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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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 pathogenicity. 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, ocular glandular, 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’-GAAATTATTGATCCCTGATTAACCTTATAAGGAGGAA-3’) and pNVU-GFP-R (5’-CTTGGTCTGACACCCCTATTGATAGCTCATCAT-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 BH1c for 3 h and then selected on BH1c agar plates containing 5 µg/mL chloramphenicol.

*In vitro* growth assay of *F. tularensis* in BH1c broth
Overnight cultures of *Francisella* strains were diluted to an OD$_{595}$ of 0.02 and then incubated under shaking in BHlc 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 BHlc broth. H$_2$O$_2$ (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 ($5 \times 10^4$ cells/well) in 48-well tissue culture dishes at a multiplicity of infection (MOI) of 1. These plates were
centrifuged for 10 min 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 stock.

Bacteria were cultivated at exponential phase and washed with PBS twice. Mice were infected intraperitoneally with approximately $1 \times 10^7$ 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$_2$O$_2$ 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$_2$O$_2$ 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 \textit{msrA} and \textit{msrB} mutants in mice

To examine the role of MsrA and MsrB in \textit{F. tularensis} on bacterial infection in mice, C57BL/6J mice were infected with the wild-type strain, \textit{msrA} mutant, or \textit{msrB} mutant. All infected mice were checked daily, with not exhibiting significant clinical signs such as, alertness, temperature, body weight, etc. The \textit{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 \textit{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$_2$O$_2$ 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$_2$O$_2$ 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

B

C

H₂O₂

t-butyl hydroperoxide

Time (hour)

OD 605
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$_2$O$_2$ (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).
Figure 2. Intracellular growth characteristics of bacteria. (A) J774 mouse macrophages were infected with wild-type *Francisella tularensis* subsp. *holaretica* LVS (WT), *msrA* mutant (Δ*msrA*), *msrB* mutant (Δ*msrB*), or *msrA* and *msrB* double mutant (Δ*msrA ΔmsrB*) 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 \( 1 \times 10^7 \) 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_ (ΔdotU) 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 BHlc plates without antibiotics at 37°C. Primers used for construction of \textit{dotU} mutant are shown in Table 1.

**Establishment of a GFP-expressing \textit{F. novicida} strain**

The \textit{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 \textit{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 \textit{gfp} gene with the \textit{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 \textit{gfp} gene with \textit{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 \textit{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 MgSO₄, 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 $1 \times 10^8$ 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 $1 \times 10^5$ 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 $1 \times 10^8$ CFU/mL in PBS. After 1, 24, 48 and 72 h the hemolymph was collected from the caudal horn and centrifuged ($6000 \times g$, 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 ΔdotU-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 $5 \times 10^6$ CFU/larva caused 50% mortality at day 5 after infection, and no silkworm survived at day 7 (data not shown), whereas $5 \times 10^5$ 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 $5 \times 10^6$ 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 $5 \times 10^6$ 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 ΔdotU 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 ΔdotU 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 ΔdotU 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, hemolymph, 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 \textit{F. novicida} for intra hemocyte growth and survival in silkworm.

The BmN4 cell line, a \textit{B. mori} ovary-derived cell line, has been used to study viral and intracellular bacterial proliferation for developing antiviral and antibacterial compounds (Orihara \textit{et al.} 2008; Iwanaga \textit{et al.} 2014; Castillo \textit{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 \textit{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* unlike 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 cecropin 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

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<th>Primer</th>
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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, $\Delta$dotU, and $\Delta$dotU+pOM5dotU 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.
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+pOM5dotU* 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.
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 \textit{msrB} mutant. The bacterial count of the \textit{msrB} mutant was significantly lower than that of the wild-type strain in the liver and spleen of mice. The bacterial count of the \textit{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 \textit{F. tularensis} in macrophages and infection in mice. To depict the complete Msr system of \textit{Francisella}, \textit{in vitro} and \textit{in vivo} analysis of all three \textit{msr} (\textit{msrA}, \textit{msrB} and \textit{msrA/B}) and their interaction of among each other is warranted.

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


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


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.


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


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


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


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


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


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

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.


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


