methionine sulfoxide reductase system in the pathogenesis of *F. tularensis*.

MATERIALS AND METHODS

Bacterial strains and culture condition

F. tularensis subsp. *holarctica* LVS, *E. coli* JM109, and *E. coli* JM109 pAcGFP (Clontech, Mountain View, CA, USA) were used in this study. *Francisella tularensis* subsp. *holarctica* LVS was obtained from the Pathogenic Microorganism Genetic Resource Stock Center, Gifu University and cultured aerobically at 37°C in brain heart infusion broth (BD, Franklin Lakes, NJ, USA) supplemented with cysteine (BHIc) (Gann et al 2010) or Brucella broth (BD) containing 1.5% agar (Wako, Osaka, Japan). Bacterial strains were maintained as glycerol stocks frozen at−80°C. *E. coli* JM109 was 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.

Construction of *F. tularensis msr* mutants

The msr mutants of *F. tularensis* were generated by group II intron insertion using the TargeTron® gene knockout system (Sigma-Aldrich, St Louis, MO, USA) modified for

Francisella species (Rodriguez *et al.* 2008), as described previously (Uda *et al.* 2014).

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

A GFP-expressing plasmid, pNVU-GFP, was constructed from a pNVU1-expressing plasmid. The tetracycline resistance gene was removed from pNVU1 by treating the plasmid with SmaI. The GFP gene containing an SD sequence was amplified from pGreenTIR using the primer pairs pNVU-GFP-F (5′-GAAATTATTGATCCCTGATTAACTTTATAAGGAGGAA-3′) and pNVU-GFP-R (5′-CTTGGTCTGACACCCCTATTTGTATAGTTCATCCATG-3′) and inserted into SmaI-digested pNVU1. pNVU-GFP was transformed and replicated in *E. coli* DH5α and purified using a Plasmid Midi Kit (Qiagen, Hilden, Germany). The 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.

***In vitro* growth assay of *F. tularensis* in BHIc broth**

Overnight cultures of *Francisella* strains were diluted to an OD_{595} of 0.02 and then incubated under shaking in BHIc broth at 37°C. The OD_{595} was measured every hour up to 14 h. Experiments were performed in triplicates.

***In vitro* oxidative stress assay**

Bacterial cultures of the mid-exponential phase were diluted to an OD_{595} of 0.02 in BHIc broth. H_2O_2 (1.8 mM) or t-butyl hydroperoxide (t-BHP; 0.25 mM) was added in the bacterial culture to create an oxidative stress condition, and then the bacterial culture was incubated at 37°C. OD_{595} was measured every hour up to 14 h. Experiments were performed in triplicates.

J774 cells culture and infection assay

The J774 mouse macrophage line was grown in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS at 37°C under an atmosphere containing 5% CO2. Bacteria were added to a monolayer of J774 cells (5×10^4 cells/well) in 48-well tissue culture dishes at a multiplicity of infection (MOI) of 1. These plates were

centrifuged for 10min at 300 × g and incubated for 30 min at 37°C. Then, cells were washed twice with PBS, and extracellular bacteria were killed by gentamicin (50 μg/mL) treatment for 30 min. To measure the intracellular growth, the cells were incubated in fresh medium at 37°C for the designated amount of time, washed three times with PBS, and then lysed with 0.1% Triton X -100 in chemically defined medium (Chamberlain, 1965). Colony forming units (CFUs) were determined by serial dilution on Brucella agar.

Fluorescence microscopy

J774 cells (1×10^5 cells/well) were grown overnight on 12-mm glass coverslips (Thermo Fisher Scientific Inc., MA, USA) in 24-well tissue culture plates and were infected with different GFP strains at an MOI of 1 for the indicated times. Samples were washed three times with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. The samples were washed with PBS three times and then washed with distilled water twice. Fluorescent images were obtained using a Fluoview FV100 confocal laser-scanning microscope (Olympus, Tokyo, Japan).

Mouse infection assay

C57BL/6J female mice (6–8 weeks old) were obtained from Kyudo Co., Ltd. (Saga, Japan). Groups of three mice per assay were used for three independent experiments. Bacterial strains were grown overnight in BHIc broth from a frozen (−80 °C) stock. Bacteria were cultivated at exponential phase and washed with PBS twice. Mice were infected intraperitoneally with approximately 1×10^7 cells of each strain in 0.2 mL of PBS. Mice were sacrificed 3 days after infection. Liver and spleen tissues were removed and homogenized in PBS after weighing. Bacteria were plated on Brucella agar after appropriate dilutions for CFU counting.

Ethics statement

All protocols for animal experiments were approved by the Animal Research Committee of Yamaguchi University (Permission number: 141). 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 all efforts were made to minimize suffering by using isoflurane anesthesia.

Statistical analyses

Student's t-test was used to make statistical comparisons between the groups. Results were considered significantly different at $P < 0.05$, as indicated by asterisks. Data are expressed as the mean of triplicate samples from three identical experiments, and the error bars represent the SD.

RESULTS

Organization of *msr* genes in the genome of *F. tularensis* subsp. *holarctica* LVS

The nucleotide sequences encoding *msrA* and *msrB* of *F. tularensis* were obtained from the National Center for Biotechnology database. FTL_1960 is a 690-bp open-reading frame annotated as *msrA* encoding the peptide methionine sulfoxide reductase A. The deduced amino acid sequence of *F. tularensis* MsrA shares 51, 36, 36, and 42% identity with MsrA from *Legionella pneumophila*, *E. coli* 0.1288, *S. typhimurium*, and *E. faecalis* JH2-2, respectively. msrA is located 369 bp downstream of FTL_1961 (hypothetical protein) and 412 bp upstream of FTL_RS09955 (Arac family transcriptional regulator). FTL_0379 is a 513-bp open-reading frame annotated as *msrB* encoding the peptide methionine sulfoxide reductase B. The amino sequence of MsrB of *F. tularensis* shares 67, 59, 51, and 61% identity with MsrB of *L. pneumophila*, *E. coli* 0.1288, *S. typhimurium*, and *E. faecalis* JH2-2, respectively. *msrB* is located 558 bp upstream of FTL_0380 (SOD Cu-Zn family protein) and 654 bp upstream of FTL_0378 (hypothetical protein).

***In vitro* growth of *msrA* and *msrB* mutants following exposure to exogenous oxidants**

For the present study, mutant strains were generated via the insertion of group II intron using the TargeTron® gene knockout system. In growth curve measurements, the mutant lacking MsrB exhibited significantly decreased bacterial growth (doubling time was approximately 1.75 h) compared to the wild-type strain (doubling time was approximately 1.5 h). When the growth pattern of the *msrA* mutant was compared with its parental strain, it showed similar characteristics (Fig. 1A). The double mutant displayed the same growth characteristics as the *msrB* mutant (Fig. 1A).

To investigate the role of the Msr proteins of *F. tularensis* via *in vitro* oxidative stress assays, *F. tularensis* strains were treated with 1.8 mM H_2O_2 and 0.25 mM t-BHP. In growth curve experiments, the *msrB* mutant exhibited a significantly lower growth rate than the wild-type strain (Fig. 1B & 1C). The *msrA* mutant did not display any growth variation compared to the wild-type strain when treated with t-BHP, but it exhibited decreased growth following H_2O_2 treatment. Strains lacking both *msrA* and *msrB* exhibited similar growth patterns as the *msrB* mutant in both cases (Fig.1B & 1C).

Growth of the *msrA* and *msrB* mutants inside macrophages

To investigate the role of MsrA and MsrB of *F. tularensis* during intracellular growth, J774 cells were infected with the wild-type strain and *msr* mutants. The number of viable bacteria was counted at 2, 24, 48, and 72 h post-infection, with the results presented in Figure 2A. Regarding the *msrB* mutant, the number of intracellular bacteria was decreased significantly at 48 and 72 h after infection about 48 and 219 times respectively. Conversely, the intracellular growth of the *msrA* mutant was not affected (Fig. 2A). When the growth characteristics of the *msrA* and *msrB* double mutant were compared with those of its parental strain, the number of viable bacteria was reduced similarly as observed for the *msrB* mutant. These results indicated that MsrB, but not MsrA, has an important role in the intracellular growth of *F. tularensis*. The intracellular growth pattern of the GFP-expressed wild-type strain and *msr* mutants in J774 cells was also examined using confocal microscopy at 2, 24, 48, and 72 h post-infection. Explosive growth of the wild-type strain and *msrA* mutant was observed at 48 and 72 h post-infection (Fig. 2B), whereas little growth was observed for the *msrB* mutant and double mutant in J774 cells (Fig. 2B).

Growth of *msrA* and *msrB* mutants in mice

To examine the role of MsrA and MsrB in *F. tularensis* on bacterial infection in mice, C57BL/6J mice were infected with the wild-type strain, *msrA* mutant, or *msrB* mutant. All infected mice were checked daily, with not exhibiting significant clinical signs such as, alertness, temperature, body weight, etc. The *msrB* mutant displayed significantly lower counts than the wild-type strain in the liver and spleen as 10 and 15 times respectively (Fig. 3). The counts of the *msrA* mutant were also lower than those of the wild-type strain in the liver, but not in the spleen.

DISCUSSION

Researchers have expressed increasing interest in the study of the methionine sulfoxide reductases MsrA and MsrB in pathogenic bacteria in recent years because of its function in repairing oxidized proteins to restore biological activity (Ezraty, Aussel and Barras 2005). These two distinct proteins share no sequence homology. The locations of the *msrA* and *msrB* gene loci in chromosomes vary among prokaryotes. The *msr* genes in several bacterial species, such as *S. aureus* and *Bacillus subtilis*, are located adjacent to each other and are co-transcribed (Singh *et al.* 2001; Ezraty, Aussel and Barras 2005). The copy numbers of *msr* genes are also variable. For example, *S. aureus* contains three copy of *msrA* and one copy of *msrB*, whereas *E. coli* contains one copy each of *msrA* and *msrB* (Ezraty, Aussel and Barras 2005). *F. tularensis* subsp. *holarctica* LVS has one copy each of *msrA* and *msrB*, but the loci are located distantly from each other.

The oxidation of proteins in the cell is one of the most important molecular consequences of oxidative stress (Dalle-Donne *et al.* 2003). The role of the Msr family proteins in defense against oxidative stress was described in *E. coli* and *Saccharomyces cerevisiae* approximately 20 years previously (Moskovitz *et al.* 1995, 1998). The

enzyme-substrate interaction of MsrA for Met-S-SO and MsrB for Met-R-SO were illustrated by several studies (Weissbach *et al.* 2002; Boschi-Muller *et al.* 2005; Weissbach, Resnick and Brot 2005; Lee *et al.* 2009). Susceptibility towards Met-S-SO or Met-R-SO among bacterial species vary greatly because in some bacteria, oxidation of methionine with S epimer is significant as compared to R epimer to protect intracellular reactive oxidative intermediates (ROI) and vice versa. Among studied pathogenic bacteria such as *S. typhimurium*, *E. coli*, and *S. aureus*, MsrA has a vital role in combating oxidative stress (Moskovitz *et al.* 1995; Denkel *et al.* 2011; Singh *et al.* 2015). The contribution of MsrB to bacterial survival in mice was also observed for *Helicobacter pylori* and *C. jejuni* (Alamuri and Maier 2004; Atack and Kelly 2008). The lack of *msrB* in *Lactobacillus reuteri* results in less efficient survival inside the gastrointestinal tract of mice (Walter *et al.* 2005). In this study, MsrA had a limited role in protecting *F. tularensis* against oxidative stress and promoting bacterial survival in mice. Although the number of msrA mutant was slightly but significantly decreased in liver, similar number of the mutant was counted in spleen and macrophage cell line. Contrarily, MsrB played a vital role in the survival of *F. tularensis* within macrophages

and mouse model. The significant role of MsrB in the survival of *Francisella* may indicate its critical effect in the neutralization of ROS within organs and macrophages. The significant role of MsrB in *Francisella* against oxidative stress in this study may be due to substrate specificity within the intracellular environment. Oxidation of methionine with R epimer in *Francisella* is probably more significant than S epimer.

The protective role of MsrB against H_2O_2 may confer resistance to *F. tularensis* against myeloperoxidase-expressing cells such as neutrophils and monocytes (Lofgren *et al.* 1984). Indeed, the *msrB* mutant was more sensitive to oxidative stressors such as H_2O_2 and t-BHP than the wild-type strain and *msrA* mutant. This result is consistent with the hypothesis that *F. tularensis* is sensitive to oxidation of the Met R epimer, explaining its lower survival within the intracellular environment. As MsrA has little to no protective effect on *F. tularensis* in the host environment, the *msrA* and *msrB* double mutant displayed a similar phenotype as the *msrB* mutant regarding both intracellular survival and sensitivity to oxidants. In this study, an *msrB*-complemented strain was also constructed by introducing an *msrB*-expressed plasmid into the *msrB* mutant. However, the *in vitro* growth of the complemented strain was inhibited in comparison

with that of the wild-type strain and *msrB* mutant (data not shown). Overexpression of *msrB* may affect bacterial growth. Therefore, analysis of complemented strain could not be performed. For further validation, complementation assay is needed.

Some bacteria such as *E. coli* and *S. typhimurium* possess an enzyme, biotin sulfoxide reductase (BisC), which can reduce oxidized methionine to methionine (Ezraty *et al.* 2005; Denkel, Rhen and Bange 2013). However, the *F. tularensis* subsp. *holarctica* genome did not contain a *bisC* homolog in a BLAST search. As ROS represent an important anti-bacterial factor, various pathways to protect against ROS are available in different bacterial species. The role of Msr proteins in bacterial virulence was well described almost a decade ago (Sasindran, Saikolappan and Dhandayuthapani 2007).

This study confirmed the role of Msr proteins, particularly MsrB, in disease production via both *in vitro* and *in vivo* experiments. In future research, determination of the biochemical activities of MsrA and MsrB is necessary to understand the mode of action of methionine sulfoxide reductases in *Francisella*.

FIGURES

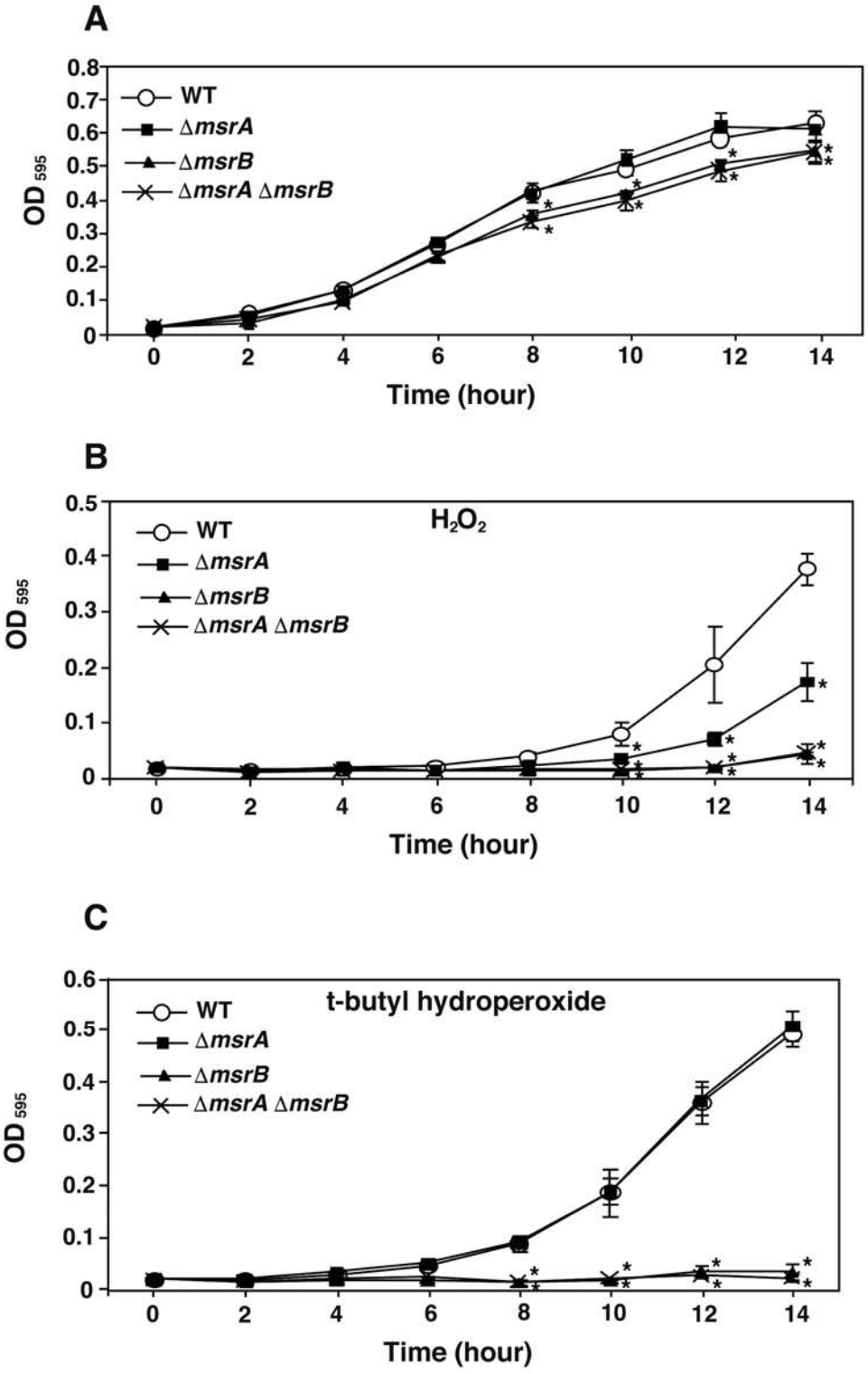
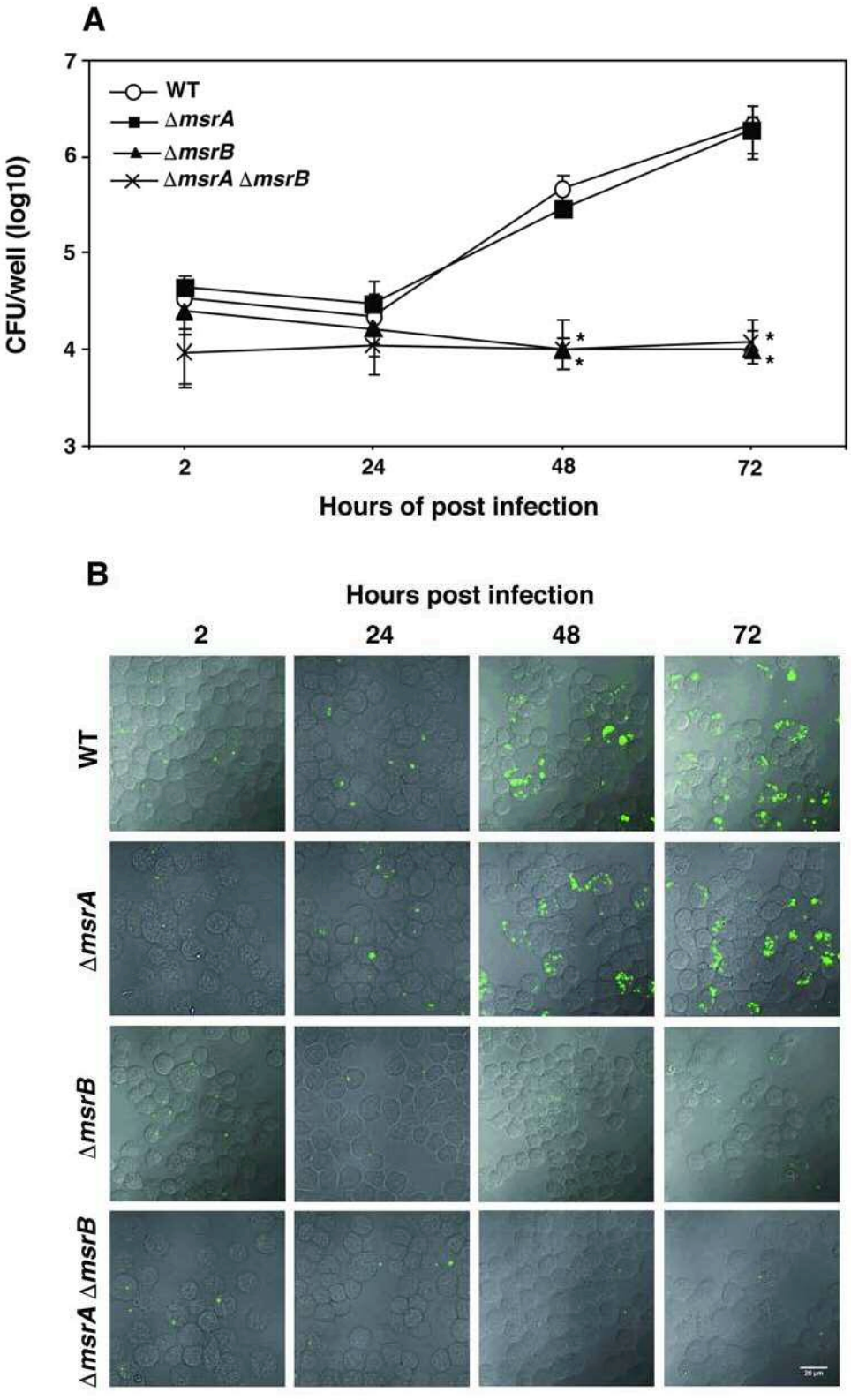

A
WT
ΔmsrA
ΔmsrB
ΔmsrA ΔmsrB
OD 595
Time (hour)
B
H2O2
C
t-butyl hydroperoxide

Figure 1. Bacterial growth *in vitro* with and without oxidative stress condition. (A) Growth curve of *Francisella tularensis* subsp. *holarctica* LVS and *msr* mutants in BHIc broth. Growth curve of wild-type *Francisella tularensis* subsp. *holarctica* LVS and *msr* mutants in BHIc broth containing 1.8 mM H_2O_2 (B) or 0.25 mM t-butyl hydroperoxide (C). Values are expressed as mean ± SD of three independent experiments. Statistically significant differences compared with the wild-type strain are indicated by asterisks (*P < 0.05).


A
7
6
5
4
3
CFU/well (log10)
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WT
ΔmsrA
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ΔmsrA ΔmsrB

Figure 2. Intracellular growth characteristics of bacteria. (A) J774 mouse macrophages were infected with wild-type *Francisella tularensis* subsp. *holarctica* LVS (WT), *msrA* mutant (Δ*msrA*), *msrB* mutant (Δ*msrB*), or *msrA* and *msrB* double mutant (Δ*msrA* Δ*msrB*) at an MOI of 1. Intracellular replication of each strain was measured 2, 24, 48, and 72 h after infection. Data points and error bars indicate the mean CFUs of representative three independent experiments and their SDs. Statistically significant differences compared with the wild-type strain are indicated by asterisks (*$P < 0.05$). (B) Intracellular growth pattern of GFP-expressed bacteria of the same strain in J774 at an MOI of 1. Infected cells were analyzed using confocal microscopy 2, 24, 48, and 72 h post-infection. Scale bar represents 20 μm.

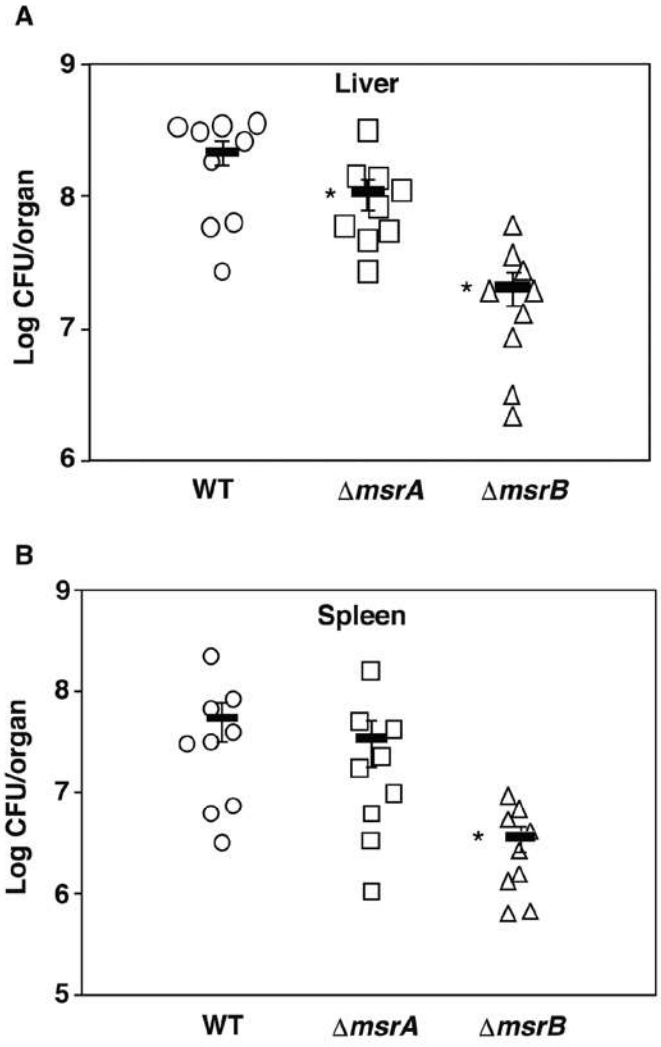

Figure 3. Bacterial growth in mice organs. Groups of three mice per assay were used. The experiment was performed three times with three mice per group. Mice were intraperitoneally infected with approximately 1×10^7 cells of each strain in 0.2 mL of PBS. CFUs were measured in the liver (A) and spleen (B) 72 h after infection. Data are presented as mean ± SEM of nine mice per group. Statistically significant differences compared with the wild-type strain are indicated by asterisks (*$P < 0.05$).

CHAPTER 2

Silkworm model for *Francisella novicida* infection

INTRODUCTION

The identification and evaluation of novel virulence factors using an animal infection model is one of the tools used to study the pathogenesis of infectious diseases. Tularemia, a highly infectious disease in humans and animals, is caused by *Francisella tularensis*, a gram-negative, facultative intracellular bacteria (Decors *et al.*). Disease severity depends on the infectious dose, route of infection, and involved strain. Because of high infectivity through aerosol transmission, it can act as a potential biological weapon; as few as 10 *F. tularensis* cells can cause disease in humans (Saslaw *et al.* 1961; DT *et al.* 2001). The bacterium is mainly found in the Northern Hemisphere, but it has also been reported in Australia and Thailand (Leelaporn *et al.* 2008; Siddaramappa *et al.* 2011). Among the different subspecies, only *F. tularensis* subsp. *tularensis* (Type A) and *F. tularensis* subsp. *holarctica* (Type B) are pathogenic to humans (Sandstrom *et al.* 1992). *F. tularensis* subsp. *novicida* (referred to here as *F. novicida*) shares significant homology with *F. tularensis* subsp. *tularensis*, is virulent in mice but non-pathogenic to immunocompetent humans, and serves as a potential surrogate organism (Kingry and Petersen 2014). Tularemia predominantly occurs in

rodents, voles, hares, and rabbits, and the causative agent has been isolated from a wide variety of sources, such as environmental water, mud, and mosquito larvae in endemic areas (Petersen, Mead and Schriefer 2009; Carvalho *et al.* 2014). The disease is transmitted by ticks, mosquitoes, direct contact with sick animals, contaminated food and water, or aerosols (Carvalho *et al.* 2014). A wide range of arthropod vectors play a crucial role in environmental persistence and disease transmission (Petersen, Mead and Schriefer 2009).

In general, mammalian models are used for the identification and evaluation of virulence factors, but the use of large numbers of mammals for infection experiments raises the question of ethics and also whether the costs warrant the use of animals other than mammals for large-scale experiments. Mammals, especially mice, have been used as infection models for *F. tularensis* subsp. *holarctica* live vaccine strain (LVS) or *F. novicida* (Elkins, Cowley and Bosio 2007; Lyons and Wu 2007). Non-mammalian models, such as the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and the zebrafish *Danio rerio* have already proven to be useful for understanding the disease process (O'Callaghan and Vergunst 2010). Insects combat

pathogens through innate immunity that shares common properties with innate immunity in mammals (Babayan and Schneider 2012). Therefore, they are a useful tool for studying host–pathogen interactions. Recently, arthropod infection models, such as wax moth (*Galleria mellonella*), fruit fly (*D. melanogaster*), cockroach (*Blaptica dubia*), and zebrafish (*D. rerio*), have been employed for understanding the host–pathogen interaction of *Francisella* (Aperis *et al.* 2007; Vonkavaara *et al.* 2008; Vojtech *et al.* 2009; Åhlund *et al.* 2010; Moule, Monack and Schneider 2010; Eklund *et al.* 2016). Among these infection models, *F. tularensis* subsp. *holarctica* LVS–*G. mellonella* has been shown to overcome some of the drawbacks of using non-mammalian infection models, such as the requirement of wax moth rearing facilities to be at 37°C and administration of the optimum infection dose.

Silkworm (*Bombyx mori*) larvae have been reported as an infection and therapeutic model for a large variety of pathogens, such as bacteria, viruses, and fungi (Kaito *et al.* 2002; Hamamoto *et al.* 2004, 2014; Castillo *et al.* 2016; Jiang *et al.* 2016; Kaito 2016; Uchida *et al.* 2016). In previous study, we reported a symbiotic relationship between silkworms and LVS (LVS does not kill silkworm) and its importance in environmental

persistence (Suzuki *et al.* 2016). As silkworms have been domestically cultivated for the past 5,000 years to produce silk, all technical information is available for rearing them at room temperature (25°C). In addition, silkworms can survive at 37°C, the optimum body temperature for humans and most pathogenic bacteria, and can therefore be used for infection experiments (Kaito 2011). The establishment of silkworm genome database provides scientists with valuable information for studying host–pathogen interaction. Silkworm rearing is economical and presents no ethical issues. Fertilized eggs can be stored for a year, and silkworms can be fed on an artificial diet throughout the year to obtain fifth instar larvae (the appropriate body size for infection), making them a suitable candidate as an infection model.

In this study, we examined silkworm–*F. novicida* interaction to develop a non-mammalian infection model for further study on *F. novicida*. We also identified a virulence factor of *F. novicida* that can kill silkworms.

MATERIALS AND METHODS

Bacterial strains and culture conditions

F. novicida U112 was obtained from the Pathogenic Microorganism Genetic Resource Stock Center, Gifu University. *F. novicida* was cultured aerobically at 37°C in brain heart infusion broth (BHIc; BD, Franklin Lakes, NJ, USA) supplemented with cysteine (Gann *et al.* 2010) or Brucella broth (BD) containing 1.5% agar (Wako Laboratory Chemicals). Bacterial strains were maintained as glycerol stock and stored at −80°C.

Construction of *F. novicida dotU* mutant

The *dotU* mutant of *F. novicida* (Δ*dotU*) was generated by group II intron insertion using the TargeTron® Gene Knockout System (Sigma-Aldrich, St. Louis, MO, USA) modified for *Francisella* species (Rodriguez *et al.* 2008), as described previously (Uda *et al.* 2014). pKEK-DotU was constructed using primers dotU-IBSXhoI, dotU-EBS1d, and dotU-EBS2. Transformation of 2 μg of pKEK-DotU was performed, and the cells were precultured in a chemically defined medium (CDM) at 30°C for 6 h. The bacterial cells were collected and cultured on BHIc plates containing 50 μg/ml of kanamycin at

30°C. Mutagenesis was confirmed using PCR to detect the 915-bp insertion. To remove the plasmids, mutants were further cultured on BHIc plates without antibiotics at 37°C. Primers used for construction of *dotU* mutant are shown in Table 1.

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

The *gfp* gene with the Shine–Dalgarno sequence was cloned from pGreenTIR (Miller and Lindow 1997) into pNVU1 to obtain pNVU-GFP. pNVU1 was linearized by SmaI. The *gfp* gene was amplified using the primers pNVU-GFP.FOR and pNVU-GFP.REV (Table 1). Ligation was performed using In-Fusion HD Cloning Kit (Takara Bio, Otsu, Japan). The *gfp* gene with the *bfr* promoter of pNVU-GFP was cloned into pOM5 (Pomerantsev, Obuchi and Ohara 2001) to obtain pOM5-GFP. pOM5 was linearized using the primers pOM5-vector.FOR and pOM5-vector.REV (Table 1). The *gfp* gene with *bfr* promoter was amplified using the primers pOM5-bfr.FOR and pOM5-GFP.REV (Table 1). Ligation was performed using In-Fusion HD Cloning Kit. Plasmids cryotransformed into *F. novicida* cells (Pavlov *et al.* 1996).

Complementation of avirulent *F. novicida dotU* mutant strain

The DotU expression vector pOM5-DotU was constructed by replacing the GFP gene of pOM5-GFP with the dotU gene. pOM5-GFP was linearized using the primers pOM5-DotU-vector.FOR and pOM5-DotU-vector.REV (Table 1). The *dotU* gene was amplified using the primers pOM5-DotU.FOR and pOM5-DotU.REV (Table 1). Ligation was performed using In-Fusion HD Cloning Kit. Plasmids were cryotransformed into *F. novicida* cells (Pavlov *et al.* 1996). Briefly, the bacterial cells were suspended in transfer buffer [0.2 M $MgSO_4$, 0.1 M Tris acetate (pH 7.5)] with 1 μg of plasmid. They were frozen in liquid nitrogen and thawed at room temperature, followed by culture in CDM. The cells were collected and cultured on BHIc plates containing 2.5 μg/ml of chloramphenicol.

Silkworm infection assay

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). For the silkworm survival

assay, the hemocoels of day 2 fifth instar larvae were inoculated with 50 μL of bacterial suspension of different doses in phosphate-buffered saline (PBS) using a 1-mL syringe equipped with a 30-gauge needle (Terumo Corp., Tokyo, Japan). After inoculation, the silkworms were incubated at room temperature with food. Subsequently, the mortality rate of the infected silkworms was assessed daily for 10 days. Survival assay of silkworm was also investigated using different raising temperature of silkworm. Control groups were injected with PBS or an equal volume of 75°C/30-min heat-killed *F. novicida*. To determine the bacterial load, 50 μL of bacterial suspension containing 1×10^8 CFU/mL in PBS was applied as before. The bacterial load was evaluated at the same time each day for 5 days after infection. To obtain bacterial counts (as CFU/mL) from whole silkworm bodies, the infected larvae were weighed and placed in disposable 15-mL centrifuge tubes before homogenizing using 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. Appropriate dilutions of the suspension samples were spread onto agar plates, and the numbers of colonies were counted. To calculate the bacterial counts

(CFUs), the volumes of the hemolymph and tissues were estimated together (1 g = 1 mL). The bacterial load in different organs was determined by larvae dissection, and the hemolymph, subcutaneous tissue, silk gland, Malpighian tubule, and reproductive organs were collected aseptically in 1.5-ml tubes. After washing thrice with PBS, the organs were chopped into small pieces, weighed, diluted in PBS, and spread on Brucella agar plates.

Fluorescence microscopy

The growth pattern of *F. novicida* strains within silkworm hemocytes was investigated by inoculating day 2 fifth instar larvae with GFP-expressed bacteria. At 1, 14, and 24 h after inoculation, hemolymph was collected and examined using a Fluoview FV100 confocal laser scanning microscope (Olympus, Tokyo, Japan) (Suzuki *et al.* 2016).

***In vivo* nodule formation assay**

In silkworms, melanized nodules mainly precipitate around the posterior end of the dorsal vessel. To check for nodule formation among strains, semi-quantitative assays

were performed as described with minor modification (Suzuki *et al.* 2011). Images were documented from the posterior end of the dorsal vessel under the same conditions using a stereoscopic microscope. The nodulation area was quantified using ImageJ 1.421 software (NIH, USA; http://rsbweb.nih.gov//ij). The relative melanized area was measured as the ratio of each group relative to that of the control.

BmN4 cell maintenance and infection assay

The BmN4 cell line derived from the ovary of *B. mori* was received from Dr. Kusakabe of the Kyushu University, Japan. The cell line was maintained in IPL-41 insect medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with heat inactivated 10% fetal bovine serum at 25°C. The BmN4 cells were plated at 1×10^5 cells/well in 48-well tissue culture plates coated with concanavalin A. Plates were coated by covering the bottom of the wells with 100 mM concanavalin A for 2 h at room temperature, followed by washing once with PBS and then completely air drying the chamber. For the infection assay, BmN4 cells were grown in 48-well tissue culture plates for 48 h before use. Cells were infected with *F. novicida* strains at a multiplicity of infection (MOI) of

10. The plate was centrifuged at 150 ×*g* for 10 min and incubated for 2 h at 25°C. Subsequently, the cells were washed twice with PBS, and extracellular bacteria were killed using gentamicin (100 μg/mL) for 30 min with shaking at 90–100 rpm. Intracellular growth was measured by incubating the cells in fresh medium at 25°C for the designated period. At the time of collection, the cells were washed thrice with PBS and then lysed with 0.1% Triton X-100 in CDM (Chamberlain 1965). CFUs were determined by serial dilution on Brucella agar.

To investigate the intracellular growth in BmN4 cells by fluorescence microscopy, cells (2×10^5 cells/well) were grown for 48 h on 12-mm glass cover slips (Thermo Fisher Scientific Inc., MA, USA) in 24-well tissue culture plates and were infected with different GFP strains at an MOI of 10 for the indicated times. Samples were washed thrice with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature, followed by washing thrice with PBS and then twice with distilled water. Fluorescent images were obtained using a Fluoview FV100 confocal laser scanning microscope (Olympus).

Immunoblotting

Day 2 fifth instar larvae were infected with 50 μL of bacterial suspension containing 1 × 10^8 CFU/mL in PBS. After 1, 24, 48 and 72 h the hemolymph was collected from the caudal horn and centrifuged (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). Immune reactions of anti-cecropin B antibody were visualized using the enhanced chemiluminescence detection system (GE Healthcare Life Science, Little Chalfont, UK) (Suzuki *et al.* 2016).

Statistical analyses

Statistical analyses were performed using one-way ANOVA with the post hoc Tukey–Kramer test. Statistically significant differences between groups were accepted at $P < 0.05$ The survival curves were estimated using the Kaplan–Meier method, and the log-rank test was used to determine significant differences between the wild-type and Δ*dotU*-infected groups ($P < 0.05$).

RESULTS

F. novicida can kill silkworms

To develop an infection model, fifth instar silkworm larvae were infected with *F. novicida* at room temperature (25°C), and the mortality rate was observed. In this study, survival rate of silkworms using different doses of *F. novicida* wild-type strain was investigated. Injection of 5×10^6 CFU/larva caused 50% mortality at day 5 after infection, and no silkworm survived at day 7 (data not shown), whereas 5×10^5 CFU/larva caused 20% and 40% mortality at days 7 and 9, respectively (data not shown). In all cases, heat-killed *F. novicida* and PBS control group showed 100% survivability. I also evaluated the killing of larvae by *F. novicida* at 37°C (the optimum temperature for *F. novicida* growth). Killing efficiency was comparatively faster at 37°C than at 25°C (rearing temperature of silkworm for this experiment). Injection of 5×10^6 CFU/larva caused 100% mortality with overnight incubation at 37°C, whereas the PBS control group silkworms survived for up to 7 days.

DotU is an essential virulence factor for survival of *F. novicida* in silkworm.

I focused on type VI secretion systems of *F. novicida* to identify pathogenic factors in silkworms. DotU, the essential component of the type VI secretion system has been selected for this study. To analyze the characteristics of DotU, *F. novicida* Δ*dotU* was developed using the TargeTron® gene knock out system, and the complemented strain for Δ*dotU* of *F. novicida* was developed using the pPOM5 expression plasmid. After injecting 5×10^6 CFU/larva of wild-type strain and *dotU* complemented strain, 50% of silkworms died (LT 50) at day 5 after infection, and no silkworm survived by day 7(Fig. 1A). On the other hand, Δ*dotU* showed approximately 90% survivability (Fig. 1A) at day 7 after infection, and these surviving silkworms successfully produced a cocoon (the next step of the life cycle of a silkworm). The recorded body weights of the silkworms revealed that Δ*dotU*-infected silkworms had a significantly increased body weight at days 2, 3, and 4 after infection compared with the wild-type strain and DotU-complemented strain (Fig. 1B). These results suggested that DotU of *F. novicida* plays an important role in the lethal pathogenicity for silkworms.

***F. novicida* can grow in different body tissues of silkworms**

The bacterial growth of *F. novicida* wild-type, Δ*dotU*, and DotU-complemented strains in the whole body of silkworms was determined by harvesting bacteria from infected larvae and obtaining bacterial load. Results showed a significantly lower bacterial load of Δ*dotU* than of wild-type and DotU-complemented strains throughout the experiment (Fig. 1C). We also investigated bacterial load in different body tissues of silkworm, such as the hemolymph, subcutaneous tissue, silk gland, Malpighian tubule, and reproductive organs. Approximately 10-fold increase in bacterial counts was observed in the hemolymph and subcutaneous tissue compared with that in other organs (Fig. 2). As with the whole body CFU count assay, the bacterial count of the Δ*dotU* mutant was significantly lower than that of its parental strain in different body tissues of silkworm, whereas the complemented strain recovered its full phenotype similar to the wild-type (Fig. 2). Intracellular growth of *F. novicida* strains in hemocytes was further confirmed by confocal microscopy using GFP-expressed bacteria. The growth pattern of wild-type and Δ*dotU* strains was assessed at 1, 14, and 24 h after infection. The wild-type strain showed extensive intracellular growth compared with that of the Δ*dotU* strain at both 14 and 24 h after infection (Fig. 3). These results suggested *F. novicida* can grow in

different body tissues of silkworms but predominantly in hemocyte and subcutaneous tissues and that DotU is an essential factor for bacterial multiplication.

F. novicida grows efficiently in BmN4 cell line

The intracellular replication of *F. novicida* was analyzed by infecting BmN4 cells, the silkworm ovary derived cell line with *F. novicida* wild-type, Δ*dotU*, and DotU-complemented strain at MOI 10. Intracellular replication was measured at 3, 24, and 48 h after infection using bacterial count. Significantly increased bacterial growth of the wild-type strain compared with that of the Δ*dotU* strain was observed at 24 and 48 h after infection (Fig. 4A). However, the DotU-complemented strain could restore its intracellular growth to that of its parental strain (Fig. 4A). Intracellular replication in BmN4 cells was further confirmed by confocal microscopy using GFP-expressed bacteria. Increased growth of the wild-type strain was observed in BmN4 cells, whereas arrested growth was observed in the Δ*dotU* strain at 24 h after infection (Fig. 4B). These results suggested that DotU plays an effective contributory role in the intracellular replication of *F. novicida* in silkworm cell lines.

***F. novicida* cannot trigger a strong early immune response in silkworms**

Melanization and nodulation are two early protective machinery of silkworm towards adverse condition. To analyze the melanization process, hemolymph was collected from *F. novicida* infected silkworms, and the optical density was recorded. Compared with their heat-killed strains, both *F. novicida* wild-type and Δ*dotU* strains did not induce significant melanization at both 1 and 18 h after infection, respectively (data not shown). For nodulation analysis, the posterior ends of the dorsal vessels were examined under the microscope. In this study, live *F. novicida* and Δ*dotU* strain did not induce significant nodules at 1 and 18 h after infection, whereas their heat-killed strains induced significant nodulation (Fig. 5A, B). Although *F. novicida* does not induce an early strong immune reaction, progressive melanization without remarkable body discoloration was recorded until death (data not shown).

Silkworm antimicrobial peptides (AMPs) are another important host defense system against pathogens that are usually produced a few hours after infection. For this study, analysis of the expression of one peptide, cecropin B of silkworm, reported to be an important insect AMP for *Francisella* (Vonkavaara *et al.* 2013). In my study, gene

expression of cecropin B was analyzed at different infection periods by immunoblotting. No expression of cecropin B was observed at 1 h after infection but expression was confirmed at 12, 24, and 48 h after infection (data not shown). No significant variation was observed in the expression of cecropin B case of Δ*dotU* strain compared with *F. novicida* wild-type strain (cause unknown).

DISCUSSION

Identification of new virulence factor and understanding of gene interaction is necessary for detail pathogenesis study of pathogen. Recently, insect model such as *G mellonella*, *D. melanogaster, B dubia* and *C. elegans* have been used as an infection model for human pathogenic bacteria including *F tularensis* (Thomsen *et al.* 2006; Aperis *et al.* 2007; Vonkavaara *et al.* 2008; Eklund *et al.* 2016). Researchers are turning their attention towards silkworms (*Bombyx mori*) larvae as an infection model now a days in both Gram positive and Gram negative bacteria such as *Staphylococcus* and *Listeria* (Castillo *et al.* 2016; Kaito 2016).

In this study to develop infection model, day 2 fifth instar larvae were infected with *F. novicida* wild-type strain, it killed larvae. Caterpillar death is dependent on the number of pathogens, and in some insects such as *G. mellonella,* it is dependent on the rearing temperature (Aperis *et al.* 2007). In this study, infectious dose and rearing temperature of silkworm greatly influenced the larval mortality. When silk worm was infected with heat killed *Francisella*, it could not kill the silk worm larvae indicating bacterial proliferation inside host body is needed for killing of silk worm. On the other hand,

silkworms infected with *Francisella tularensis* subsp. *holarctica* live vaccine strain (LVS) and *Escherichia coli* have been reported to survive for 7 days (Suzuki *et al.* 2016). LVS persists in the environment in arthropod vectors, whereas environmental existence of *F. novicida* does not depend on arthropod vectors and but on a different type of protective mechanism, such as biofilm (van Hoek 2013). The infection of silkworms by *F. novicida* causes death, whereas that by LVS protects silkworms against infection by other organisms (Suzuki *et al.* 2016).

In next steps, I focused on factors involving killing of silk worm. The type VI secretion system of *Francisella* is reportedly involved in intracellular growth in both mammalian and insect cell lines (Read *et al.* 2008; Bröms *et al.* 2012). For this, *dotU*, the core component of the type VI secretion system (Bröms *et al.* 2012) was disrupted. The DotU deletion mutant failed to kill silk worm, whereas, complemented strain of Δ*dotU* showed virulence characteristics as like as wild-type. When body weight and CFU count was checked from infected silkworm, significantly lower body weight and decreased number of bacteria were counted throughout the experiment period in case of DotU deficient strain compared with wild- type and complemented strain. These results

suggest that DotU is an essential virulence factor for survival of *F. novicida* with in silkworm body.

Bacterial load in different body tissues such as, hemoloymph, subcutaneous tissue, silk gland, malpighian tubule and reproductive organs were checked in different infection time period. About 10 fold increased bacterial number was observed in hemolymph and subcutaneous tissue compared with other organs. In another infection experiment in silkworms, LVS showed similar bacterial load on all 7 days of the experiment but *E. coli* showed reduced bacterial load at every subsequent day after infection (Suzuki *et al.* 2016). As like whole body CFU count assay, Δ*dotU* mutant displayed significantly lower bacterial count compared with its parental strain, whereas, complemented strain recovered its full phenotype as like wild-type. DotU deletion mutant of *Francisella* is incapable of phagosomal escape (Broms et al 2012). To understand the effect of DotU in intracellular multiplication, growth of GFP expressed bacteria was examined in hemocyte of infected silkworm by confocal microscopy. Robust growth of wild-type strain was observed at both 14h and 24h of infection, whereas, Δ*dotU* mutant showed

interrupted growth. This result suggests that DotU is an essential virulence factor of *F. novicida* for intra hemocyte growth and survival in silkworm.

The BmN4 cell line, a *B. mori* ovary-derived cell line, has been used to study viral and intracellular bacterial proliferation for developing antiviral and antibacterial compounds (Orihara *et al.* 2008; Iwanaga *et al.* 2014; Castillo *et al.* 2016). In our study, significant bacterial growth was observed in BmN4 in case of wild-type strain, whereas, arrested bacterial growth was observed in case of DotU deletion mutant at 24h and 48h of post infection. However, DotU complemented strain restored its phenotype. The intracellular growth was confirmed by confocal microscopy. These result of cell line infection assay further emphasizing the role of dotU in silkworm infection.

Melanization and nodulation are the two primary defenses of insects toward pathogenic bacteria (Cerenius and Söderhäll 2004). Infection in silkworms causes the activation of prophenoloxidase, resulting in melanin formation that causes a dark discoloration of the body as a visible phenotype (Kaito *et al.* 2002; Tanaka and Yamakawa 2011). Melanin binds with the pathogens and kills them by depriving them of nutrients and by producing reactive oxygen or nitrogen molecules (Chen and Chen

1995; Tang 2009). These reactions start immediately after infection. Through the nodulation process, hemocytes engulf and kill the invading pathogen (Suzuki *et al.* 2011). In my study melanization and nodulation reactions were not prominent in silkworm by live *F. novicida* infection compared with heat killed *Francisella* unlikely other bacteria such as *Micrococcus luteus* (Suzuki *et al.* 2011). Melanization and nodulation reactions in some bacterial infection such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* leads to dark body discoloration of silkworm (Suzuki *et al.* 2011, 2016). No significant body discoloration of silkworm was observed in this live *F. novicida* infection compared with heat killed bacteria at early stage of infection. However, progressive melanization of hemolymph was observed at later stage of *F. novicida* infection that might be the cause of blackish discoloration of body color of silk worm larvae. No induction variation was observed in case of DotU deletion mutant compared with wild-type. Further investigation is needed regarding the mechanisms of *Francisella* in silkworms that might be involved in the inhibition of early steps of the innate immune response, as observed in LVS(Suzuki *et al.* 2016).

Silkworm has 14 toll isotypes, some of which are expressed several hours after

infection, that transmit signals for the production of AMPs (Cheng *et al.* 2008; Tanaka *et al.* 2008). Expression analysis of one AMP, cecropin B, which is reported to be an essential AMP in other insect against *Francisella* infection (Vonkavaara *et al.* 2013) revealed marked expression from 12 h of post infection but unable to protect silkworm. Similar level of cecropin B expression was observed in case of DotU deletion mutant. The contribution of cecrμμopin B alone might not optimum to protect the bacterial growth within silkworm larvae. Further investigation is necessary to determine the specific contributory AMPs involved in *F. novicida* infection.

This study, established silkworm model for *F. novicida* infection confirming growth of bacteria in different body tissues of silkworm especially within hemocyte and also growth in silkworm ovary derived cell line, BmN4. DotU of *Francisella* has already been proven as a virulence factor in mammals, and it was also found to be an essential virulence factor in silkworm infection model. Therefore, this silkworm infection model is a useful tool for identification and evaluation of new virulence factors of *Francisella novicida*.

TABLES AND FIGURES

Table 1 Primers used in this study

Primer	Sequence	Target
pNVU-GFP.FOR	GAAATTATTGATCCCTGATTAACTTTATAAGGAGGAA	pNVU-GFP
pNVU-GFP.REV	CTTGGTCTGACACCCCTATTTGTATAGTTCATCCATG	
pOM5-bfr.FOR	AGTCTCTTCTCTAGAGATCCATACCCATGATGGT	pOM5-GFP
pOM5-GFP.REV	TGATCTTTTCTACGGCTATTTGTATAGTTCATCCATGCCATGT	
pOM5-vector.FOR	CCGTAGAAAAGATCAAAGGATCTTCTTGAGATC	
pOM5-vector.REV	TCTAGAGAAGAGACTGACAAGCTTTTAAAAGACT	
dotU-IBSXhoI	AAAACTCGAGATAATTATCCTTAATAGACAATTCGGTGCGCCCAGATAGGGTG	pKEK-DotU
dotU-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCAATTCGACTAACTTACCTTTCTTTGT	
dotU-EBS2	TGAACGCAAGTTTCTAATTTCGGTTTCTATCCGATAGAGGAAAGTGTCT	
pOM5-DotU-vector.FOR	CCGTAGAAAAGATCAAAGGATCTTCTTGAGATC	pOM5-DotU
pOM5-DotU-vector.REV	ATGTTTTTCCTCCTTATAAAGTTAATCACTAGGCC	
pOM5-DotU.FOR	AAGGAGGAAAAACATATGAAAGACTTTAAAGAGATAGAAATTATTCTAGATATTATAAAAACT	
pOM5-DotU.REV	TGATCTTTTCTACGGTTACCAGCTTAATAAAATTAGTAAGCTTAAAAGAAACAGTCCT	

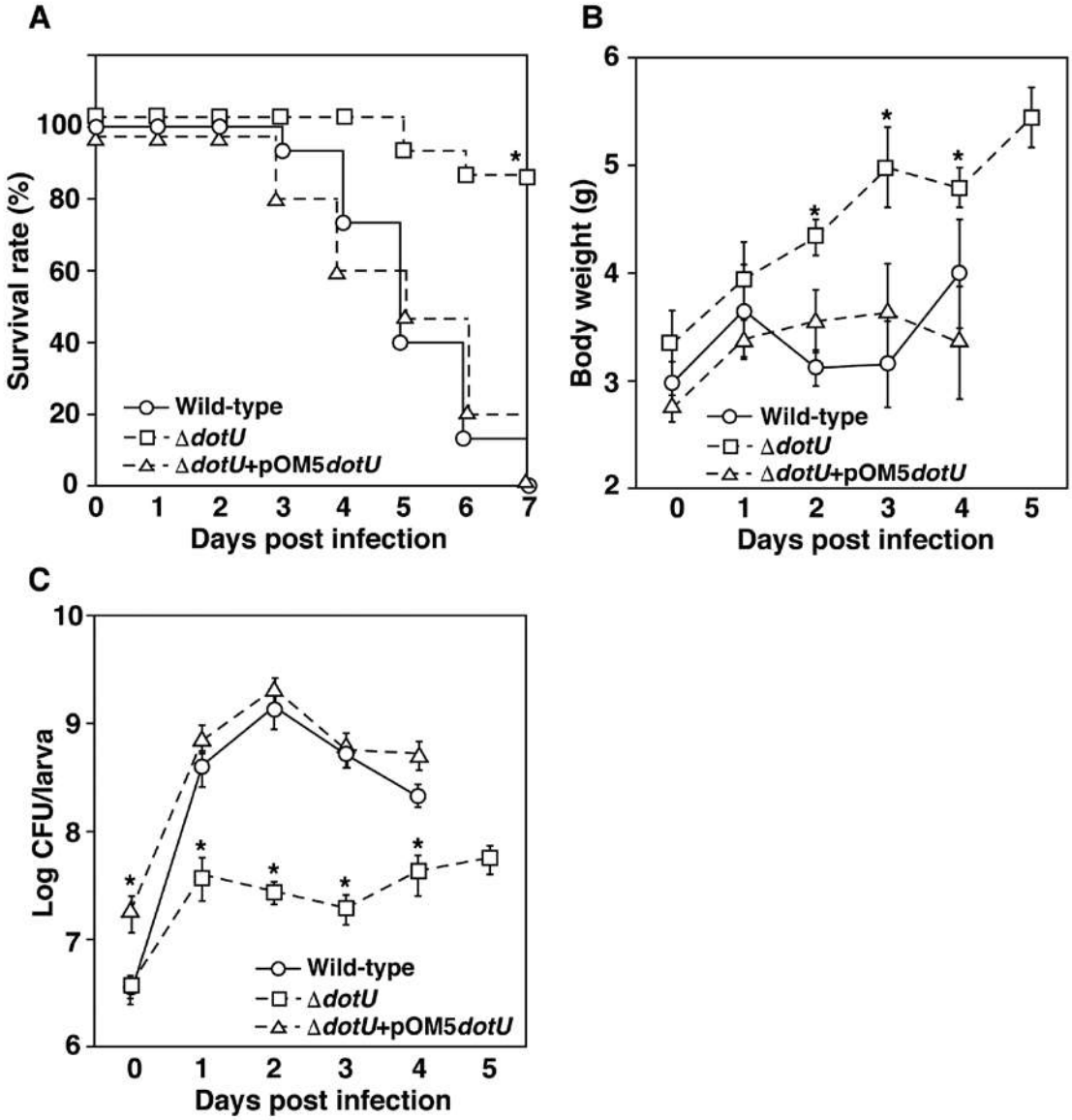
A
Survival rate (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Days post infection
Wild-type
Δ*dotU*
Δ*dotU*+pOM5*dotU*
*
B
Body weight (g)
6
5
4
3
2
0 1 2 3 4 5
Days post infection
Wild-type
Δ*dotU*
Δ*dotU*+pOM5*dotU*
*
C
Log CFU/larva
10
9
8
7
6
0 1 2 3 4 5
Days post infection
Wild-type
Δ*dotU*
Δ*dotU*+pOM5*dotU*
*

Fig. 1. Infection of silkworm with *F. novicida*. (A) Survival rate of silkworms. Fifteen silkworms fifth instars larvae were infected with *F. novicida* wild-type, Δ*dotU*, and DotU-complemented strain (Δ*dotU*+pOM5*dotU*), and the survival rate was calculated at the indicated time point. (B) Silkworms were infected with *F. novicida* wild-type, Δ*dotU*, and Δ*dotU*+pOM5*dotU* strains. Body weight of each strain was measured at the indicated time period. (C) Larvae were infected with indicated bacteria. Bacterial numbers were counted from whole silkworm bodies at designated time points. (A, B & C) 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 $P < 0.05$, and they are indicated by asterisks (*).

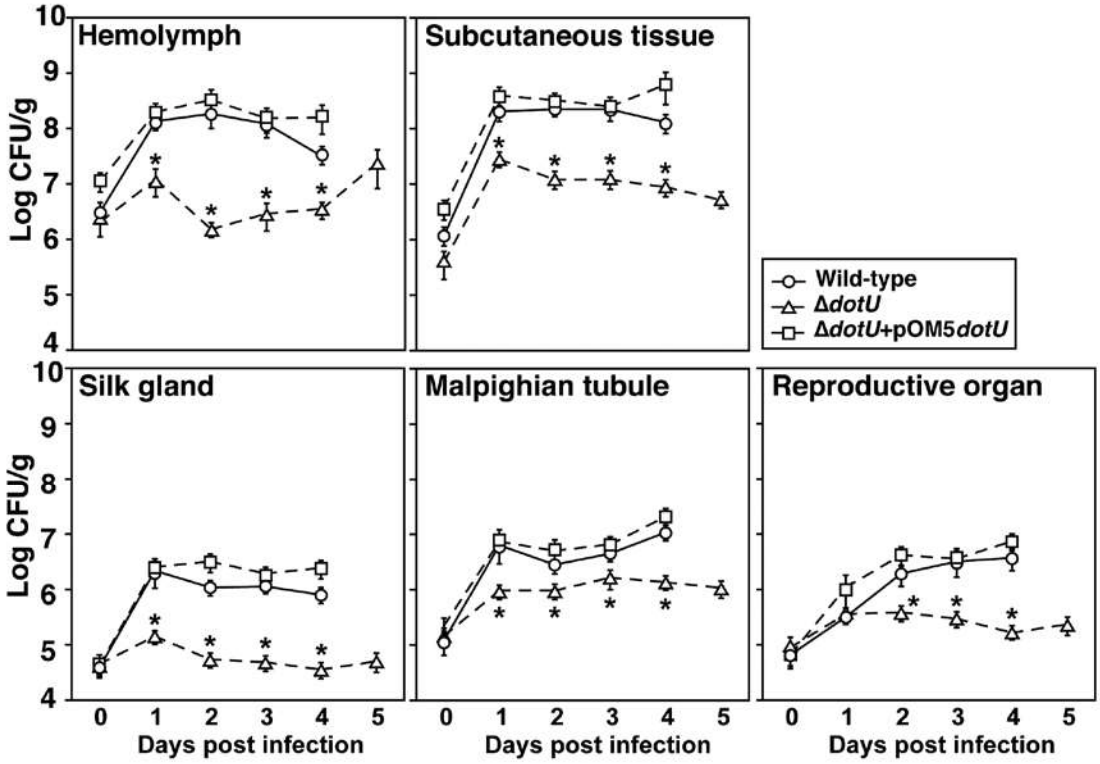

Fig. 2. Growth of *F. novicida* strains in the hemolymph, subcutaneous tissue, silk gland, Malpighian tubule, and reproductive organs of silkworm larvae. Fifth instar silkworm larvae were injected with *F. novicida* wild-type, Δ*dotU*, and Δ*dotU*+pOM5*dotU* strains at 5 $\times 10^6$ CFU/larva. Larvae were dissected aseptically, and the hemolymph, subcutaneous tissue, silk gland, Malpighian tubule, and reproductive organs were collected and homogenized to determine the CFU count at a designated time period after spreading on Brucella agar. 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 $P < 0.05$, and they are indicated by asterisks (*).

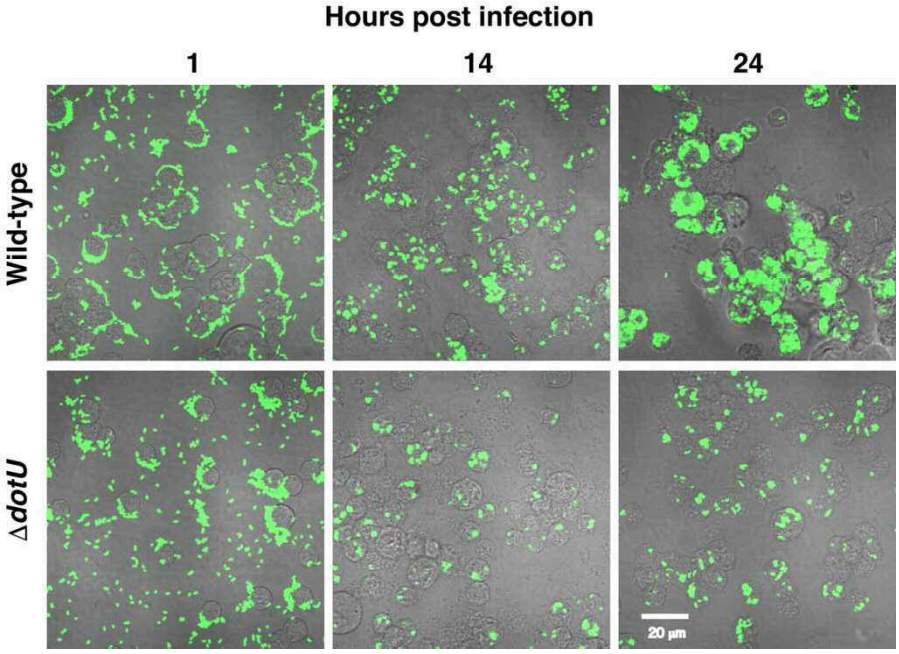

Fig. 3. Intracellular growth of GFP-expressed bacteria within silkworm hemocyte. Fifth instar silkworm larvae were infected with GFP-expressed *F. novicida* wild-type and Δ*dotU* strains at 5×10^6 CFU/ larva. Hemocytes containing *F. novicida* strains (green) were observed by confocal laser scanning microscopy at 1, 14, and 24 h after inoculation. Scale bar represents 20 μm.

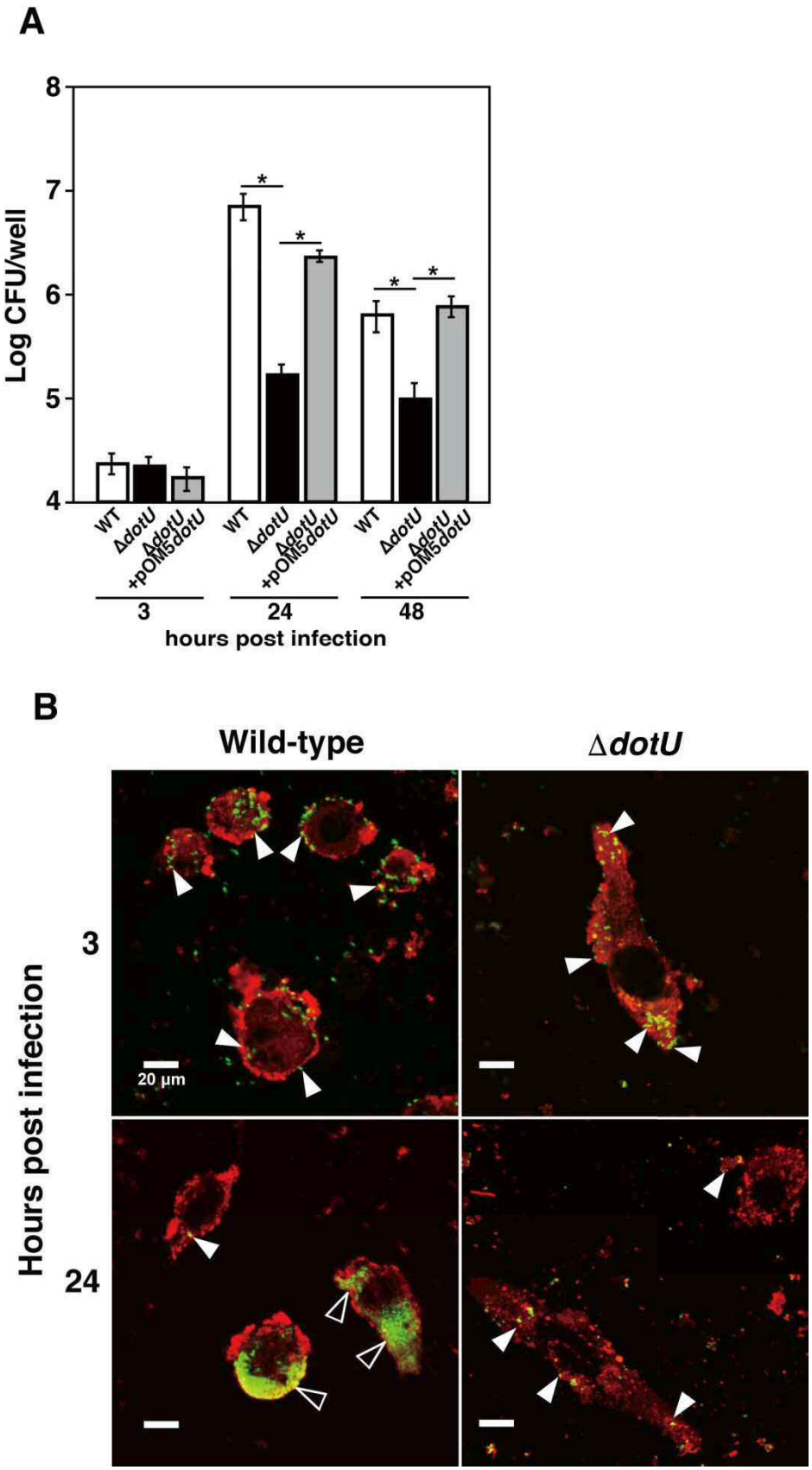
A
Log CFU/well
8
7
6
5
4
*
*
*
*
WT
ΔdotU
ΔdotU +pOM5dotU
3
24
48
hours post infection
B
Wild-type
ΔdotU
Hours post infection
3
24
20 μm

Fig. 4. Intracellular growth of *F. novicida* in BmN4 cells. (A) *F. novicida* replicates intracellularly in BmN4 cells. The BmN4 cell line was infected with *F. novicida* wild-type (WT), Δ*dotU*, and Δ*dotU*+pOM5*dotU* strains at an MOI of 10. Bacterial invasion and intracellular replication by each strain were measured at 3, 24, and 48 h after infection. The data represent averages based on triplicate samples from three identical independent experiments, and the error bars represent the standard deviations. Significant differences were accepted at $P < 0.05$, and they are indicated by asterisks (*). (B) The BmN4 cell line was infected with GFP-expressed *F. novicida* wild-type and Δ*dotU* strains. The wild-type strain BmN4 cells (red) containing *F. novicida* strains (green, white arrow heads) were observed by confocal laser scanning microscopy at 3 and 24 h after inoculation. Explosive bacterial replication is indicated by open arrowheads. Scale bar represents 20 μm. The experiment was replicated thrice independently.

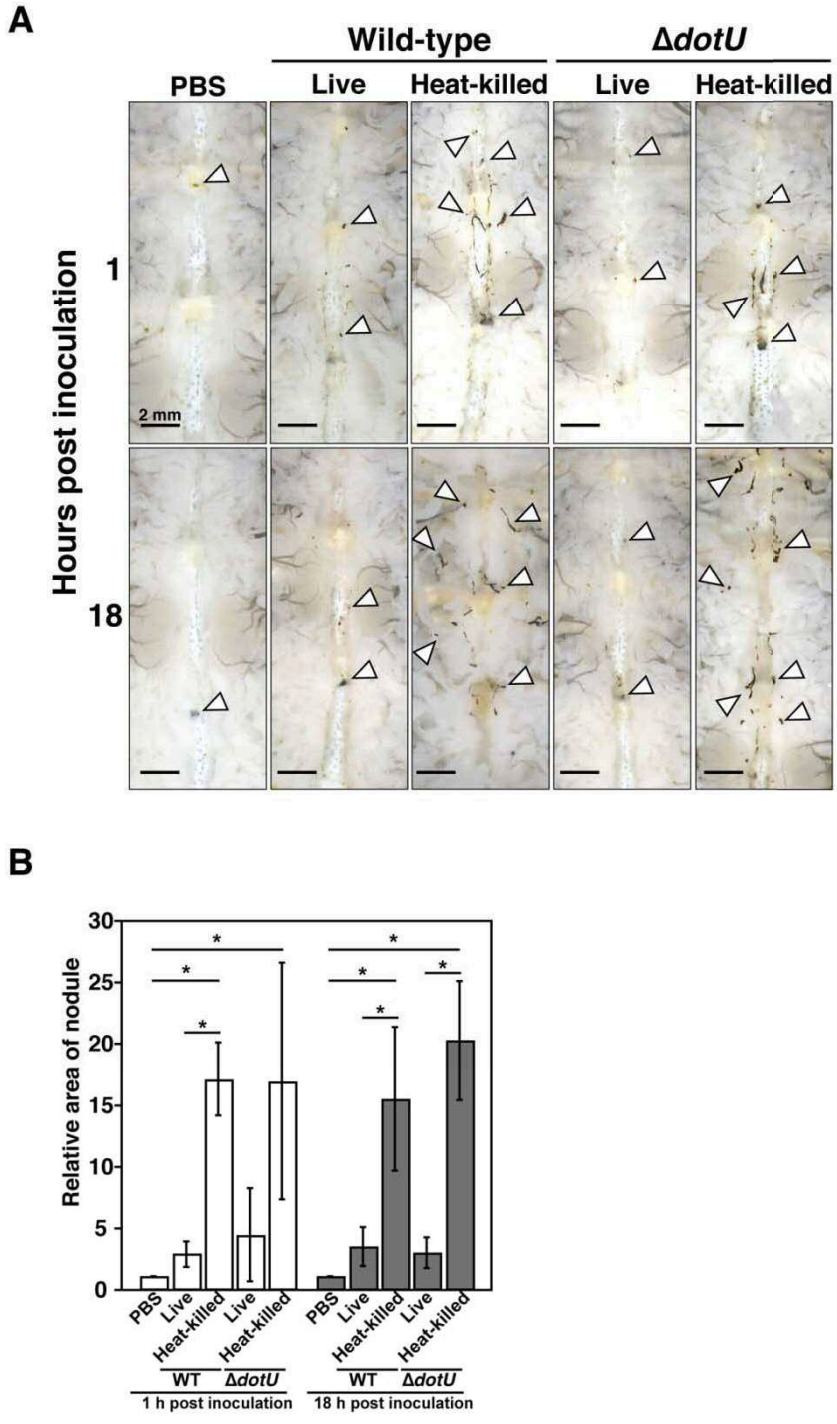
A
Wild-type
ΔdotU
PBS
Live
Heat-killed
Live
Heat-killed
Hours post inoculation
1
18
2 mm
B
Relative area of nodule
30
25
20
15
10
5
0
PBS
Live
Heat-killed
Live
Heat-killed
WT
ΔdotU
1 h post inoculation
18 h post inoculation

Fig. 5. Immune response of silkworm to *F. novicida*. (A) Fifth instar silkworm larvae were infected with *F. novicida* wild-type (WT) and Δ*dotU* strains at 5×10^6 CFU/ larva. Nodule formation around the dorsal vessel was documented at 1 and 18 h after infection. Arrowheads indicate nodule formation. (B) Silkworms were infected with indicated bacteria. The total area of nodule formation was calculated using an area measurement tool. The relative melanized area is shown compared with that in PBS-inoculated control group. The data represent the averages of 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 (*).

CONCLUSION

Study of tularemia has been started since 1911 when it was first used as a bioterrorism agent (Dennis *et al.* 2001), but the essential mechanism and virulence factors enabling *Francisella* inside host has not been completely understood. For successful control of *Francisella* infection in nature, and also to develop a strong protective measure against bioterrorist attack detail pathogenesis study of *Francisella* is necessary. In this study, I focused on methionine sulfoxide reductase system of *Francisella tularensis* LVS strain, and development of silkworm infection model for *Francisella novicida* strain.

In chapter 1, I analyzed the role of two methionine sulfoxide reductase (Msr) of *Francisella tularensis* LVS strain *in vitro* and *in vivo* oxidative stress condition. *F. tularensis* carries *msrA, msrB* and *msrA/B* in different parts of its chromosome. In this chapter, I concentrated on MsrA and MsrB of methionine sulfoxide reductases system. For this, single and double mutants of *msrA* and *msrB* were constructed, and the characteristics of these mutants were investigated. The *msrB* mutant exhibited decreased *in vitro* growth, exogenous oxidative stress resistance, and intracellular growth in macrophages, whereas the *msrA* mutant displayed little difference with wild-type strain.

The double mutant exhibited the same characteristics as the *msrB* mutant. The bacterial count of the *msrB* mutant was significantly lower than that of the wild-type strain in the liver and spleen of mice. The bacterial count of the *msrA* mutant was lower than that of the wild-type strain in the liver, but not in the spleen, of mice. These results suggest that MsrB has an important role in the intracellular replication of *F. tularensis* in macrophages and infection in mice. To depict the complete Msr system of *Francisella*, *in vitro* and *in vivo* analysis of all three *msr* (*msrA, msrB* and *msrA/B*) and their interaction of among each other is warranted.

In chapter 2, I established a silkworm (*Bombyx mori*) infection model for *F. novicida.* The role of arthropods in the environmental persistence and transmission of *Francisella* is not fully discovered. Recently, our laboratory demonstrated an endosymbiotic relationship between LVS and silkworms that helps *Francisella* to survive in the environment (Suzuki *et al.* 2016). Subsequently, I investigated the role *F. novicida* U112 strain in silkworms and found that it could kill silkworms. This result suggested that silkworms do not act as reservoirs for *F. novicida*, unlike that for LVS. In this study, I developed a novel silkworm model for *F. novicida* infection and determined that DotU

is an essential virulence factor involved in silkworm mortality. We also confirmed the growth of *F. novicida* in different silkworm body tissues and the silkworm ovary-derived cell line BmN4. The findings of this study revealed that DotU, a known virulence factor of *Francisella* in mammals, is also an important growth factor in silkworms, suggesting that this *B. mori–F. novicida* infection model is suitable for studying human pathogens. In addition, silkworms have some advantageous characteristics over other insects, such as no biting, no escaping, and their suitability for injecting the target bacterial dose. This *B. mori–F. novicida* infection model provides a new approach as an alternative to using an expensive mammalian model for studying host–pathogen interactions, virulence screening of transposon insertion mutants, identification and evaluation of gene functions to obtain an understanding of bacterial virulence and evaluation of therapeutic effects of medicine.

Altogether, in this study I demonstrated contributory role of msrB in oxidative stress condition both *in vitro* condition and mouse infection model. From the experience of using mouse infection model I found that mouse maintenance is expensive for laboratory use. Considering the cost and issues raised by the animal welfare association,

I decided to develop a cheap and non mammalian infection model for *Francisella* infection. Then I started to develop an infection model on silkworm and got succeeded for *Francisella novicida* infection. This model can be a suitable infection model as an alternative of mammalian infection model for large scale *Francisella novicida* experiment.

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REFERENCES

Åhlund MK, Rydén P, Sjöstedt A *et al.* Directed screen of *Francisella novicida* virulence determinants using *Drosophila melanogaster*. *Infect Immun* 2010;**78**:3118–28.

Alamuri P, Maier RJ. Methionine sulphoxide reductase is an important antioxidant enzyme in the gastric pathogen *Helicobacter pylori*. *Mol Microbiol* 2004;**53**:1397–406.

Aperis G, Burgwyn Fuchs B, Anderson CA *et al. Galleria mellonella* as a model host to study infection by the *Francisella tularensis* live vaccine strain. *Microbes Infect* 2007;**9**:729–34.

Atack JM, Kelly DJ. Contribution of the stereospecific methionine sulphoxide reductases MsrA and MsrB to oxidative and nitrosative stress resistance in the food-borne pathogen *Campylobacter jejuni*. *Microbiology* 2008;**154**:2219–30.

Babayan SA, Schneider DS. Immunity in society: Diverse solutions to common problems. *PLoS Biol* 2012;**10**:10–2.

Bakshi CS, Malik M, Regan K *et al.* Superoxide dismutase B gene (*sodB*)-deficient

mutants of *Francisella tularensis* demonstrate hypersensitivity to oxidative stress and attenuated virulence. *J Bacteriol* 2006;**188**:6443–8.

Binesse J, Lindgren H, Lindgren L *et al.* Roles of reactive oxygen species-degrading enzymes of *Francisella tularensis* Schu S4. *Infect Immun* 2015;**83**:2255–63.

Boschi-Muller S, Olry A, Antoine M *et al.* The enzymology and biochemistry of methionine sulfoxide reductases. *Biochim Biophys Acta - Proteins Proteomics* 2005;**1703**:231–8.

Bröms JE, Meyer L, Sun K *et al.* Unique Substrates Secreted by the Type VI Secretion System of *Francisella tularensis* during Intramacrophage Infection. *PLoS One* 2012;**7**, DOI: 10.1371/journal.pone.0050473.

Carvalho CL, Lopes de Carvalho I, Zé-Zé L *et al.* Tularaemia: A challenging zoonosis. *Comp Immunol Microbiol Infect Dis* 2014;**37**:85–96.

Castillo Y, Suzuki J, Watanabe K *et al.* Effect of Vitamin A on *Listeria monocytogenes* infection in a silkworm model. *PLoS One* 2016;**11**:1–14.

Cerenius L, Söderhäll K. The prophenoloxidase-activating system in invertebrates. *Immunol Rev* 2004;**198**:116–26.

Chamberlain RE. Evaluation of Live Tularemia Vaccine Prepared in a Chemically Defined Medium. *Appl Microbiol* 1965;**13**:232–5.

Chen CC, Chen CS. *Brugia bahangi*: Effects of melanization on the uptake of nutrients by microfilariae *in vitro*. *Exp Parasitol* 1995;**81**:72–8.

Cheng TC, Zhang YL, Liu C *et al.* Identification and analysis of Toll-related genes in the domesticated silkworm, *Bombyx mori*. *Dev Comp Immunol* 2008;**32**:464–75.

Clemens DL, Horwitz MA. Uptake and intracellular fate of *Francisella tularensis* in human macrophages. *Ann N Y Acad Sci* 2007;**1105**:160–86.

Dalle-Donne I, Rossi R, Giustarini D *et al.* Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta* 2003;**329**:23–38.

Decors A, Lesage C, Jourdain E *et al.* Outbreak of tularaemia in brown hares (Lepus europaeus) in France. *Euro Surveill* **16**.

Denkel LA, Horst SA, Rouf SF *et al.* Methionine sulfoxide reductases are essential for virulence of *Salmonella typhimurium*. *PLoS One* 2011;**6**, DOI: 10.1371/journal.pone.0026974.

Denkel LA, Rhen M, Bange F-C. Biotin sulfoxide reductase contributes to oxidative

stress tolerance and virulence in *Salmonella enterica* serovar *typhimurium*. *Microbiology* 2013;**159**:1447–58.

Dennis DT, Inglesby T V., Henderson DA *et al.* Tularemia as a Biological Weapon. *JAMA* 2001;**285**:2763.

Dieppedale J, Sobral D, Dupuis M *et al.* Identification of a putative chaperone involved in stress resistance and virulence in *Francisella tularensis*. *Infect Immun* 2011;**79**:1428–39.

Douglas T, Daniel DS, Parida BK *et al.* Methionine Sulfoxide Reductase A (MsrA) Deficiency Affects the Survival of *Mycobacterium smegmatis* within Macrophages. *J Bacteriol* 2004;**186**:3590–8.

DT D, TV I, DA H *et al.* Tularemia as a biological weapon: Medical and public health management. *JAMA* 2001;**285**:2763–73.

Eklund BE, Gilson T, Mahdi O *et al.* The orange spotted cockroach (*Blaptica dubia*, Serville 1839) is a permissive experimental host for *Francisella tularensis*. *DoiOrg* 2016:1–33.

Elkins KL, Cowley SC, Bosio CM. Innate and adaptive immunity to *Francisella*. *Ann N*

Y Acad Sci 2007;**1105**:284–324.

Evans ME, Gregory DW, Schaffner W *et al.* Tularemia: a 30-year experience with 88 cases. *Medicine (Baltimore)* 1985; **64**;251-69.

Ezraty B, Aussel L, Barras F. Methionine sulfoxide reductases in prokaryotes. *Biochim Biophys Acta - Proteins Proteomics* 2005;**1703**:221–9.

Ezraty B, Bos J, Barras F *et al.* Methionine sulfoxide reduction and assimilation in *Escherichia coli*: new role for the biotin sulfoxide reductase BisC. *J Bacteriol* 2005;**187**:231–7.

Guina T, Radulovic D, Bahrami AJ *et al.* MglA regulates *Francisella tularensis* subsp. *novicida* (*Francisella novicida*) response to starvation and oxidative stress. *J Bacteriol* 2007;**189**:6580–6.

Hamamoto H, Kurokawa K, Kaito C *et al.* Quantitative evaluation of the therapeutic effects of antibiotics using silkworms infected with human pathogenic microorganisms. *Antimicrob Agents Chemother* 2004;**48**:774–9.

Hamamoto H, Urai M, Ishii K *et al.* Lysocin E is a new antibiotic that targets menaquinone in the bacterial membrane. *Nat Chem Biol* 2014;**11**:127–33.

van Hoek ML. Biofilms An advancement in our understanding of *Francisella* species. *Virulence* 2013;**4**:833–46.

Iwanaga M, Tsukui K, Uchiyama K *et al.* Expression of recombinant proteins by BEVS in a macula-like virus-free silkworm cell line. *J Invertebr Pathol* 2014;**123**:34–7.

Jiang L, Peng Z, Guo Y *et al.* Transcriptome analysis of interactions between silkworm and cytoplasmic polyhedrosis virus. *Sci Rep* 2016;**6**:1–10.

Kaito C. Isolation of mammalian pathogenic bacteria using silkworms. *Drug Discov Ther* 2011;**5**:66–70.

Kaito C. Understanding of bacterial virulence using the silkworm infection model. *Drug Discov Ther* 2016;**10**:30–3.

Kaito C, Akimitsu N, Watanabe H *et al.* Silkworm larvae as an animal model of bacterial infection pathogenic to humans. *Microb Pathog* 2002;**32**:183–90.

Kingry LC, Petersen JM. Comparative review of *Francisella tularensis* and *Francisella novicida*. *Front Cell Infect Microbiol* 2014;**4**:1–12.

Lee WL, Gold B, Darby C *et al. Mycobacterium tuberculosis* expresses methionine sulphoxide reductases A and B that protect from killing by nitrite and hypochlorite.

Mol Microbiol 2009;**71**:583–93.

Leelaporn A, Yongyod S, Limsrivanichakorn S *et al.* Emergence of *Francisella novicida* bacteremia, Thailand. *Emerg Infect Dis* 2008;**14**:1935–7.

Lenco J, Pavkova I, Hubalek M *et al.* Insights into the oxidative stress response in *Francisella tularensis* LVS and its mutant Δ*iglC1* + 2 by proteomics analysis. *FEMS Microbiol Lett* 2005;**246**:47–54.

Lindgren H, Shen H, Zingmark C *et al.* Resistance of *Francisella tularensis* strains against reactive nitrogen and oxygen species with special reference to the role of KatG. *Infect Immun* 2007;**75**:1303–9.

Lofgren S, Tarnvik A, Thore M *et al.* A wild and attenuated strain of *Francisella tularensis* differ in susceptibility to hypochlorous acid: A possible explanation of their different handling by polymorphonuclear leukocytes. *Infect Immun* 1984;**43**:730–4.

Lyons CR, Wu TH. Animal models of *Francisella tularensis* infection. *Ann N Y Acad Sci* 2007;**1105**:238–65.

Mc Gann P, Rozak DA, Nikolich MP *et al.* A novel brain heart infusion broth supports

the study of common *Francisella tularensis* serotypes. *J Microbiol Methods* 2010;**80**:164–71.

McCaffrey RL, Schwartz JT, Lindemann SR *et al.* Multiple mechanisms of NADPH oxidase inhibition by type A and type B *Francisella tularensis*. *J Leukoc Biol* 2010;**88**:791–805.

Meibom KL, Charbit A. The unraveling panoply of *Francisella tularensis* virulence attributes. *Curr Opin Microbiol* 2010;**13**:11–7.

Melillo AA, Mahawar M, Sellati TJ *et al.* Identification of *Francisella tularensis* live vaccine strain CuZn superoxide dismutase as critical for resistance to extracellularly generated reactive oxygen species. *J Bacteriol* 2009;**191**:6447–56.

Miller WG, Lindow SE. An improved GFP cloning cassette designed for prokaryotic transcriptional fusions. *Gene* 1997;**191**:149–53.

Mörner T and Addison E. Tularemia. In ES Williams and IK Barker, editors, I*nfectious Diseases of Wild Mammals*, chapter 18.Iowa State University Press, Ames, Iowa, USA, 3 edition, 2001. Doi: 10.1002/9780470344880.ch18.

Moskovitz J, Flescher E, Berlett BS *et al.* Overexpression of peptide-methionine

sulfoxide reductase in *Saccharomyces cerevisiae* and human T cells provides them with high resistance to oxidative stress. *Proc Natl Acad Sci* 1998;**95**:14071–5.

Moskovitz J, Rahman MA, Strassman J *et al. Escherichia coli* peptide methionine sulfoxide reductase gene: regulation of expression and role in protecting against oxidative damage. *J Bacteriol* 1995;**177**:502–7.

Moule MG, Monack DM, Schneider DS. Reciprocal analysis of *Francisella novicida* infections of a *Drosophila melanogaster* model reveal host pathogen conflicts mediated by reactive oxygen and imdregulated innate immune response. *PLoS Pathog* 2010;**6**:73–4.

O'Callaghan D, Vergunst A. Non-mammalian animal models to study infectious disease: worms or fly fishing? *Curr Opin Microbiol* 2010;**13**:79–85.

Orihara Y, Hamamoto H, Kasuga H *et al.* A silkworm-baculovirus model for assessing the therapeutic effects of antiviral compounds: Characterization and application to the isolation of antivirals from traditional medicines. *J Gen Virol* 2008;**89**:188–94.

Pavlov VM, Mokrievich AN, Volkovoy K *et al.* Cryptic plasmid pFNL10 from *Francisella novicida* -like F6168: The base of plasmid vectors for *Francisella*

tularensis. *FEMS Immunol Med Microbiol* 1996;**13**:253–6.

Petersen JM, Mead PS, Schriefer ME. *Francisella tularensis*: an arthropod-borne pathogen. *Vet Res* 2009;**40**:7.

Pomerantsev AP, Obuchi M, Ohara Y. Nucleotide Sequence, Structural Organization, and Functional Characterization of the Small Recombinant Plasmid pOM1 That Is Specific for *Francisella tularensis*. *Plasmid* 2001;**46**:86–94.

Read A, Vogl SJ, Hueffer K *et al. Francisella* Genes Required for Replication in Mosquito Cells. *J Med Entomol* 2008;**45**:1108–16.

Rodriguez SA, Yu JJ, Davis G *et al.* Targeted inactivation of *Francisella tularensis* genes by group II introns. *Appl Environ Microbiol* 2008;**74**:2619–26.

Sandstrom G, Sjostedt A, Forsman M *et al.* Characterization and classification of strains of *Francisella tularensis* isolated in the central Asian focus of the Soviet Union and in Japan. *J Clin Microbiol* 1992;**30**:172–5.

Sasindran SJ, Saikolappan S, Dhandayuthapani S. Methionine sulfoxide reductases and virulence of bacterial pathogens. *Future Microbiol* 2007;**2**:619–30.

Saslaw S, Eigelsbach HT, Wilson HE *et al.* Tularemia vaccine study. I. Intracutaneous

challenge. *Arch Intern Med* 1961;**107**:689–701.

Siddaramappa S, Challacombe JF, Petersen JM *et al.* common acestry and novel genetic traits of *Francisella novicida*-like isolates from North America and Australia as revealed by comparative genomic analyses. *Appl Environ Microbiol* 2011;**77**:5110–22.

Singh VK, Moskovitz J, Wilkinson BJ *et al.* Molecular characterization of a chromosomal locus in *Staphylococcus aureus* that contributes to oxidative defence and is highly induced by the cell-wall-active antibiotic oxacillin. *Microbiology* 2001;**147**:3037–45.

Singh VK, Vaish M, Johansson TR *et al.* Significance of four methionine sulfoxide reductases in *Staphylococcus aureus*. *PLoS One* 2015;**10**:1–20.

Sjödin A, Svensson K, Öhrman C *et al.* Genome characterisation of the genus *Francisella* reveals insight into similar evolutionary paths in pathogens of mammals and fish. *BMC Genomics* 2012;**13**:268.

Suzuki A, Yoshizawa Y, Tanaka S *et al.* Extra- and intracellular signaling pathways regulating nodule formation by hemocytes of the silkworm, *Bombyx mori*

(Lepidoptera : Bombicidae). 2011;**56**:49–56.

Suzuki J, Uda A, Watanabe K *et al.* Symbiosis with *Francisella tularensis* provides resistance to pathogens in the silkworm. *Sci Rep* 2016;**6**:31476.

Tanaka H, Ishibashi J, Fujita K *et al.* A genome-wide analysis of genes and gene families involved in innate immunity of *Bombyx mori*. *Insect Biochem Mol Biol* 2008;**38**:1087–110.

Tanaka H, Yamakawa M. Regulation of the innate immune responses in the silkworm, *Bombyx mori*. *Invertebr Surviv J* 2011;**8**:59–69.

Tang H. Regulation and function of the melanization reaction in *Drosophila*. *Fly (Austin)* 2009;**3**:105–11.

Thomsen LE, Slutz SS, Tan M *et al. Caenorhabditis elegans* Is a Model Host for *Listeria monocytogenes*. 2006;**72**:1700–1.

Uchida R, Namiguchi S, Ishijima H *et al.* Therapeutic effects of three trichothecenes in the silkworm infection assay with *Candida albicans*. *Drug Discov Ther* 2016;**10**:44–8.

Uda A, Sekizuka T, Tanabayashi K *et al.* Role of pathogenicity determinant protein C

(PdpC) in determining the virulence of the *Francisella tularensis* subspecies *tularensis* SCHU. *PLoS One* 2014;**9**:1–13.

Vojtech LN, Sanders GE, Conway C *et al.* Host immune response and acute disease in a zebrafish model of *Francisella* pathogenesis. *Infect Immun* 2009;**77**:914–25.

Vonkavaara M, Pavel STI, Hölzl K *et al. Francisella* is sensitive to insect antimicrobial peptides. *J Innate Immun* 2013;**5**:50–9.

Vonkavaara M, Telepnev M V., Rydén P *et al. Drosophila melanogaster* as a model for elucidating the pathogenicity of *Francisella tularensis*. *Cell Microbiol* 2008;**10**:1327–38.

Walter J, Chagnaud P, Tannock GW *et al.* A high-molecular-mass surface protein (Lsp) and methionine sulfoxide reductase B (MsrB) contribute to the ecological performance of *Lactobacillus reuteri* in the murine gut. *Appl Environ Microbiol* 2005;**71**:979–86.

Weissbach H, Etienne F, Hoshi T *et al.* Peptide methionine sulfoxide reductase: Structure, mechanism of action, and biological function. *Arch Biochem Biophys* 2002;**397**:172–8.

Weissbach H, Resnick L, Brot N. Methionine sulfoxide reductases: History and cellular role in protecting against oxidative damage. *Biochim Biophys Acta - Proteins Proteomics* 2005;**1703**:203–12.

Zhao C, Hartke A, La Sorda M *et al.* Role of methionine sulfoxide reductases A and B of *Enterococcus faecalis* in oxidative stress and virulence. *Infect Immun* 2010;**78**:3889–97.