Study on the crucial factors related to the pathogenicity and transmission of *Francisella tularensis.*

（野兎病菌の病原性と伝播を決定する重要因子に関する研究）

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Table of contents

General introduction

Zoonotic diseases account for approximately 60% of all human infectious diseases and are responsible for approximately 2.7 million deaths per year in the world (Salyer et al., 2017) Also, it is estimated that the zoonotic diseases are the cause of approximately 75% emerging disease. The SARS-CoV-2 virus, which has caused tremendous damage and sacrifice to human society, has also been suspected to be driven from wildlife [1]. Thus, understanding the causes and mechanisms of zoonotic diseases and preparing for them is an urgent issue imposed on human society. In this research, we aimed to elucidate the transmission and pathogenicity mechanisms of *Francisella tularensis*, a pathogenic bacterium that causes the zoonotic disease tularemia.

F. tularensis is gram-negative, a facultative intracellular bacteria and the causative agent of zoonotic disease tularemia [2]. This bacterium is mainly carried by wild rabbits and rodents, and can be infected to humans by contact with these infected animals [3]. The most important characteristics of *F. tularensis* is its high pathogenicity to humans. There are primarily three factors that determine the high risk of *F. tularensis*: the low infectious dose required, its ease of aerosolization, and its high fatality rate [4]. Although a transmission from person to person is not observed, tularemia is classified as category A bioterrorism agents by Centers for Disease Control and Prevention (CDC) from these features [5]. Category A is the most high-level category containing relatively well-known pathogens such as anthrax (*Bacillus anthracis*) and Ebola hemorrhagic fevers (Ebola virus). As such, *F. tularensis* has potential risks not only as a typical zoonotic disease but also as a biological weapon.

The history of *Francisella* research is long. In Japan, tularemia has been known as an endemic disease for a long time and causative bacteria (*F. tularensis*) were identified

almost simultaneously with its discoveries in the world. *F. tularensis* subsp. *holarctica* has been found mainly in the Tohoku district, and approximately 1,400 cases of tularemia observed in Japan since 1924 [6]. Thus, this bacterium has been known for over 100 years. However, many questions about the detailed mechanisms of its pathogenicity and maintenance in natural environment remain unanswered. This is evidenced by the fact that the absence of any effective vaccines to prevent *Francisella* infection at present. In this Ph.D. theses, we attempted to identify the important factors contributing to the characteristic phenotypes of *Francisella* by transposon mutant strain library screening.

In Chapter I, we focused on the mechanisms by which *Francisella* is maintained in arthropod vectors. It has been reported that arthropods, particularly ticks, act as vectors for transmission of *Francisella* [7]. These facts indicate that tularemia is a zoonotic disease transmitted by arthropod vectors, establishing an infection cycle between wildlife and arthropods in nature. We showed that the *F. holarctica* LVS strain is maintained in the silkworm (*Bombyx mori*) and forms a symbiotic relationship in previous research [8]. On the other hand, interestingly, we also revealed that *F. tularensis* subsp. *novicida* (*F. novicida*), *Francisella* subspecies has a low pathogenicity to humans derived from environmental water, is lethal to silkworms. The differences in pathogenicity toward silkworm between strains can be inferred to reflect variations in *Francisella*'s adaptation to arthropod vectors. Such phenotypes may provide important insights into the maintenance of *Francisella* in the natural environment. Therefore, this chapter mention about the identification of important factor, which supports growth and survival of *Francisella* in arthropod vector. We infected silkworms with more than 750 *F. novicida* mutant strains and conducted the screening of important factors based on the survival of the silkworms. Several genes were identified through screening. Eventually, a gene

deletion mutant strain was constructed for one of these genes to analyze a detailed examination.

In chapter II, we focused on the ability of *Francisella* to suppress/evade the host immune system following intracellular infection in human macrophage cells. Pathogenic intracellular bacteria, such as *Francisella*, *Legionella*, and *Listeria*, exhibit diverse unique strategy to survive and replicate in host immune cells [9]. Immunosuppression is one of these strategies, and it is suggested that the capability to suppress host immune activation, especially innate immunity, by *Francisella* is associated with its high pathogenicity [10]. The factors that determine the immune evasion activity of *Francisella* have been largely unidentified. While the existence of factors that involve in immune evasion and suppression has been reported [11–13], each discovery has been sporadic, and there has been no attempt to elucidate these comprehensive mechanisms. Therefore, comprehensive identification of responsible factors and explaining their mechanisms will provide us significant insights regarding the pathogenicity of *Francisella*. In this chapter, following chapter I, the scale of the mutant strain library was expanded to over 3500 strains to perform a more large-scale screening. *Francisella* is known for its ability to evade the immune system by escaping detection by human Toll-like receptors, which are the major receptor responsible for detecting bacterial components [10]. Therefore, the production of tumor necrosis factor-alpha (TNF-α) cytokine by U937 cells, human-macrophage-like cells differentiated from human monocyte cell line, during *Francisella* infection was used as an indicator in screening. Culture supernatants from U937 cells infected with *F. novicida* transposon mutants were collected, and the production levels of TNF-α were quantified using ELISA to screen for mutant strains that showed increased production.

As a result of screening, we identified multiple genes responsible for immunosuppressive activities and performed further analysis to elucidate the function of one specific gene.

Chapter I

Identification of membrane-bound lytic murein transglycosylase A (MltA) as a growth factor for *Francisella novicida* in a silkworm infection model.

Abstract

Francisella tularensis, the causative agent of tularemia, is transmitted by arthropod vectors within mammalian hosts. The detailed mechanisms contributing to growth and survival of *Francisella* within arthropod remain poorly understood. To identify novel factors supporting growth and survival of *Francisella* within arthropods, a transposon mutant library of *F. tularensis* subsp. *novicida* (*F. novicida*) was screened using an *F. novicida*–silkworm infection model. Among 750 transposon mutants screened, the *mltA*-encoding membrane-bound lytic murein transglycosylase A (MltA) was identified as a novel growth factor of *F. novicida* in silkworms. Silkworms infection with an *mltA* deletion mutant (Δ*mltA*) resulted in a reduction in the number of bacteria and prolonged survival. The Δ*mltA* strain exhibited limited intracellular growth and cytotoxicity in BmN4 silkworm ovary cells. Moreover, the Δ*mltA* strain induced higher expression of the antimicrobial peptide in silkworms compared to the wild-type strain. These results suggest that *F. novicida* MltA contributes to the survival of *F. novicida* in silkworms via immune suppression-related mechanisms. Intracellular growth of the Δ*mltA* strain was also reduced in human monocyte THP-1 cells. These results also suggest the contribution of MltA to pathogenicity in humans and utility of the *F. novicida*–silkworm infection model to explore *Francisella* infection.

Introduction

Francisella tularensis is a facultative intracellular pathogen and the causative agent of tularemia [2]. It shows high infectivity via aerosol transmission; as few as 10 *F. tularensis* bacterial cells can cause disease in humans [14]. As such, *F. tularensis* may be deployed as a potential biological weapon and is currently classified as a category A bioterrorism agent by the US Centers for Disease Control and Prevention (CDC) [15]. Among the different subspecies, only *F. tularensis* subsp. *tularensis* (Type A) and *F. tularensis* subsp. *holarctica* (Type B) are highly pathogenic to humans [16]. *F. tularensis* subsp. *novicida*, referred to here as *F. novicida*, shares significant homology with *F. tularensis* subsp. *tularensis*, as it is virulent in mice but nonpathogenic to immunocompetent humans. As such, *F. novicida* serves as a surrogate organism for experimental studies [17]. Although *F. tularensis* has been identified in a wide range of organisms, including mammals, birds, amphibians, and invertebrates [18], the disease tularemia predominantly occurs in rodents, voles, hares, rabbits, and humans [19]. Interactions between *F. tularensis* and arthropod hosts play major roles in supporting its life cycle [20]. The pathogen is transmitted between animals including humans by arthropod vectors such as deer flies, horse flies, ticks, and mosquitoes [19]. The routes of infection to humans are varied, e.g. inhalation, contact with infection animals or contaminated tissues [2]. Arthropods vectors are also considered as one of main infection routes of *F. tularensis* [19]. However, the detailed mechanisms of the growth and survival of *F. tularensis* in arthropod vectors remain poorly understood.

Mammalian infection models are typically used for the identification and evaluation of virulence factors; however, large-scale experiments using mammals raise the issue of ethics and feasibility. To address this issue, we have developed a non-mammalian infection model using the silkworm, *Bombyx mori*. As silkworms have been domestically

cultivated for the past 5000 years to produce silk, there are many lineages, which are well-defined and maintained, and tools for genetic engineering of these organisms are also readily available. Silkworms survive at 37°C; this is the optimal temperature for humans and one that supports the growth of most pathogens [21]. The body size of silkworm larva (3–85 mm) and the period of the larvae (25 days) facilitate injection of pathogens and experimental repetitions. Therefore, *B. mori* larvae have been used as infection models for experiments focused on a large variety of agents, such as bacteria, viruses, and fungi [22–25]. Recently, non-mammalian infection models, including the wax moth (*Galleria mellonella*), fruit fly (*Drosophila melanogaster*), cockroach (*Blaptica dubia*), and zebrafish (*Danio rerio*), have all been employed as models to promote understanding of host–pathogen interactions associated with *Francisella* [26–30]. Compared with these infection models, the silkworm model has advantages with respect to body size, breeding period, breeding equipment, temperature, cost, and availability of genetic information. We have developed both symbiotic and pathogenic silkworm infection models. Infection with *F. tularensis* subsp. *holarctica* LVS (LVS), the bacteria fail to proliferate and are maintained in the silkworm in a symbiotic relationship [8]. By contrast, *F. novicida* proliferates in silkworms and promotes a lethal infection; these results underscore the pathogenic properties of *F. novicida* in silkworm [31].

We herein carried out a large-scale screening of a *F. novicida* transposon mutant library using the *F. novicida*–silkworm infection model. Membrane-bound lytic murein transglycosylase A (MltA) was identified as a novel factor, which supports growth and survival of *Francisella* in arthropod species.

Materials and Methods

Bacterial strains and culture conditions

F. novicida U112 was obtained from the Pathogenic Microorganism Genetic Resource Stock Center (Gifu University, Japan). *F. novicida* was cultured aerobically at 37 °C in a chemically defined medium (CDM) [32], brain heart infusion broth (Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with cysteine (BHIc) [33], or BHIc containing 1.5% agar (Wako Laboratory Chemicals, Osaka, Japan). All experiments were conducted in compliance with the institutional biosecurity guidelines and were approved by Yamaguchi University.

Cell culture

Human monocytic THP-1 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum at 37 ºC in an atmosphere containing 5% CO_2. Silkworm ovary-derived BmN4 cells were grown in Sf-900 III SFM medium (Thermo Fisher, Waltham, MA) at 27 ºC.

Silkworms

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

Plasmid construction, transformation, and transfection

Table 2 lists the primer sets and templates used to construct the plasmids used in this

study. Polymerase chain reaction (PCR) was performed using KOD-Plus-Neo polymerase (Toyobo, Osaka, Japan), and ligation was performed using an In-Fusion HD Cloning Kit (Takara Bio, Shiga, Japan). Plasmids were used to transform *F. novicida* via electroporation. Specifically, bacterial cells were suspended 0.5 M sucrose with 2 μg of plasmid DNA and were electroporated using a Bio-Rad micropulser (Bio-Rad, Hercules, CA) at 3.0 kV, 10 μF, and 600 Ω with 0.2 cm cuvette; transformants were precultured in CDM medium overnight. To select the transformed bacteria, the pre-incubated bacteria were cultured on BHIc agar plates containing 30 μg/ml kanamycin or 2.5 μg/ml chloramphenicol.

Construction of a transposon mutant library

The transposon mutant library was constructed using the Ez-Tn5 transposon system (Epicentre, Madison, WI) as previously reported [34]. Briefly, the multiple cloning site of pMOD3 was opened by digestion with Hind III and EcoRI, and the kanamycin resistance cassette of pKEK1140 [35] was ligated into these sites to generate pMOD3-FtKm. The transposon moiety of pMOD3-FtKm was amplified by PCR, purified, mixed with transposase according to the instruction manual, and then used to transform *F. novicida* via electroporation. Transformed bacteria were cultured on BHIc plates containing 30 μg/ml kanamycin.

Sequence analysis of transposon mutants

pMOD3 harbors the *E. coli* R6Kγ origin of replication. The genomes of *F. novicida* transposon mutants were purified using a PureLink Genomic DNA Mini Kit (Thermo Fisher) and digested with a combination of restriction enzymes, such as XhoI, BglII,

EcoRI, SalI, NotI, and BamHI. The ends of the digested DNAs were then blunted using a DNA Blunting Kit (Takara Bio) and ligated using Ligation High Ver. 2 (Toyobo). The ligated DNA was used to transform One Shot PIR1 Chemically Competent *E. coli* (Thermo Fisher). The transformed *E. coli* were selected for kanamycin resistance, and the plasmid DNAs were purified. Sequence analysis was performed using the primer described in the instruction manual for the Ez-Tn5 transposon system.

Construction of *F. novicida* mutants

A deletion mutant of *dotU* (Δ*dotU*) was previously constructed [36] through group II intron insertion using a TargeTron Gene Knockout System (Sigma-Aldrich), which was modified for *Francisella* species [35]. A deletion mutant of *mltA* (Δ*mltA*) was generated via homologous recombination using the *Francisella* suicide vector pFRSU [36]. The upstream and downstream regions of *mltA* (1.5 kbp each) were cloned into the BamHI site of pFRSU to generate pFRSU-mltA. The pFRSU-mltA vector (2 μg) was used to transform *F. novicida*; transformants were selected on BHIc plates containing 30 μg/ml kanamycin. Isolated bacteria were cultured in BHIc without antibiotics overnight and then plated on BHIc plates containing 5% sucrose. The deletion of the *mltA* gene was confirmed via PCR.

Green fluorescent protein (GFP)- and MltA-expressing *F. novicida* strains

A GFP-expressing plasmid pOM5-GFP was constructed according to published procedures [36]. The *F. novicida* chromosomal *mltA* gene with its native promoter region (300 bp upstream) was cloned into pOM5 to generate pOM5-MltA. pOM5-MltA and pOM5-GFP were used to transform the wild-type or Δ*mltA* strains of *F. novicida* via

electroporation.

Silkworm infection assay

Hemocoels of day 2 fifth instar larvae were inoculated with 50 µl of bacterial suspension in BHIc medium at 5 × 10^6 colony-forming unit (CFU)/larva 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 free access to food. The mortality rate of the infected silkworms was assessed daily for 9 days. The bacterial load was evaluated at the same time each day for 4 days after infection. To obtain bacterial counts (CFU/larva) from whole silkworm bodies, the infected larvae were placed in disposable 15 ml centrifuge tubes and homogenized in 3 ml phosphate-buffered saline (PBS) using Biomasher SP (Funakoshi Co., Ltd, Tokyo, Japan) followed by centrifugation at 300 × g for 30 s. Appropriate dilutions of the suspension samples were spread onto BHIc agar plates to facilitate colony counts.

Intracellular growth assay

THP-1 cells (4 × 10^5 cells/well) were incubated in a 48-well tissue culture plate with 200 nM phorbol myristate acetate (PMA) for 48 h. Then, *F. novicida* strains were added at a multiplicity of infection (MOI) = 1. Next, plates were centrifuged for 10 min at 300 × *g* and incubated for 1 h at 37°C. The cells were washed three times with RPMI 1640 medium, and extracellular bacteria were killed with gentamicin at 50 µg/ml for 1 h. The cells were then incubated in fresh medium at 37°C for the times indicated. To measure intracellular growth, the cells were washed with PBS and then lysed with 0.1% Triton X-100 in CDM. The number of CFUs was determined on BHIc agar plates via plating serial

dilutions of cultures. Similar methods were used to determine growth in BmN4 cells (3×10^5 cells/well), which were incubated in a 48-well tissue culture plate overnight at 27°C in Sf-900 III SFM medium.

Fluorescence microscopy

THP-1 cells (4×10^5 cells/well) were incubated with 200 nM PMA for 48 h on 12 mm glass coverslips in 24-well tissue culture plates. GFP-expressing *F. novicida* strains were introduced at MOI = 1. Plates were centrifuged for 10 min at 300 × *g* and incubated for 1 h at 37°C. The cells were washed three times with RPMI 1640 medium, and extracellular bacteria were eliminated with gentamicin at 50 μg/ml for 1 h. The cells were then incubated in fresh medium at 37°C for the times indicated. Cells were fixed with 4% paraformaldehyde at room temperature for 30 min. A FluoView FV100 confocal laser scanning microscope (Olympus, Tokyo, Japan) was used to obtain images of the cells. Similarly, BmN4 cells (3×10^5 cells/well) were grown on 12 mm glass coverslips in Sf-900 III SFM in 24-well tissue culture plates and were incubated overnight at 27°C; a similar procedure was performed. To examine the hemocytes, the hemocoels of day 2 fifth instar larvae were inoculated with 50 μl of bacterial suspension at 5×10^6 CFU/larva. At indicated times after inoculation, hemolymph was collected via an incision in the caudal horn and observed using a FluoView FV100 confocal laser scanning microscope.

Trypan blue exclusion assay

BmN4 cells (3×10^5 cells/well) were incubated in a 24-well tissue culture plate overnight and were then infected with *F. novicida* strains at MOI = 1. The plates were then centrifuged for 10 min at 300 × *g* and incubated for 1 h. Subsequently, the cells were

washed three times with Sf-900 III SFM medium, and extracellular bacteria were eliminated with gentamicin at 50 μg/ml for 1 h. Cells were provided with fresh medium and grown for 48 h at 27°C. Cells were then resuspended in an equivalent volume of 0.5% trypan blue solution (Nacalai Tesque, Kyoto, Japan) to facilitate counting of live (trypan blue negative) or dead (trypan blue positive) cells using a hemocytometer. Cell death was determined as the percentage of dead (blue) cells in the grid square.

Lactate dehydrogenase (LDH) release assay

THP-1 cells (4×10^5 cells/well) were incubated in a 48-well tissue culture plate with 200 nM PMA for 48 h. Cells were infected with *F. novicida* strains at MOI = 0.01. The plates were centrifuged for 10 min at 300 × g and incubated for 1 h and then washed three times with RPMI 1640 medium, and extracellular bacteria were eliminated with gentamicin (50 μg/ml for 1 h). Cells were then incubated in fresh medium at 37°C for 48 h. Release of LDH into culture medium was measured using an LDH Cytotoxicity Detection Kit (Takara Bio).

Immunoblotting

Hemocoels of day 2 fifth instar larvae were inoculated with 50 μl of bacterial suspension at OD_{595} = 0.05 in BHIc medium using a 1 ml syringe equipped with a 30-gauge needle. After 6, 12, and 24 h, the hemolymph was collected from the caudal horn. The proteins in 0.5 μl of hemolymph were separated by SDS-PAGE on a 4%–12% Bis-Tris Gel (Thermo Fisher) and were transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated overnight with anti-cecropin B antibody (1:1000; ab27571; Abcam, Cambridge, UK) at 4 °C. The membrane

was then incubated with horseradish peroxidase-conjugated secondary antibody (0.01 μg/ml) at room temperature and was developed using an enhanced chemiluminescence detection system (GE Healthcare Life Science, Little Chalfont, UK).

Statistical analysis

Multiple comparisons using the Tukey–Kramer test were used to evaluate the significance of differences between groups. The Kaplan–Meier method was used to generate survival curves, and the log-rank test was used to evaluate the significance of differences compared with the wild type strain; $P < 0.05$ indicates a significant difference.

Results

Identification of genes required for the growth of *F. novicida* in arthropods.

We previously demonstrated that *F. novicida* can replicate in silkworms and ultimately promote death [31]. To identify novel factors that support growth of *F. novicida* in arthropods, silkworms were infected with a transposon mutant library previously developed [34]; strains with reduced pathogenicity for silkworms were identified. The screening was performed by infecting three silkworms with each transposon mutant of *F. novicida*. Among 750 characterized transposon mutants, we identified 26 mutants that failed to kill at least 1 of the 3 silkworms; these were identified as strains with reduced pathogenicity. To confirm the reduced pathogenicity of these mutants, 10 silkworms were infected with each of these 26 transposon mutants. A Δ*dotU* strain, lacking a gene encoding part of the type VI secretion system, was used as a negative control [8]. Among the 26 mutants, we identified 8 mutants that permitted more than 80% survival; these were identified as strains with minimal pathogenicity in the silkworm infection model

(Figure 1). To identify the genes responsible for the pathogenicity, transposon insertion sites of the mutant strains were evaluated; seven unique genes were identified (Table 1). In this study, we focused on the characteristics and pathogenicity associated with *mltA* (*FTN_1286*), the gene encoding hypothetical membrane-bound lytic transglycosylase MltA.

Characteristics of an *mltA* deletion mutant.

To analyze the effect of MltA in *Francisella* infection, a Δ*mltA* strain of *F. novicida* was constructed via homologous recombination. The known functions of MltA are related to degradation of cell wall peptidoglycan (PG); cell morphology and growth rates of the mutant strains were evaluated. The Δ*mltA* cells all maintained the characteristic rod shape in the early-exponential phase of liquid culture and were otherwise indistinguishable from the wild-type strain (Figure 2A). The growth rates of Δ*mltA* strain and of a complemented (Δ*mltA*/*mltA*) strain in liquid culture medium were slightly slower than those of the wild-type strain and of the transposon mutant at mid-exponential phase; there were no significant differences at late- exponential phase (Figure 2B).

Role of MltA in promoting multiplication and virulence of *F. novicida* in silkworm.

To explore the role of *mltA* in promoting virulence, silkworms were infected with the Δ*mltA* strain. All silkworms infected with the wild-type strain were dead within 7 days; by contrast, 80%–90% of the silkworms infected with the Δ*mltA* or transposon mutant strains remained viable at 9 days after infection (Figure 3A). Complementation (Δ*mltA*/*mltA*) restored virulence; survival of silkworms infected with this strain was comparable to that of the wild-type strain (Figure 3A). The number of bacterial cells in

whole silkworms was also determined. As expected, the wild-type and complemented strains increased in number through 1 to 2 days post infection in whole silkworms; no growth of the Δ*mltA* or transposon mutant strains was detected (Figure 3B). A similar tendency was observed when analyzing the silkworm hemolymph. Specifically, when silkworms were infected with a GFP-expressing wild-type *F. novicida*, the bacterial population detected in the hemolymph increased at 24 to 48 h post infection (Figure 3C). This was not observed in response to infection with GFP-expressing Δ*mltA* strain (Figure 3C). Taken together, these results indicate that MltA is important for growth and virulence of *F. novicida* in silkworms.

Effect of MltA on intracellular growth and cytotoxicity of *F. novicida* in BmN4 cells.

To confirm the role of MltA in promoting intracellular growth of *F. novicida* in silkworm cells, *F. novicida* strains were used to infect to cultures of the silkworm ovary BmN4 cell line (Grace, 1967). The wild-type strain grew intracellularly in BmN4 cells from 2 to 48 h post infection; by contrast, the Δ*mltA* and transposon mutant strains showed comparatively limited growth under these conditions. The complemented strain restored the capacity for intracellular growth (Figure 4A, B). To investigate a role for MltA in promoting cytotoxicity, BmN4 cells were infected with *F. novicida* strains and cell death was evaluated by the trypan blue exclusion assay. Among our results, the Δ*mltA* strain was significantly less cytotoxic than either the wild-type or complemented strains (Figure 4C). These results suggest that MltA is necessary for intracellular growth and is a critical factor underlying the cytotoxicity of *F. novicida* within silkworm cells.

Immune suppression of silkworm promoted by MltA.

F. tularensis suppresses host immune systems of both mammals and arthropods [8,37]. One of the main immune responses of arthropods when confronted with pathogens is the production of antimicrobial peptides (AMPs) (Chen and Lu, 2018). To explore the impact of MltA on the immune responses in silkworms, we examined expression levels of the major AMP, cecropin B, by immunoblotting. We found that the induction of cecropin B expression was accelerated in response to infection with the Δ*mltA* strain. The Δ*mltA*-induced expression of cecropin B was detected as early as 6 h post infection, and no response was observed in response to infection with the wild-type strain at this time point. Cecropin B was detected at 12 h post infection with both the wild-type and Δ*mltA* strains, although expression was stronger in the Δ*mltA*-infected silkworms. At 24 h post infection, expression levels were nearly indistinguishable (Figure 5). These results suggest that *F. novicida* promotes host immune suppression at the early stages of infection via an MltA-dependent mechanism.

Impact of MltA on the pathogenicity of *F. novicida* in human macrophage cell line.

Intracellular growth of *Francisella* in macrophages is closely related to its pathogenicity in humans [38]. To determine whether *mltA* plays an important role in human virulence, cells of the human monocyte cell line, THP-1, were infected with the wild-type and mutant strains of *F. novicida*. The wild-type strain multiplied intracellularly within THP-1 cells at 2 to 48 h post infection (Figure 6A, B). The Δ*mltA* and transposon mutant strains failed to grow in THP-1 cell culture; their numbers decreased during this time period (Figure 6A, B). Complementation restored the capacity for intracellular growth during 2 to 24 h post infection. To explore the role of MltA in promoting cytotoxicity in human macrophages, THP-1 cells were infected with *F. novicida* strains and cytotoxicity was

measured using the LDH release assay. Infection with the Δ*mltA* and transposon mutant strains showed less cytotoxicity than that with the wild-type and complemented strains (Figure 6C). These results suggest that MltA is essential for intracellular growth of *F. novicida* in human macrophages.

Discussion

Francisella is transmitted to mammalian hosts by arthropod vectors [20]; as such, a careful analysis of the unique growth mechanisms in arthropods is important for our understanding of the full life cycle of *Francisella*. In this study, we identified a novel factor, which promotes growth and survival of *F. novicida* in arthropods using the silkworm infection model. We previously developed two distinct silkworm infection models. The first uses LVS. In this infection model, LVS was maintained and the infected silkworms survived, resulting in symbiotic relationship [8]. The second features *F. novicida*; *F. novicida* proliferates within the silkworms and ultimately promotes their death [31]. As bacterial symbiosis and growth within arthropods are considered to be related processes in the *Francisella* life cycle, symbiotic factors that predominate in LVS infection and growth factors associated with *F. novicida* infection may ultimately cooperate to promote survival of *Francisella* in arthropods. We identified herein critical factors underlying bacterial growth in the *F. novicida*–silkworm infection model; specifically, we identified seven distinct genes that were critical for *F. novicida* growth in silkworms by screening a transposon mutant library.

To date, arthropod infection models, such as wax moth (*G. mellonella*) and fruit fly (*D. melanogaster*), have been used to explore features of the *Francisella*–arthropod interaction [26–29]. Several of the aforementioned seven genes (*iglB*, *slt*) identified based on screening using the silkworm infection model include those previously identified in *G. mellonella* or *D. melanogaster* infection models [28,39]. Several of these genes have already been reported to encode pathogenic factors related to *Francisella* infection in humans. Despite the previous screenings, MltA encoded by *mltA* had not been reported until now as a pathogenic factor in human infection or a bacterial growth factor in arthropods.

Although many reports point to bacterial-derived lytic transglycosylases (LTs) as closely associated with pathogenicity [40], information about the function of LTs associated with *Francisella* infection in mammals or arthropods is limited [34,41]. Given these previous findings, we proceeded on the assumption that *mltA* might be involved in proliferation of *F. novicida* in arthropods and that this function might be revealed in our silkworm infection model.

To analyze functions of MltA in *F. novicida*, we generated a Δ*mltA* strain. Infection with the wild-type strain killed silkworms, while that with the Δ*mltA* or associated transposon mutant strains permitted longer-term survival. Intracellular growth of the Δ*mltA* and transposon mutant strains was reduced in lymphocyte and in the silkworm-derived cell line BmN4 when compared with growth of the wild-type and complemented strains. These results indicate that MltA is a critical factor and is required to support intracellular growth in silkworms. In addition, bacterial numbers of Δ*mltA* and transposon mutant strains in BmN4 cells at early stage of infection (2 h post infection) tended to be decreased compared with that of the wild-type and complemented strains, indicating that MltA of *F. novicida* may affect the invasion ability to silkworm cells.

LTs are known to be involved in cell division and membrane stability of other bacteria [40]; as such, we considered the possibility that the Δ*mltA* and/or the transposon mutant strains might exhibit growth defects in the culture medium. Bobie et al. [42] reported that a Δ*mltA* strain of *Neisseria meningitidis* exhibited delayed growth in vitro. In our study, we found that the Δ*mltA* strain showed similar morphology to the wild-type strain but grew at slightly decreased rates in liquid culture medium. Interestingly, the transposon mutant exhibited no growth delay, and *mltA* complementation failed to restore the growth rate. These data suggest that growth delay may be a structural result associated

with the gene deletion. For example, the deletion of the *mltA* gene may have an impact on the expression of neighboring genes. Of note, although growth of Δ*mltA* strain was only slightly delayed in liquid culture, its growth was significantly inhibited in silkworms and in cultures of BmN4 and THP-1 cells. Likewise, while growth of the transposon mutant was similar to that of the wild-type strain in liquid culture, its growth in silkworms, BmN4 cells, and THP-1 cells was markedly reduced. Moreover, despite the slightly delayed growth of the complemented strain when evaluated in liquid culture, complementation restored the capacity for growth in silkworms, BmN4 cells, and THP-1 cells. From these results, we concluded that diminished proliferation of the Δ*mltA* strain in both silkworms and cell lines could not be attributed to factors promoting growth delay in liquid culture.

MltA is a membrane-bound lytic murein transglycosylase, which belongs to the LT enzyme family. LTs show same substrate specificity as the muramidases (lysozymes), and catalyze the cleavage of β-1,4-glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid residues of PG, but resulted in the different formation of product with 1,6-anhydromuramyl residues [43]. LTs are lytic enzymes that target PG and promote appropriate cell division and membrane stability [44]. LTs also create spaces within the PG to facilitate assembly of macromolecular complexes including flagella, pili, and secretion systems larger than the size of PG pores [45]. Specifically, LTs were associated with the activity of the type IV secretion system in both *Brucella abortus* and *Helicobacter pylori* [46,47] and with the type VI secretion system of *Escherichia coli* [48]. *Francisella* spp. have a type VI secretion system, which plays an important role in promoting growth within arthropods; this secretion system was previously reported to be critical for intracellular growth in a mosquito cell line [49]. In addition, we previously showed that the type VI

secretion system was directly involved in proliferation of *Francisella* within silkworms [31]. In *Acinetobacter*, soluble lytic transglycosylase (SLT) is required in order to form type IV pili, which are associated with bacterial pathogenicity [50]. In *F. novicida*, peptidase and chitin-binding proteins are secreted from the type IV pili apparatus [51]. These findings suggest that proteins secreted from type VI secretion system or type IV pili may contribute to the intracellular growth and/or to the immunosuppressive activity associated with *F. novicida* infection.

While we could not fully clarify the detailed mechanisms via which *F. novicida* MltA promotes growth and survival in silkworms, our results suggest a critical role played by its capacity to suppress host immune responses. Previous studies have reported that *Francisella* spp. are generally capable of suppressing host immune responses, thus enabling them to survive within the host [10]. Our previous studies in silkworms showed that both *F. novicida* and LVS were capable of inhibiting host immune activity [8,31]. Arthropods, including silkworms, possess Toll pathways to overcome the pathogen infection [52,53]. Pathogen-associated molecular pattern molecules, including lipopolysaccharide (LPS) and PG, were captured by recognition proteins, resulting in immune responses that include expression of AMPs [54]. Cecropin B is one of major AMPs effective against both Gram-positive and Gram-negative bacteria [55]; cecropin B was shown to be effective against *F. novicida* in a *D. melanogaster* infection model [39]. In this study, expression of cecropin B was suppressed during the early stages, at 6–12 h post infection with the wild-type strain. By contrast, although the bacterial number was diminished, the Δ*mltA* strain induced high levels of expression of cecropin B compared to that observed in response to infection with the wild-type strain. These results suggest that limited survival of the Δ*mltA* strain may be attributed to the fact that it does not

suppress expression of AMPs in infected silkworms. MltA has been associated with PG remodeling, LPS synthesis, and the production of outer membrane vesicles (OMVs) in various bacterial species [56–58]. In *Neisseria gonorrhoeae*, LTs plays an important role in escape of the bacterium from TLR recognition via control of OMVs production [59]. These findings in earlier works may suggest that MltA of *Francisella* modulates exposure of LPS, PG, and OMVs and thus avoids host recognition by modifying membrane structures.

In this study, MltA was identified as a growth factor in the silkworm infection model. MltA also contributed to intracellular growth and cytotoxicity in human macrophages. Among the seven genes identified as growth factors in silkworms, four genes encoding the major facilitator superfamily (MFS) transport protein, intracellular growth locus protein B, gamma-glutamyl transpeptidase, and SLT were previously identified as promoting pathogenicity in mammalian hosts [60–62]. These results suggest that many of the factors that promote growth and survival in silkworms may also be involved in intracellular proliferation in mammal cells. This finding is consistent with the suggestion made by Akimana and Kwaik [63], specifically that the mechanisms associated with the *Francisella* pathogenicity may depend on systems that are common to or at least similar among arthropods and mammals. As such, we conclude that the *F. novicida*–silkworm infection model is useful as an infection model for mammals, including humans.

In conclusion, our research has highlighted the importance of MltA of *F. novicida* as a novel factor that promotes bacterial growth in silkworms and similarly contributes to pathogenicity in human cells. Because highly pathogenic *F. tularensis* subspecies possess *mltA*, it might be a potential target for the control of arthropod-mediated transmission of *F. tularensis*.

Figure Legends

Figure 1. Screening of a transposon mutant library using a silkworm infection model. Each group of silkworms (n = 10) was infected with either the wild-type (WT), negative control (Δ*dotU*), or transposon mutant strains of *F. novicida* at 5×10^6 CFU/larva. Survival was determined at each time point as indicated.

Figure 2. Characteristics of the *mltA* deletion mutant. (A) The GFP-expressing wild-type (WT) and Δ*mltA* strains of *F. novicida* at early-exponential phase in BHIc medium. High-magnification images are as shown in the box frame. Scale bar = 50 μm. (B) The wild-type (WT), transposon mutant (C15-5), Δ*mltA*, and the complemented (Δ*mltA/mltA*) strains of *F. novicida* were cultured in BHIc medium. Absorbance was measured at 595 nm. The data represent the averages and standard error of three identical experiments. Differences from the wild-type strain were determined via multiple comparison methods and are indicated by asterisks; $^{**}P < 0.01$, $^{*}P < 0.05$.

Figure 3. Role of MltA in multiplication and virulence of *F. novicida* in the silkworm infection model. (A) Each group of silkworms (n = 13) was infected with the wild-type (WT), transposon mutant (C15-5), Δ*mltA*, or the complemented (Δ*mltA/mltA*) strains of *F. novicida* at 5×10^6 CFU/larva. Survival was evaluated at the time points; differences from the wild-type strain were evaluated via log-rank test; ** $P < 0.01$. (B) Each group of silkworms (n = 15) was infected with the wild-type (WT), transposon mutant (C15-5), Δ*mltA*, and the complemented (Δ*mltA/mltA*) strains of *F. novicida* at 5×10^6 CFU/larva. Bacterial numbers in silkworms were determined at 0 (immediately after infection), 1, 2,

3, 4 days post infection. These data represent the averages and standard error of bacterial numbers in three different silkworms. Differences from the wild-type strain were determined via multiple comparisons and are indicated by asterisks; ** $P < 0.01$. (C) Silkworms were infected with the GFP-expressing wild-type (WT) or Δ*mltA* strains of *F. novicida*. Hemolymph was harvested at time points indicated and observed by confocal laser scanning microscopy. Scale bar = 20 μm.

Figure 4. Impact of MltA on intracellular growth and cytotoxicity of *F. novicida* in BmN4 cells. (A) BmN4 cells were infected with the wild-type (WT), transposon mutant (C15-5), Δ*mltA*, or the complemented (Δ*mltA/mltA*) strains of *F. novicida* at MOI = 1 followed by treatment with 50 μg/ml gentamicin for 1 h. The cells were disrupted with 0.1% Triton X-100 and plated on BHIc agar at the time points indicated. (B) BmN4 cells were infected with the GFP-expressing wild-type (WT) or Δ*mltA* strains of *F. novicida* at MOI = 1 and treated 50 μg/ml gentamicin for 1 h. The cells were fixed and evaluated by confocal laser scanning microscopy at indicated time points. Scale bar = 20 μm. (C) BmN4 cells were infected with the wild-type (WT), transposon mutant (C15-5), Δ*mltA*, and the complemented (Δ*mltA/mltA*) strains of *F. novicida* at MOI = 1 and treated 50 μg/ml gentamicin for 1 h and incubated for 48 h. Cell survival was evaluated by trypan blue exclusion assay. (A, C) The data represent the averages and standard error of three identical experiments. Differences from the wild-type strain were determined via multiple comparisons and are indicated by asterisks; ** $P < 0.01$, *$P < 0.05$.

Figure 5. MltA-mediated host immune suppression in the silkworm infection model. Hemolymph plasma samples from silkworms infected with the wild-type (WT) or the Δ*mltA* strains of *F. novicida* were collected at post-infection time points as indicated. Cecropin B expression was determined by immunoblotting. Representative data from three independent experiments are as shown.

Figure 6. Impact of MltA on pathogenicity of *F. novicida* in a human macrophage cell line. (A) THP-1 cells were infected with the wild-type (WT), transposon mutant (C15-5), Δ*mltA*, or the complemented (Δ*mltA/mltA*) strains of *F. novicida* at MOI = 1 and treated 50 μg/ml gentamicin for 1 h. Cells were disrupted with 0.1% Triton X-100 and plated on BHIc agar at the times indicated. (B) THP-1 cells were infected with the GFP-expressing wild-type (WT) or Δ*mltA* strains of *F. novicida* at MOI = 1 and treated 50 μg/ml gentamicin for 1 h. Cells were fixed and evaluated by confocal laser scanning microscopy at the time points as indicated. Scale bar = 20 μm. (C) THP-1 cells were infected with the wild-type (WT), transposon mutant (C15-5), Δ*mltA*, or the complemented (Δ*mltA/mltA*) strains of *F. novicida* at MOI = 0.01 and treated 50 μg/ml gentamicin for 1 h. Cells were incubated for 48 h and cytotoxicity following infection was measured using the LDH assay. (A, C) The data represent the averages and standard error of three identical experiments. Differences from the wild type strain were evaluated via multiple comparisons and are indicated by asterisks; ** $P < 0.01$, *$P < 0.05$.

Tables and Figures

Table 1. Sequence analysis of transposon mutants.

Strain	Locus_tag	Gene name	Product
A20-4	FTN_0496	*slt*	Soluble lytic murein transglycosylase
A22-1	FTN_0420	*purD*	SAICAR synthetase/phosphoribosylamine-glycine ligase
B17-6	FTN_1323	*iglB*	Intracellular growth locus protein B
B22-5	FTN_1641	*ampG*	MFS transport protein
B23-6	FTN_1641	*ampG*	MFS transport protein
C4-4	FTN_0177	*purH*	AICAR transformylase/IMP cyclohydrolase
C15-5	FTN_1286	*mltA*	Membrane-bound lytic murein transglycosylase
D3-7	FTN_1159	*ggt*	Gamma-glutamyl transpeptidase

Table 2. Primer sets and templates.

Vector	Primer	Sequence	Template (Reference)
pMOD3-FtKm	pMOD3-kanR.FOR	CATCGTGGCCGGATCGATCTTTTGGGTTGTCACTCATCGTATT	pKEK1440 [35]
	pMOD3-kanR.REV	ATTAACCAATTCTGATTAGAAAAACTCATCGAGCATCAAATGAAACT	
	pMOD3-kanR-vector.FOR	TCAGAATTGGTTAATTGGTTGTAACACTGG	pMOD3
	pMOD3-kanR-vector.REV	GATCCGGCCACGATGCG	
pFRSU-mltA	mItA-up1.5.FOR	TAGAACTAGTGGATCCCAAGTCGAAGTCTTCACCGC	*F. novicida* genome
	mIitA-up1.5.REV	GTTTTGTAATAAAAAAATACCAATAATTTCTTGAAAATACAGAATTAATCACTATC	
	mltA-down1.5.FOR	TTTTTATTACAAAACAGATACTATTATAATTGGTGCTGGGATTT	*F. novicida* genome
	mltA-down1.5.REV	GCAGCCCGGGGGATCACTTTTTTCAGCATTTACGATAACAACATAATCACC	
pOM5-MItA	pOM5-mltA.FOR	AGTCTCTTCTCTAGAGCTCTTAAAGCCTCATCTTTAAATGATCACAAC	*F. novicida* genome
	pOM5-mltA.REV	TGATCTTTTCTACGGTCAGTCATTTGGTAAAAGTATCCATAGTTTGCC	
	pOM5-vector-liner.FOR	CCGTAGAAAAGATCAAAGGATCTTCTTGAGATC	pOM5-IglE [36]
	pOM5-vector-liner.REV	TCTAGAGAAGAGACTGACAAGCTTTTAAAAGACT	

Figure 1

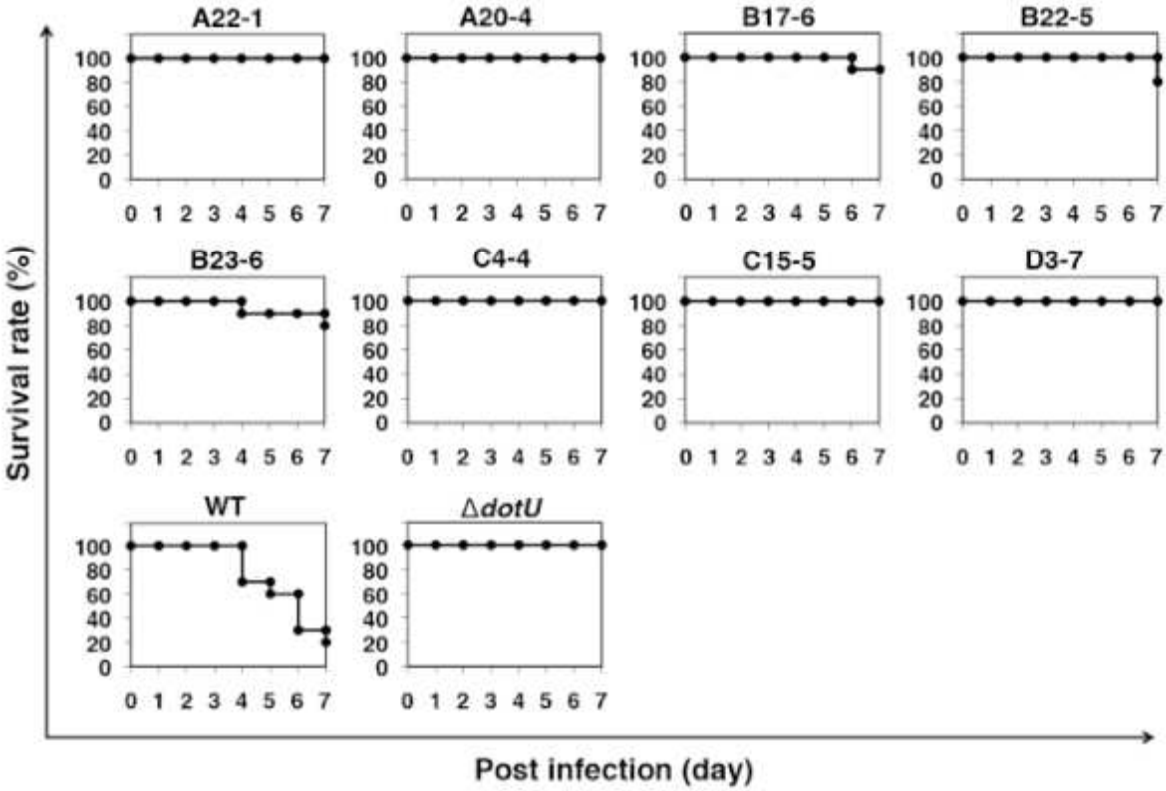
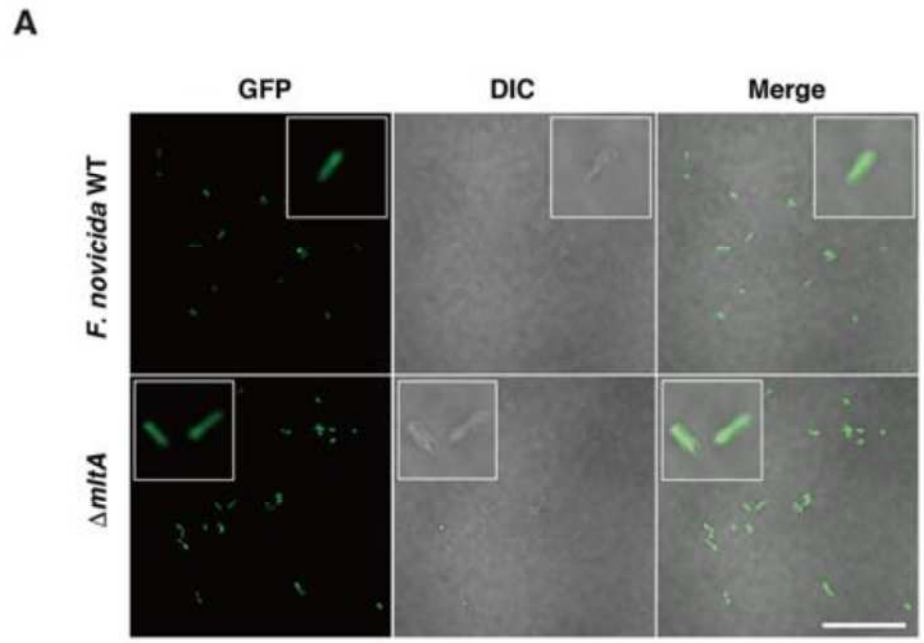

A22-1
A20-4
B17-6
B22-5
B23-6
C4-4
C15-5
D3-7
WT
ΔdotU
Survival rate (%)
Post infection (day)
0
20
40
60
80
100
0 1 2 3 4 5 6 7

Figure 2


A
GFP
DIC
Merge
F. novicida WT
ΔmltA

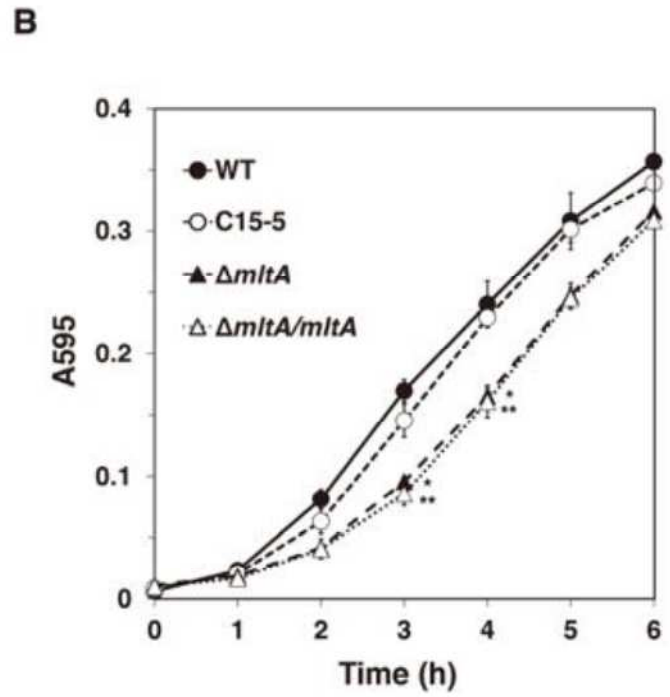

B
A595
Time (h)
WT
C15-5
ΔmltA
ΔmltA/mltA
0
0.1
0.2
0.3
0.4
0
1
2
3
4
5
6

Figure 3

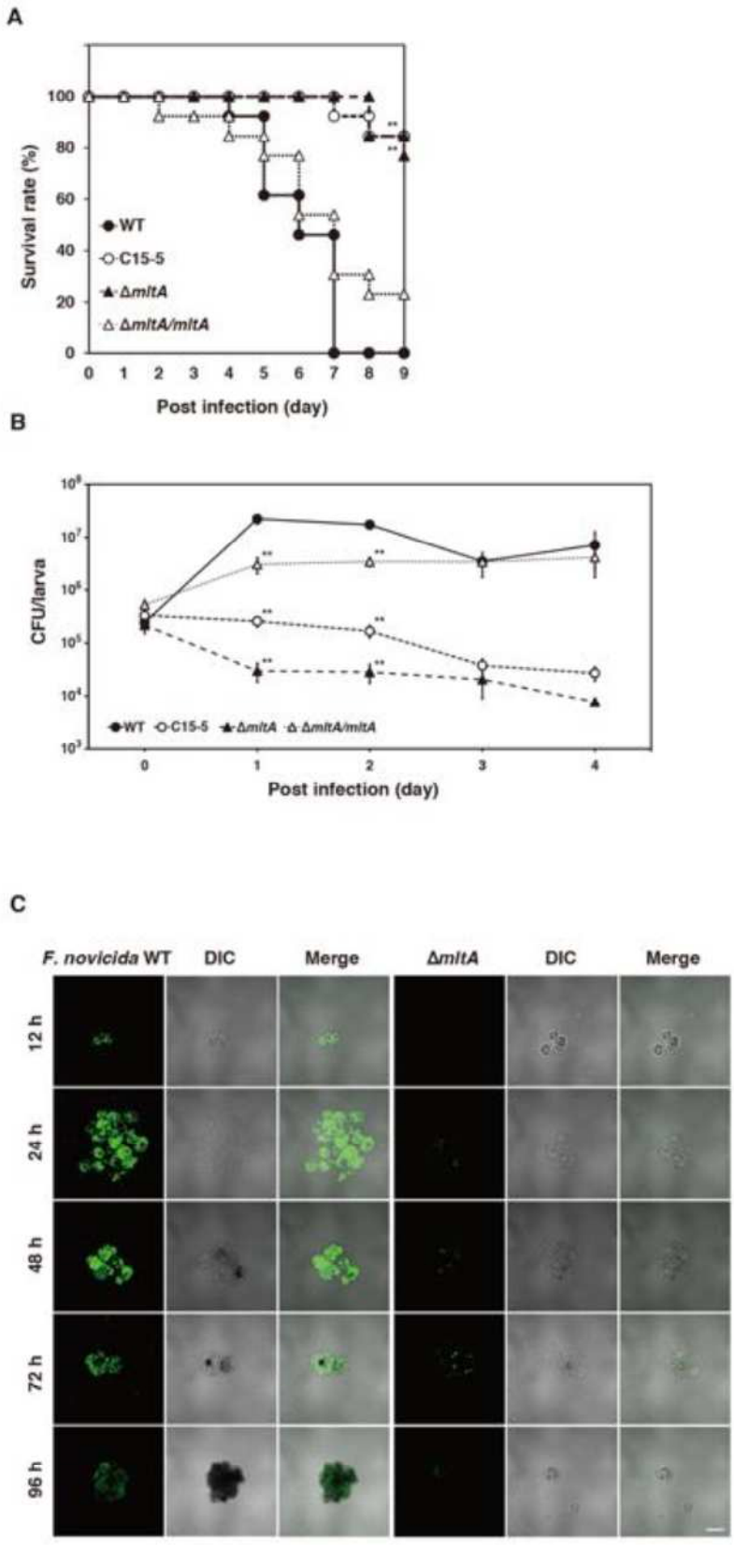

A
Survival rate (%)
100
80
60
40
20
0
WT
C15-5
ΔmltA
ΔmltA/mltA
0 1 2 3 4 5 6 7 8 9
Post infection (day)
B
CFU/larva
WT C15-5 ΔmltA ΔmltA/mltA
0 1 2 3 4
Post infection (day)
C
F. novicida WT
DIC
Merge
ΔmltA
DIC
Merge
12 h
24 h
48 h
72 h
96 h

Figure 4

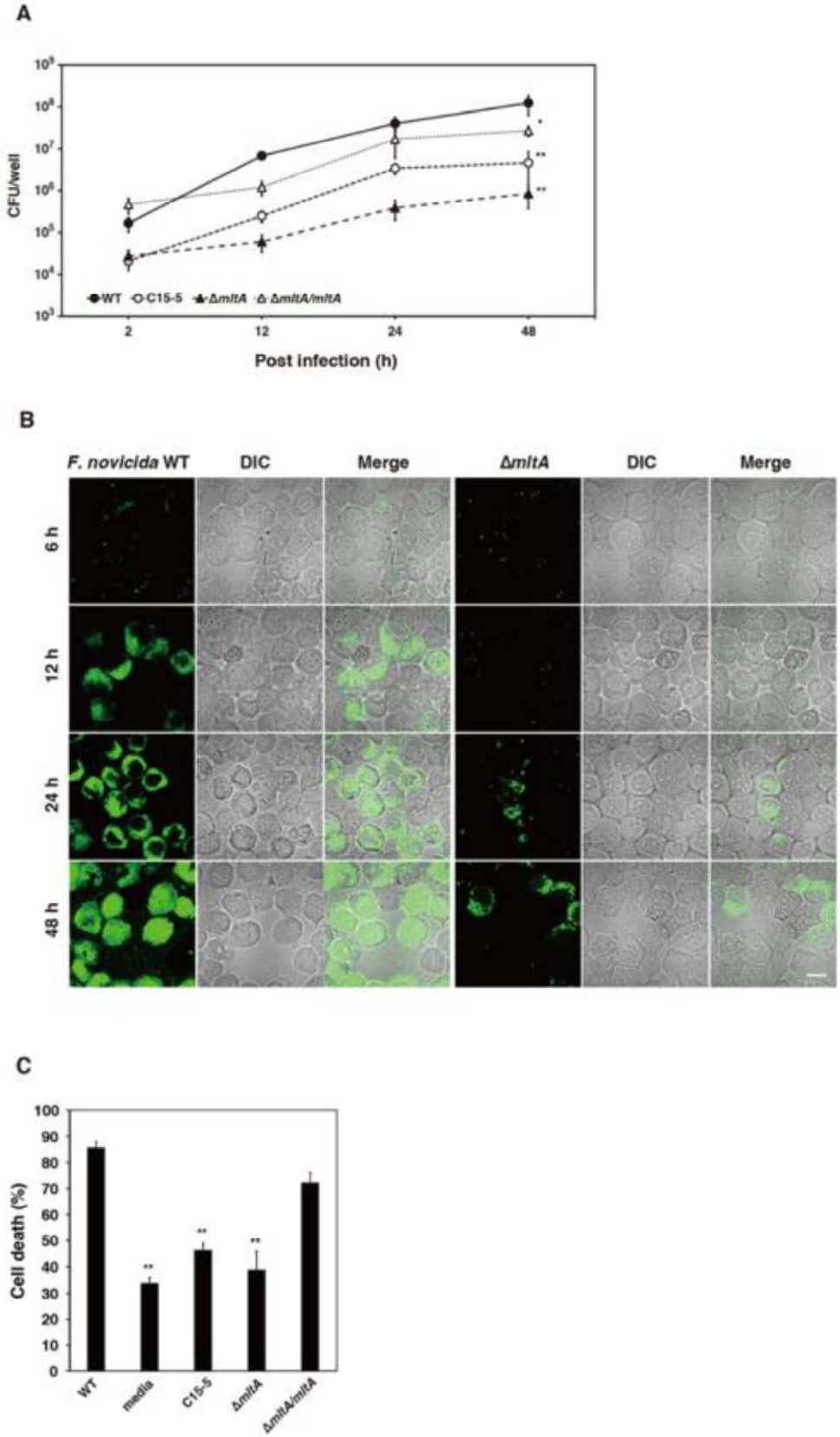

A
CFU/well
WT
C15-5
ΔmltA
ΔmltA/mltA
Post infection (h)
B
F. novicida WT
DIC
Merge
ΔmltA
DIC
Merge
6 h
12 h
24 h
48 h
C
Cell death (%)
WT
media
C15-5
ΔmltA
ΔmltA/mltA

Figure 5

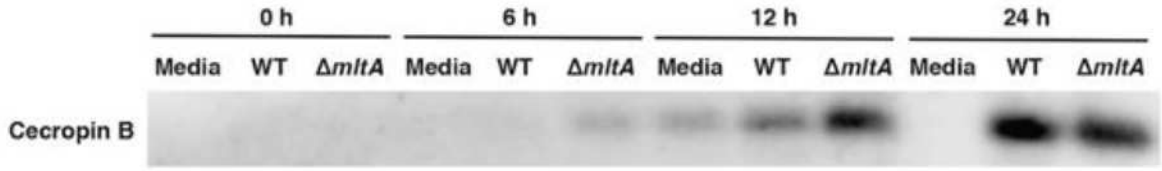
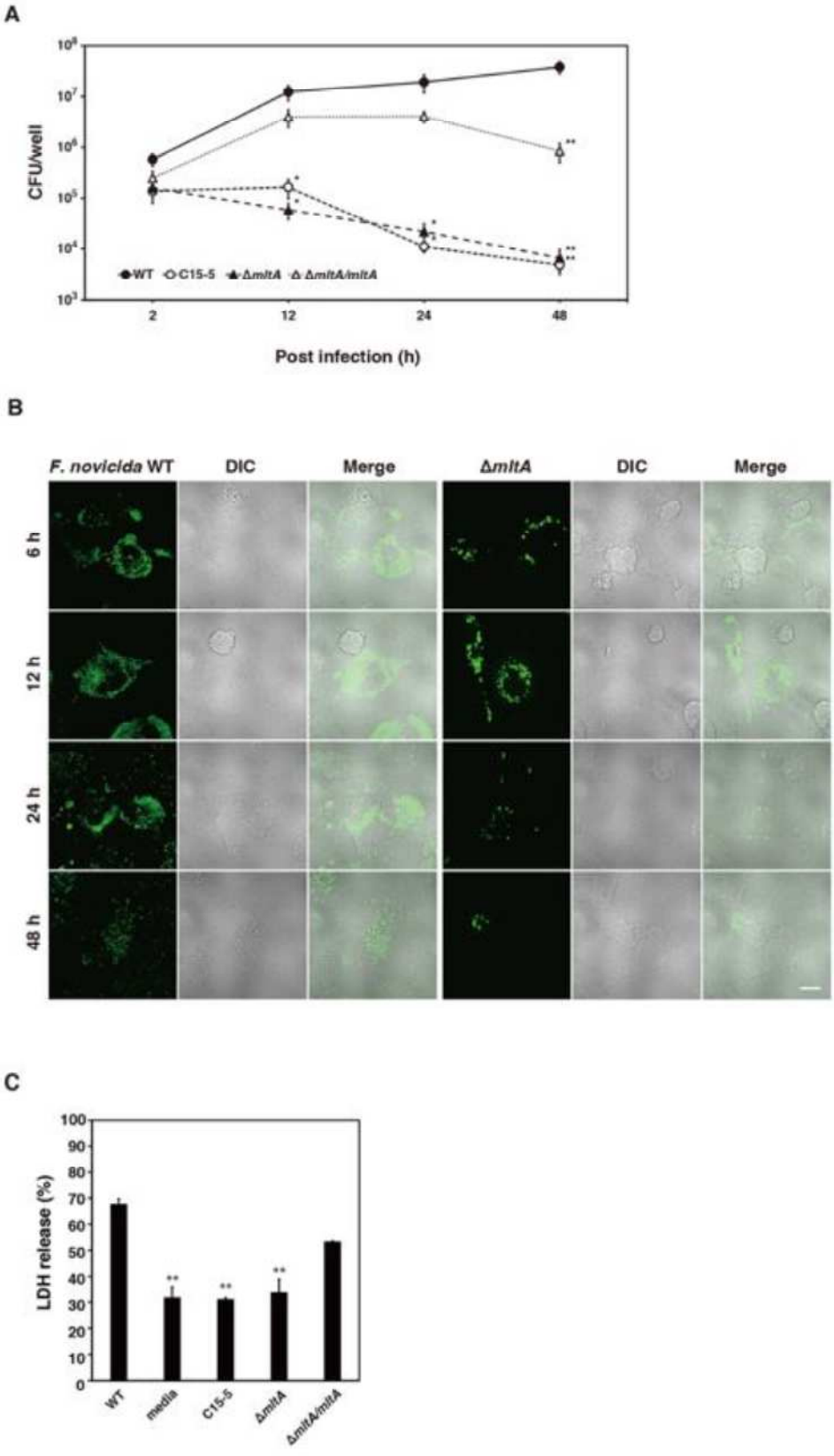

0 h
6 h
12 h
24 h
Media
WT
ΔmltA
Media
WT
ΔmltA
Media
WT
ΔmltA
Media
WT
ΔmltA
Cecropin B

Figure 6


A
CFU/well
WT
C15-5
ΔmltA
ΔmltA/mltA
2
12
24
48
Post infection (h)
B
F. novicida WT
DIC
Merge
ΔmltA
DIC
Merge
6 h
12 h
24 h
48 h
C
LDH release (%)
WT
media
C15-5
ΔmltA
ΔmltA/mltA

Chapter II

Identification of *pyrC* gene as an immunosuppressive factor in *Francisella novicida* infection.

Abstract

Francisella tularensis, a bacterial causative agent of the zoonosis tularemia, is highly pathogenic to humans. The pathogenicity of this bacterium is characterized by intracellular growth in immune cells, like macrophages, and host immune suppression. However, the detailed mechanism of immune suppression by *F. tularensis* is still unclear. To identify the key factors causing *Francisella*-mediated immunosuppression, large-scale screening using a transposon random mutant library containing 3552 mutant strains of *F. tularensis* subsp. *novicida* (*F. novicida*) was performed. Thirteen mutants that caused stronger tumor necrosis factor (TNF)-α production in infected U937 human macrophage cells than the wild-type *F. novicida* strain were isolated. Sequencing analysis of transposon insertion sites revealed 10 genes, including six novel genes, as immunosuppressive factors of *Francisella*. Among these, the relationship of the *pyrC* gene, which encodes dihydroorotase in the pyrimidine biosynthesis pathway, with *Francisella*-mediated immunosuppression was investigated. The *pyrC* deletion mutant strain (Δ*pyrC*) induced higher TNF-α production in U937 host cells than the wild-type *F. novicida* strain. The Δ*pyrC* mutant strain was also found to enhance host interleukin-1β and interferon (IFN)-β production. The heat-inactivated Δ*pyrC* mutant strain could not induce host TNF-α production. Moreover, the production of IFN-β resulting from Δ*pyrC* infection in U937 cells was repressed upon treatment with the stimulator of interferon genes (STING)-specific inhibitor, H-151. These results suggest that *pyrC* is related to the immunosuppressive activity and pathogenicity of *Francisella* via the STING pathway.

Introduction

Tularemia is a vector-borne zoonosis with severe symptoms, including fever, lymphadenitis, cutaneous lesions, and primary pulmonary involvement in humans [64]. *Francisella tularensis*, the causative agent of tularemia, is a gram-negative, facultative, and intracellular bacterial pathogen. Tularemia is highly contagious, with subcutaneous infection with as few as 10 bacterial cells and aerosol-mediated inhalation of as few as 25 bacterial cells being able to cause human infection [65]. Accordingly, highly virulent *F. tularensis* strains have been classified as a Category-A agents of potential bioterrorism by the Centers for Disease Control and Prevention and are required to be handled and contained in BSL-3 laboratories [66]. *F. tularensis* is divided into four subspecies, subsp. *tularensis*, *holarctica*, *mediasiatica*, and *novicida*. Among them, only *Francisella tularensis* subsp. *tularensis* (*F. tularensis*) and *Francisella tularensis* subsp. *holarctica* (*F. holarctica*) cause tularemia in human [16]. *F. novicida* infection in humans is considerably rare and can be handled in a BSL-2 laboratory. Most cases of human *F. novicida* infection have involved patients who were immunocompromised or had an underlying disease. However, *F. novicida* has over 98% identity to *F. tularensis* at the DNA level and shows many characteristics similar to *F. tularensis* with regards to its life cycle within macrophages and pathogenicity in mice [17,67]. Therefore, *F. novicida* has been used as the model bacterium for research on *Francisella* pathogenicity.

While *Francisella* research has a long history, the detailed molecular mechanisms of infection by *Francisella* subspecies remain unknown. *Francisella* can replicate in immunocompetent cells, such as macrophages, neutrophils, and dendritic cells, which are essentially responsible for the elimination of pathogens from the body [68]. To survive inside host cells, *Francisella* species employ various strategies. *Francisella* enters these

cells via phagocytosis, escapes digestion by phagolysosomes and autophagosomes, and finally replicates in the cytoplasm [69,70]. Further, *Francisella* suppresses or evades host pattern recognition receptors (PRRs), which usually initiate the innate immune response to exclude pathogens [37,71,72]. Although some of the factors responsible for *Francisella*'s immunosuppressive abilities were identified in previous research [12,73,74], the detailed mechanisms involved in *Francisella*-mediated immunosuppression remain to be elucidated.

Francisella grows in nutrient-limited host cells, and this nutrient limitation is closely related to its pathogenicity [75]. To date, a lot of genes responsible for the uptake or biosynthesis of nutrients, such as amino acids, carbon, vitamins, and bases, were reported as crucial factors for the intracellular replication of *Francisella* [76–78]. Especially, nucleotide biosynthesis is essential for the survival and virulence of bacterial pathogens, including intracellular bacteria, such as *Salmonella*, *Listeria*, *Brucella*, and *Francisella* [79]. Since pyrimidine nucleotides are essential for all organisms, almost all bacterial species have a *de novo* pyrimidine biosynthesis pathway, which has highly conserved enzymatic steps. The *de novo* pyrimidine biosynthesis pathway for synthesizing uridine 5′-monophosphate consists of six steps and employs enzymes encoded by *carA*/*B* and *pyrB-F* [80]. The *pyrC* gene encodes a putative dihydroorotase that converts carbamoyl N-Carbamoyl-L-aspartate into 4,5-dihydroorotate in the *Francisella* pyrimidine biosynthesis pathway. Although some reports suggest pyrimidine biosynthesis is important for the intracellular growth of *Francisella*, it is not well understood how pyrimidine biosynthesis is involved in immunosuppression by *Francisella* [81,82].

In this study, we performed large-scale screening of a *F. novicida* transposon mutant library to search for the key factors involved in the immunosuppression mechanisms of

Francisella. We identified *pyrC* as a novel *F. novicida* factor suppressing host innate immune responses and evaluated the immunological characteristics of host cells infected with *F. novicida*.

Materials and Methods

Bacterial strains and culture conditions

F. novicida U112 was obtained from the Pathogenic Microorganism Genetic Resource Stock Center (Gifu University, Gifu, Japan). *F. novicida* was cultured aerobically at 37°C in a chemically defined medium (CDM) [32] or brain heart infusion broth (Becton, Dickinson and Company, NJ, USA) supplemented with 0.1% cysteine (BHIc) [33] or BHIc containing 1.5% agar (Wako Laboratory Chemicals, Osaka, Japan). All experiments were conducted in compliance with the institutional biosecurity guidelines and were approved by Yamaguchi University.

Cell culture

Human monocytic U937 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum at 37 ºC in an atmosphere containing 5% CO_2.

Plasmid construction, transformation, and transfection

Table 2 lists the primer sets and templates used to construct the plasmids used in this study. Polymerase chain reaction (PCR) was performed using KOD-Plus-Neo polymerase (Toyobo, Osaka, Japan), and ligation was performed using an In-Fusion HD Cloning Kit (Takara Bio, Shiga, Japan). Plasmids were used to transform *F. novicida* via electroporation. Specifically, bacterial cells were suspended in 0.5 M sucrose with 2 μg of plasmid DNA and were electroporated using a Bio-Rad micropulser (Bio-Rad, Hercules, CA, USA) at 3.0 kV, 10 μF, and 600 Ω with a 0.2 cm cuvette. The transformants were pre-incubated in BHIc medium overnight. To select transformed bacteria, the pre-

incubated bacteria were cultured on BHIc agar plates containing 30 μg/ml kanamycin or 2.5 μg/ml chloramphenicol.

Construction of a transposon mutant library

The transposon mutant library was constructed using the Ez-Tn5 transposon system (Epicentre Lucigen, Madison, WI, USA), as previously reported [34]. Briefly, the multiple cloning site of pMOD3 was linearized by digestion with Hind III and EcoRI, and the kanamycin resistance cassette of pKEK1140 [35] was ligated into the Hind III and EcoRI sites to generate pMOD3-FtKm. The transposon moiety of pMOD3-FtKm was amplified by PCR, purified, mixed with transposase according to the manufacturer's instructions, and then used to transform *F. novicida* via electroporation. Transformed bacteria were cultured on BHIc plates containing 30 μg/ml kanamycin.

Sequence analysis of transposon mutants

pMOD3 harbors the *E. coli* R6Kγ origin of replication. The genomes of *F. novicida* transposon mutants were purified using a PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, MA, USA) and digested with a combination of restriction enzymes, including XhoI, BglII, EcoRI, SalI, NotI, and BamHI. The ends of the digested DNAs were then blunted using a DNA Blunting Kit (Takara Bio) and ligated using Ligation High Ver. 2 (Toyobo). The ligated DNA was used to transform λpir Chemically Competent *E. coli* (Thermo Fisher Scientific). The transformed *E. coli* were selected for kanamycin resistance, and plasmid DNAs were purified. Sequence analysis was performed using the primer described in the manufacturer's instructions for the Ez-Tn5 transposon system.

Construction of *F. novicida* mutants

The *dotU* homolog (*FTN_1316*) deletion mutant (Δ*dotU*) was previously constructed [36] through group II intron insertion using a TargeTron Gene Knockout System (Sigma-Aldrich), which was modified for *Francisella* species (Rodriguez et al., 2008). The *pyrC* gene (*FTN_0024*) deletion mutant (Δ*pyrC*) was generated via homologous recombination using the *Francisella* suicide vector pFRSU [36]. The upstream and downstream regions of *pyrC* (1.5 kbp each) were cloned into the BamHI site of pFRSU to generate pFRSU-pyrC. The pFRSU-pyrC vector (2 μg) was used to transform *F. novicida*; transformants were selected on BHIc plates containing 30 μg/ml kanamycin. Isolated bacteria were cultured in BHIc without antibiotics overnight and then plated on BHIc plates containing 5% sucrose. The deletion of the *pyrC* gene was confirmed via PCR.

Green fluorescent protein (GFP)- and PyrC-expressing *F. novicida* strains

A GFP-expressing plasmid, pOM5-GFP, was constructed according to published procedures [36]. The *F. novicida* chromosomal *pyrC* gene was cloned into pOM5 to generate pOM5-pyrC. To construct GFP-expressing strains and *pyrC* complemented strains, pOM5-pyrC and pOM5-GFP were used to transform the wild-type strain or the Δ*pyrC* mutant strain of *F. novicida* via electroporation.

Intracellular growth assay

U937 cells (1×10^5 cells/well) were incubated in a 48-well tissue culture plate with 100 nM phorbol myristate acetate (PMA) for 48 h. Then, *F. novicida* strains were added at a multiplicity of infection (MOI) of 1. Next, the plates were centrifuged for 10 min at 300

× *g* and incubated for 1 h at 37°C. The cells were then washed three times with RPMI 1640 medium, and extracellular bacteria were killed with gentamicin at 50 μg/ml for 1 h. The cells were then incubated in fresh medium at 37°C for the indicated time durations in figure legends. To measure intracellular growth, the cells were washed with PBS and then lysed with 0.1% Triton X-100 in CDM. The CFUs were determined on BHIc agar plates by plating serial dilutions of cultures.

Fluorescence microscopy

U937 cells (1×10^5 cells/well) were incubated with 100 nM PMA for 48 h on 12 mm glass coverslips in 24-well tissue culture plates. GFP-expressing *F. novicida* strains were infected at an MOI of 1. Plates were then centrifuged for 10 min at 300 × *g* and incubated for 1 h at 37°C. The cells were washed three times with RPMI 1640 medium, and extracellular bacteria were eliminated using gentamicin at 50 μg/ml for 1 h. The cells were then incubated in fresh medium at 37°C for the indicated time durations in figure legends. Cells were fixed with 4% paraformaldehyde at room temperature for 30 min. A FluoView FV100 confocal laser scanning microscope (Olympus, Tokyo, Japan) was used to obtain images of the cells.

RNA isolation and qPCR analysis

U937 cells (4×10^5 cells/well) were incubated in a 12-well tissue culture plate with 100 nM PMA for 48 h. The medium was exchanged with fresh pre-incubated RPMI 1640 medium one-hour prior to infection. Cells were infected with *F. novicida* strains at MOI = 1 or stimulated with 100 ng/ml of lipopolysaccharide (LPS) derived from *E. coli* (O127:B8) (Sigma-Aldrich), or 10 ng/ml 2′3′-cGAMP (InvivoGen, CA, USA). The plates

were centrifuged for 10 min at 300 × g and incubated for indicated time the indicated time durations in figure legends. Cells were carefully washed twice with PBS, and total RNA was collected using NucleoSpin RNA kit (Takara Bio). RNA was quantified by determining absorption at 260 nm using NanoDrop 2000 (Thermo Fisher Scientific). Next, qPCR was performed using the RNA-direct Realtime PCR Master Mix (Toyobo) with an RNA concentration of 50 ng per 20 μl reaction. The *HPRT1* amplicon was used as an endogenous control to normalize all mRNA expression data. The relative expression levels of genes in various conditions compared with those in the BHIc medium-treated control were calculated using the relative quantification method (ΔΔCt method) [83]. Used primer sets are shown in Table S1.

ELISA

U937 cells (1×10^5 cells/well) were pre-incubated in a 48-well tissue culture plate with 100 nM PMA for 48 h. After exchanging the medium with pre-incubated fresh RPMI medium 1 h prior to infection, cells were infected with bacterial strains (*F. novicida* transposon mutant strains or deletion mutant strains) at an MOI of 1 or stimulated with 100 ng/ml of LPS derived from *E. coli* (O127:B8) or 10 ng/ml of 2′3′-cGAMP (InvivoGen). Heat-inactivation of each strain was performed by incubating the bacterial suspension in a heat block at 90°C for 5 min. In the case of STING inhibition, a STING-specific inhibitor, H-151 was used [84]. U937 cells were treated with H-151 (final concentration 0.5 μM) or the same volume of DMSO 2 h prior to infection. After incubation for the indicated time durations in figure legends, concentrations of tumor necrosis factor (TNF)-α and interleukin (IL)-1β in the supernatants were measured using ELISA MAX Standard Kit (Biolegend, CA, USA) according to the manufacturer's

instructions. Interferon (IFN)-β in the supernatants were measured using VeriKine Human Interferon Beta ELISA Kit (PBL Assay Science, Piscataway, NJ, USA).

Statistical analysis

Student's *t* test or multiple comparisons using the Tukey–Kramer test and Dunnett's test were used to evaluate the significance of differences compared with the wild-type strain; $P < 0.05$ indicates a significant difference.

Results

Ten genes were identified as immunosuppressive factors of *F. novicida.*

To identify novel immunosuppressive factors of *F. novicida* in human macrophages, we expanded a previously constructed *F. novicida* transposon mutant library consist of 750 strains [34] up to 3552 strains. TNF-α is a cytokine produced through a broad range of innate immune signaling pathways, including Toll-like Receptor (TLR) 4- and TLR2-mediated pathways, and is reported to be suppressed by *Francisella* infection [85,86]. To identify genes responsible for immunosuppression by *F. novicida*, U937 cells were infected with a mutant library, and the transposon mutants inducing excessive TNF-α production compared with the wild-type strain were selected through ELISA using the culture supernatant. In the 1st screening, the cut-off was set to a 1.5-fold increase in TNF-α production. In the 2nd screening, U937 cells were infected with mutant strains selected via the first screening, and their TNF-α production was measured three times. Finally, 13 mutants that increased TNF-α production in U937 cells were identified (Figure 1). The Δ*slt* mutant strain, lacking the gene encoding soluble lytic transglycosylase, was used as a positive control [34]. To determine the genes responsible for *Francisella*-mediated immunosuppression, the transposon insertion sites of the selected mutant strains were evaluated by sequence analysis; 10 unique genes were identified (Table 1). In this study, we focused on *pyrC* (*FTN_0024*), the gene putatively encoding dihydroorotase, and the effect of this gene on immunosuppression by *Francisella*.

The *pyrC* deletion mutant evokes the innate immune responses of host U937 macrophage cells.

To estimate the effect of *pyrC* in immunosuppression by *F. novicida*, we constructed a

Δ*pyrC* mutant strain of *F. novicida* via homologous recombination. ELISA showed that the Δ*pyrC* mutant strain induced significantly higher levels of TNF-α production in the cell culture supernatant of U937 cells than the wild-type strain (Figure 2A). Although not significantly different, the *pyrC* complemented strain tended to show decreased levels of TNF-α induction compared with the Δ*pyrC* mutant strain. The *pyrC* complemented strain showed equivalent levels of TNF-α induction to the wild-type strain (Figure 2A). Next, we measured the induction of IL-1β and IFN-β, which are important cytokines for *Francisella* infection, in the Δ*pyrC* mutant strain [87]. As in the case of TNF-α, the Δ*pyrC* mutant strain-infected U937 cells showed significantly higher levels of IL-1β and IFN-β production than the wild-type strain, and *pyrC* complementation decreased these levels to that observed upon infection with the wild-type strain (Figure 2B, 2C). The mRNA expression levels of *TNF*, *IL1B*, and *IFNB1* in *Francisella*-infected U937 cells were also examined using real-time PCR. Similar results to those obtained via ELISA were observed for the mRNAs of all these genes (Figures 2D, 2E, 2F). These results indicate that the Δ*pyrC* mutant strain strongly evokes host innate immune responses compared to the wild-type strain, suggesting that *pyrC* is a critical factor for the immunosuppression of *F. novicida*.

The *pyrC* gene is important for the intracellular growth of *F. novicida* in U937 cells.

Next, we examined whether Δ*pyrC* is involved in the intracellular growth of *F. novicida*. The Δ*pyrC* mutant strain entered the stationary phase slightly earlier than wild-type strain in BHIc medium and failed to grow in CDM (Figures 3A, 3B). The GFP-expressing wild-type strain of *F. novicida* grew intracellularly in U937 cells from 2 to 48 h post infection, while the GFP-expressing Δ*pyrC* mutant strain did not show

remarkable intracellular growth during the same period through fluorescence microscopy (Figure 3C). To support this finding, the number of Δ*pyrC* and transposon mutant strain cells were significantly decreased to approximately 1/10th that of wild-type strain cells within U937 cells. On the contrary, the Δ*pyrC* mutant strain showed higher intracellular growth compared to the strain deficient in the type VI secretion system (Δ*dotU*), which was used as a negative control. The complemented strain restored the ability of the Δ*pyrC* mutant strain to grow intracellularly (Figure 3D). These results suggest that *pyrC* is not essential but is important for the intracellular growth of *F*. *novicida* in host cells.

TNF-α induction by the Δ*pyrC* mutant strain is abolished by heat treatment.

Host cells infected with bacteria produce TNF-α due to the recognition of various bacterial ligands, such as LPS, peptidoglycan, and nucleotides, by PRRs [88,89]. To identify Δ*pyrC* mutant strain ligands responsible for the induction of TNF-α in the Δ*pyrC* mutant strain infected U937 cells, U937 cells were treated with heat-inactivated *F. novicida* mutant strains. TNF-α induction by all heat-inactivated *F*. *novicida* strains, including the Δ*pyrC* mutant strain, was decreased to the same level as that by the negative control; however, LPS retained the ability to induce TNF-α with or without heat inactivation (Figure 4). This result suggests that TNF-α production by *F. novicida* strains, including the Δ*pyrC* mutant strain, is induced through biological activities of *F. novicida*, such as internalization by or proliferation in host cells, and not through heat-stable ligands, such as LPS, peptidoglycan, and nucleotides.

IFN-β induction in U937 cells infected with the Δ*pyrC* mutant strain is mediated by

the STING pathway.

Because IFN-β production is induced by recognition of cytosolic DNA by the cyclic-di-nucleotide sensor STING [90,91] pathway, we next examined whether *F. novicida pyrC* affects the STING pathway. U937 cells treated with a STING inhibitor H-151 were infected with the Δ*pyrC* mutant strain, and the IFN-β levels induced in them were measured using ELISA. H-151 showed no significant effect on the growth of both the wild-type and the Δ*pyrC* mutant strain in BHIc medium (Figure 5A). IFN-β levels in the supernatant of H-151-treated U937 cells infected with the Δ*pyrC* mutant or transposon mutants and stimulated with the STING agonist 2′3′-cGAMP were significantly decreased compared to those in the supernatant of the DMSO control cells (Figure 5B). Contrarily, H-151-treated U937 cells showed no significant difference in IFN-β induction upon infection with Δ*pyrC* mutant or transposon mutants compared to the wild-type and *pyrC* complemented strains. These results indicate that *pyrC* is involved in the suppression of IFN-β through the STING pathway.

Discussion

Intracellular bacteria, including *Francisella*, have refined their strategy to escape the host immune system and survive in host cells. To date, the importance of immunosuppression and immune evasion in *Francisella* infection has been well recognized, but their detailed mechanisms are poorly understood. To our knowledge, a large-scale gene screening of *Francisella* mutants focusing on *Francisella* immunosuppressive properties has not been performed yet. Therefore, here, we developed a transposon mutant library of *F. novicida* consisting of 3552 mutants. This library seems to cover the 1731 protein coding genes of the *F. novicida* U112 strain [67]. Among the 3552 mutant strains in our library, strains that induced higher levels of host immune response than the wild-type *F. novicida* strain were isolated. Ten genes were determined to be immunosuppression-related. Among these 10 genes, four genes, *FTN_0756* (*fopA*), *FTN_1286* (*mltA*), *FTN_0757* (*cas9*), and *FTN_0496* (*slt*), were previously reported as immunosuppressive factors of *Francisella* [11,13,34,92], ensuring the reliability of the screening method employed in this study. The remaining 6 genes, *FTN_1641* (*ampG*), *FTN_1199* (*capA*/*B*) *FTN_1548* (*yfgL*), *FTN_0917* (*dacB*), *FTN_0611* (*kdsA*), and *FTN_0024* (*pyrC*), were newly identified as *Francisella* immunosuppressive factors in this study. Because *ampG*, *capA*/*B, yfgL*, *dacB*, *and kdsA* are involved in creating the structure of bacterial cells, disruption of these genes can result in the leakage of ligands for intracellular receptor inflammasomes, such as LPS, peptidoglycan, and nucleotides [11,93,94]. In contrast, the gene *pyrC* putatively encodes dihydroorotase, which converts carbamoyl N-Carbamoyl-L-aspartate into 4,5-dihydroorotate through the *Francisella* pyrimidine biosynthesis pathway [80]. It was previously reported that another pyrimidine biosynthesis gene *pyrF* is involved in immune suppression by the *F. tularensis* LVS [81]. Although these results indicate that

pyrimidine biosynthesis pathway is strongly related to immunosuppression by *Francisella*, the detailed mechanisms through which the pyrimidine biosynthesis pathway affects immunosuppression by *Francisella* remain unclear. Therefore, we focused on *pyrC* in this study. In this screening method, *pyrF* was not identified as immune suppressive factor. This may indicate that 3552 mutants are not enough to cover the 1731 protein coding genes of *F. novicida* or there is a bias in the insertion site of transposon.

To confirm the effects of *pyrC* on immunosuppression, a *pyrC* deletion mutant was generated. The Δ*pyrC* mutant strain induced a high level of TNF-α production in infected U937 macrophage cells compared to that in the wild-type strain, and the complemented strain restored the immunosuppressive property of *F. novicida*. TNF-α is produced through a broad range of innate immune signaling pathways, including TLR signaling pathways activated by various bacterial ligands, such as LPS, peptidoglycan, and nucleotides [88,89]. In host cells infected by *Francisella*, the production of inflammatory cytokines, such as TNF-α and IL-6, is induced by the recognition of *Francisella* by TLR2, followed by the recognition of *Francisella* DNA by TLR 9 [10]. Because *pyrC* is related to pyrimidine biosynthesis, it may suppress immune responses by modifying nucleotides recognized by TLRs, such as TLR9. However, as discussed below, heat-inactivated *F. novicida* strains, including the Δ*pyrC* mutant strain, failed to induce TNF-α production, indicating that biological activities of *F. novicida*, such as its phagosomal escape, are necessary for it to induce TNF-α and other cytokines.

Our data also revealed that the disruption of the pyrimidine biosynthesis pathway by *F. novicida* induces higher levels of IL-1β and IFN-β production in U937 macrophage cells. IL-1β is secreted when its precursor is expressed through the activation of TLR or type I IFN signaling followed by cleavage with caspase-1 activated through recognition

by inflammasomes [93,95]. In *F. novicida* infections, infected host cells exhibit robust inflammasome activation and IL-1β secretion compared with *F. holarctica* LVS and *F. tularensis* Schu S4 infections [71,96]. In this study, we found that IL-1β production was suppressed in host cells infected by wild-type *F. novicida* and increased in host cells infected with the Δ*pyrC* mutant strain. This result is inconsistent with those of previous reports by Horzempa's and Schulert, which indicate that several genes of *F. tularensis* Schu S4 and *F. holartica* LVS strains are related to the suppression of IL-1β production [81,97]. Our results, however, show that the expression of IL-1β mRNA was suppressed upon host cell infection by the wild-type strain of *F. novicida* and increased upon host cell infection with the Δ*pyrC* mutant strain. Taken together with the fact that the IL-1β precursor is expressed through TLR or type I IFN signaling, the suppression of IL-1β secretion by *pyrC* cannot be attributed to the inhibition of inflammasomes but to the inhibition of TLR or type I IFN signaling.

Type I IFNs are secreted through STING-related pathways. The cGAS-STING pathway, known as an intracellular DNA sensor, recognizes bacterial DNA and induces type I IFNs [98]. STING is also activated by direct recognition of cyclic-di-nucleotides released by bacteria [99]. Type I IFN secretion resulting from *Francisella* infection exacerbates the infection. It was reported that type I IFNs suppress host immunity by inhibiting IL-17A expression of γδT cell, and deficiency in type Ⅰ IFN-related molecules, such as cGAS, STING, IFNAR1, and IRF3, in mice result in resistance to *Francisella* infection [91,95,100]. However, bacterial factors involved in the modulation of host type I IFNs and their mechanisms of action are incompletely understood. In this study as well, INF-β was suppressed in U937 cells infected with *F. novicida,* and *pyrC* was related to this suppression. To elucidate the mechanisms involved in IFN-β

suppression, the STING inhibitor H-151 was used. H-151 decreased the induction of IFN-β in U937 cells infected by the Δ*pyrC* mutant strain, indicating that *pyrC* is involved in the suppression of pathways related to STING. IFN-β production through STING in *Francisella* infected cells has been observed in human cell line and mouse infection models [90,101]. Several studies revealed that IFN-β secretion by the STING-dependent detection of *F. novicida* in host cell cytoplasm boosts inflammasome activation, IL-1β release, and pyroptosis by promoting gene expression of gamma guanylate proteins, ZBP1, and pyrin [95,102–104]. In our investigation, IFN-β and IL-1β were induced in U937 cells infected with the Δ*pyrC* mutant strain, not only at the protein level, but also at the mRNA level. Because STING pathway activation was found to induce IL-1β mRNA expression in a type I IFN-dependent manner [105], our findings suggest that *pyrC* is crucial for IFN-β suppression followed by the suppression of IL-1β secretion.

To determine the ligands that induce TNF-α in U937 cells infected with the Δ*pyrC* mutant strain, we treated U937 cells with heat-inactivated *Francisella* mutant strains, including the Δ*pyrC* mutant strain. TNF-α production decreased to control levels in all the heat-inactivated strains. This result indicates that TNF-α production is not caused by the recognition of *F. novicida* by heat-resistant ligands, such as LPS, peptidoglycan, and nucleotides, present outside the cells, but by biological responses of *F. novicida*, such as intracellular proliferation. A previous report on *Pseudomonas aeruginosa* indicated that uracil controls biofilm formation via quorum-sensing, and *P. aeruginosa* mutants lacking genes involved in uracil biosynthesis could not form biofilms [106]. A biofilm is a structured community of microbial cells in a matrix formed by extracellular polymeric substances (EPS). The EPS consist of polysaccharides, nucleic acids (extracellular DNA and RNA), proteins, lipids, and other biomolecules [107]. It has also been shown that biofilms formed

by *Mycobacterium avium* and *P. aeruginosa* and the extracellular DNA of these bacteria have the potential to induce TNF-α production in host cells infected by them [108,109]. In the Δ*pyrC* mutant strain-infected U937 cells, the production of the TNF-α, IL-1β, and IFN-β cytokines was increased in response to the recognition of bacterial nucleic acids compared with that in wild-type strain-infected U937 cells. These results suggest that *pyrC* may be involved in the coordination or modification of ligands, such as extracellular DNA in host cytosol, protecting *Francisella* from TLR or STING recognition and allowing it to grow intracellularly by suppressing immune responses.

Intracellular growth is one of the most important abilities determining *Francisella*'s pathogenicity, a lot of genes involved in the intracellular growth of *Francisella* have been identified in previous studies [82,110]. PyrC is required for *de novo* pyrimidine biosynthesis [111]. Mutants of *Francisella* genes involved in the pyrimidine pathway (e.g., *carA*, *pyrB*, *pyrD*, and *pyrF*) become uracil auxotrophs and show deficient growth on complete medium [81,82,112]. Several reports indicate that these pyrimidine pathway-related mutants can grow within epithelial cells but not macrophage cells [81,82]. In addition, Schulert et al showed that a pyrimidine biosynthesis pathway transposon mutant of *F. novicida* was eliminated by monocyte-derived macrophages, in part via phagosomes [97]. In our study, the Δ*pyrC* mutant showed decreased growth in culture medium, and decreased but constant intracellular growth in U937 cells compared with that of the wild-type strain. *Francisella* mutants that is deficient in the phagosomal escape-related factor such as *mglA* or type VI secretion system, have no ability to induce IL-1β secretion [90,113,114]. These results indicate that the Δ*pyrC* mutant strain can enter the host cytoplasm and grow intracellularly. Although the intracellular bacterial number of the Δ*pyrC* mutant strain was relatively low, it increased the production of cytokines compared with that in cells

infected with the wild-type strain, suggesting strongly that *pyrC* contributes to the suppression of host immune responses.

In summary, we performed here, a large-scale screening to search for factors responsible for immunosuppression by *F. novicida* in human macrophage cells. Ten genes were determined to be responsible for the immunosuppression. Among them, *pyrC* was identified as a novel *F. novicida* immunosuppressive factor and was immunologically characterized. Although further studies are needed to elucidate the detailed mechanisms by which *pyrC* is involved in host immunosuppression by *Francisella*, research on pyrimidine metabolic pathways involving *pyrC* may provide new insight into *Francisella* immunosuppression and pathogenicity and into the mechanisms by which host cells recognize intracellular bacteria.

Figure Legends

Fig 1. Screening of the *F. novicida* transposon mutant library using a TNF-α ELISA assay

U937 cells were infected with *F. novicida* transposon mutants at an MOI of 1 or stimulated with 100 ng /ml of *E. coli* LPS- or same volume BHIc-containing medium. After 5 h of infection, TNF-α released into the cell supernatant was measured by ELISA. Data shows averages and standard deviations from three independent experiments. Transposon mutant strains that exhibited a 1.5-fold increase in TNF-α production compared with the wild-type strain are shown.

Fig 2. Cytokine induction by the *F. novicida pyrC* deletion mutant

U937 cells were stimulated with 100 ng /ml of *E. coli* LPS-containing medium or 10 ng/ml 2′3′-cGAMP- or same volume BHIc-containing medium or infected with *F. novicida* strains (MOI = 1). Cell supernatants were collected and concentrations of TNF-α at 5 h post infection (p.i.) (A), IL-1β at 12 h p.i. (B), and IFN-β at 24 h p.i. (C) were measured by ELISA. Total RNA was collected, and mRNA expression of *TNFA* at 5 h p.i. (D), *IL1B* at 5 h p.i. (E), and *IFNB1* at 12 h p.i. (F) was measured by qPCR; relative expression normalized to that in the same volume BHIc-containing medium treatment control are shown. Data shows averages and standard deviations from three independent experiments. Differences compared with the wild-type strain were determined by Dunnett's multiple comparison and are indicated by asterisks $**P < 0.01$, $*P < 0.05$.

Fig 3. Intracellular growth of the *pyrC* deletion mutant

The wild-type (WT) and the Δ*pyrC* mutant strain of *F. novicida* were cultured in BHIc (A) or CDM medium (B). Absorbances were measured at 595 nm. Data shows averages and standard deviations from three independent experiments. Differences compared with the wild-type strain at 12 h were determined by the Student's *t* test and significances were indicated by asterisks; $^{**}P < 0.01$, $^{*}P < 0.05$. (C) U937 cells were infected with GFP-expressing *F. novicida* strains at an MOI of 1 and treated with 50 μg/ml of gentamicin for 1 h. At 2–48 h post infection, the cells were fixed and observed by FV1000 confocal laser scanning microscopy. Scale bar: 20 μm. (D) U937 cells were infected with *F. novicida* strains at an MOI of 1 and treated with 50 μg/ml of gentamicin for 1 h. At indicated time points post infection, cells were disrupted with 0.1% Triton X-100 and plated on BHIc agar; colony forming units were then counted. Data shows averages and standard deviations from three independent experiments. Differences compared with the wild-type strain were determined by Dunnett's multiple comparison and are indicated by asterisks $^{**}P < 0.01$, $^{*}P < 0.05$.

Fig 4. TNF-α induction by heat-inactivated *F. novicida pyrC* deletion mutant

U937 cells were stimulated with 100 ng /ml of *E. coli* LPS- or same volume BHIc-containing medium or infected with *F. novicida* strains (MOI = 1). Heat inactivation of the bacterial suspension was performed at 90°C for 5 min. At 5 h post infection, cell supernatants were collected, and TNF-α concentrations were measured by ELISA. Data shows averages and standard deviations from three independent experiments. The differences among various conditions were analyzed by Tukey–Kramer multiple comparison and are indicated by asterisks $^{**}P < 0.01$, $^{*}P < 0.05$.

Fig 5. IFN-β induction by the *F. novicida pyrC* deletion mutant under STING inhibition

(A) The wild-type (WT) and the Δ*pyrC* mutant strain of *F. novicida* were cultured in BHIc medium containing 0.5 μM H-151 or the same volume of dimethyl sulfoxide (DMSO). Absorbances were measured at 595 nm. Data shows averages and standard deviations from three independent experiments. Differences compared with each DMSO condition at 12 h were determined by the Student's t test and significances were indicated by asterisks; $^{**}P < 0.01$, $^{*}P < 0.05$, n.s. = not significant.

(B) U937 cells were treated the H-151 STING inhibitor 2 h prior to infection and then stimulated with 10 ng/ml 2′3′-cGAMP, BHIc medium or infected with *F. novicida* strains (MOI = 1). At 24 h post infection, cell supernatants were collected, and concentrations of IFN-β were measured by ELISA. Data shows averages and standard deviations from three independent experiments. The differences among various conditions were determined by Tukey–Kramer multiple comparison and are indicated by asterisks $^{**}P < 0.01$, $^{*}P < 0.05$.

Tables and Figures

Table 1. The results of screening and sequence analysis of transposon mutants.

Strain	TNF-α (pg/ml) mean ± S.D.	Locus	Name	Putative protein
Wild-type (WT)	432.39 ± 131.00	-	--	
A11-6	2912.36 ± 1332.87	*FTN_0756*	*fopA*	OmpA family protein
B22-5	2489.22 ± 1021.12	*FTN_1641*	*ampG*	Major Facilitator Superfamily protein
C15-5	2635.81 ± 1576.51	*FTN_1286*	*mltA*	MltA specific insert domain protein
E12-3	1153.80 ± 797.89	*FTN_1199*	*capA/B*	Poly-gamma-glutamate system family protein
H31-3	946.36 ± 303.20	*FTN_0757*	*cas9*	CRISPR-associated endonuclease Cas9
I11-2	1138.33 ± 445.28	*FTN_1548*	*yfgL*	Outer membrane assembly lipoprotein YfgL
J17-1	2068.55 ± 942.58	*FTN_0917*	*dacB*	D-alanyl-D-alanine carboxypeptidase/D-alanyl-D-alanine-endopeptidase
J21-3	2925.88 ± 1726.95	*FTN_0496*	*slt*	Transglycosylase SLT domain protein
L8-8	3024.12 ± 1718.24	*FTN_0496*	*slt*	Transglycosylase SLT domain protein
L32-4	2094.21 ± 1281.19	*FTN_0611*	*kdsA*	3-deoxy-D-manno-octulosonic acid 8-phosphate synthase
M14-8	970.04 ± 320.70	*FTN_0757*	*cas9*	CRISPR-associated endonuclease Cas9
M20-7	1290.48 ± 310.36	*FTN_0024*	*pyrC*	Dihydroorotase, multifunctional complex type domain protein
O7-8	2285.06 ± 352.58	*FTN_0496*	*slt*	Transglycosylase SLT domain protein
Δ*slt*	1767.43 ± 351.55	*FTN_0496*	*slt*	Transglycosylase SLT domain protein
LPS (O127:B8)	1491.96 ± 455.53	-	-	-

Table 2 Primer sets and template

Plasmid construction.

Vector	Primer	Sequence	Template (Reference)
pMOD3-FtKm	pMOD3-kanR.FOR	CATCGTGGCCGGATCG ATCTTTTGGGTTGTCAC TCATCGTATT	pKEK1440 [35]
	pMOD3-kanR.REV	ATTAACCAATTCTGATT AGAAAAACTCATCGAG CATCAAATGAAACT	
	pMOD3-kanR-vector.FOR	TCAGAATTGGTTAATTG GTTGTAACACTGG	pMOD3
	pMOD3-kanR-vector.REV	GATCCGGCCACGATGC G	
pFRSU-pyrC	FTN_0024_up1.5.FOR	GCAGCCCGGGGGATCG ATTTCAACCAATACCAA TAATGACCTTGTCAG	*F.novicida* genome
	FTN_0024_up1.5.REV	TGATAGCATTTTTCCTC CTTTATAATCTACCCAA ATTATAATGAACCG	
	FTN_0024_down1.5.FOR	GGAAAAATGCTATCAG CTACTAATGTTGTAGGG AAA	
	FTN_0024_down1.5.REV	TAGAACTAGTGGATCG GGGAATCAGCACATTA GCGATTAG	
	FTN_0024_check.FOR	ACCCACATCATCATTTT GCCA	
	FTN_0024_check.REV	GGATGAACTCCTAGCA CACCA	
pOM5-pyrC	pOM5_PyrC.FOR	AGCAAGGAGAAGTCAA TGTCAAATCAAAGTTTA CTTATCAAAAATGCTAC GGTTGTAAATGAG	pOM5-IglC (Nakamura *et al*., 2019)
	pOM5_PyrC.REV	TGATCTTTTCTACGGTC AAAACTCATGATTAAA CTCTAAACATTGTCCTC TTTGATCGC	

Real-time q-PCR.

GENE	Primer	Sequence
HPRT1	Human HRPT1.FOR	GACCAGTCAACAGGGGACAT
	Human HRPT1.REV	AACACTTCGTGGGGTCCTTTTC
TNF	Human TNF.FOR	CCTCTCTCTAATCAGCCCTCTG
	Human TNF.REV	GAGGACCTGGGAGTAGATGAG
IL1B	Human IL1B.FOR	AGCTACGAATCTCCGACCAC
	Human IL1B.REV	CGTTATCCCATGTGTCGAAGAA
IFNB1	Human IFNB1.FOR	TTGACATCCCTGAGGAGATTAAGC
	Human IFNB1.REV	TTAGCCAGGAGGTTCTCAACAATAG

Figure 1

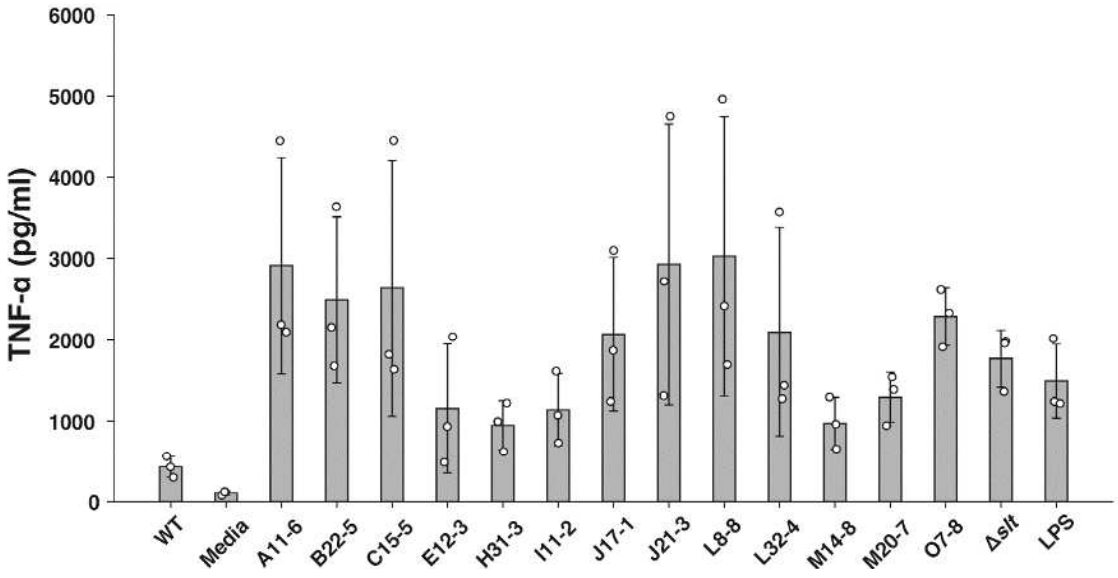

TNF-α (pg/ml)
6000
5000
4000
3000
2000
1000
0
WT
Media
A11-6
B22-5
C15-5
E12-3
H31-3
I11-2
J17-1
J21-3
L8-8
L32-4
M14-8
M20-7
O7-8
Δ*slt*
LPS

Figure 2

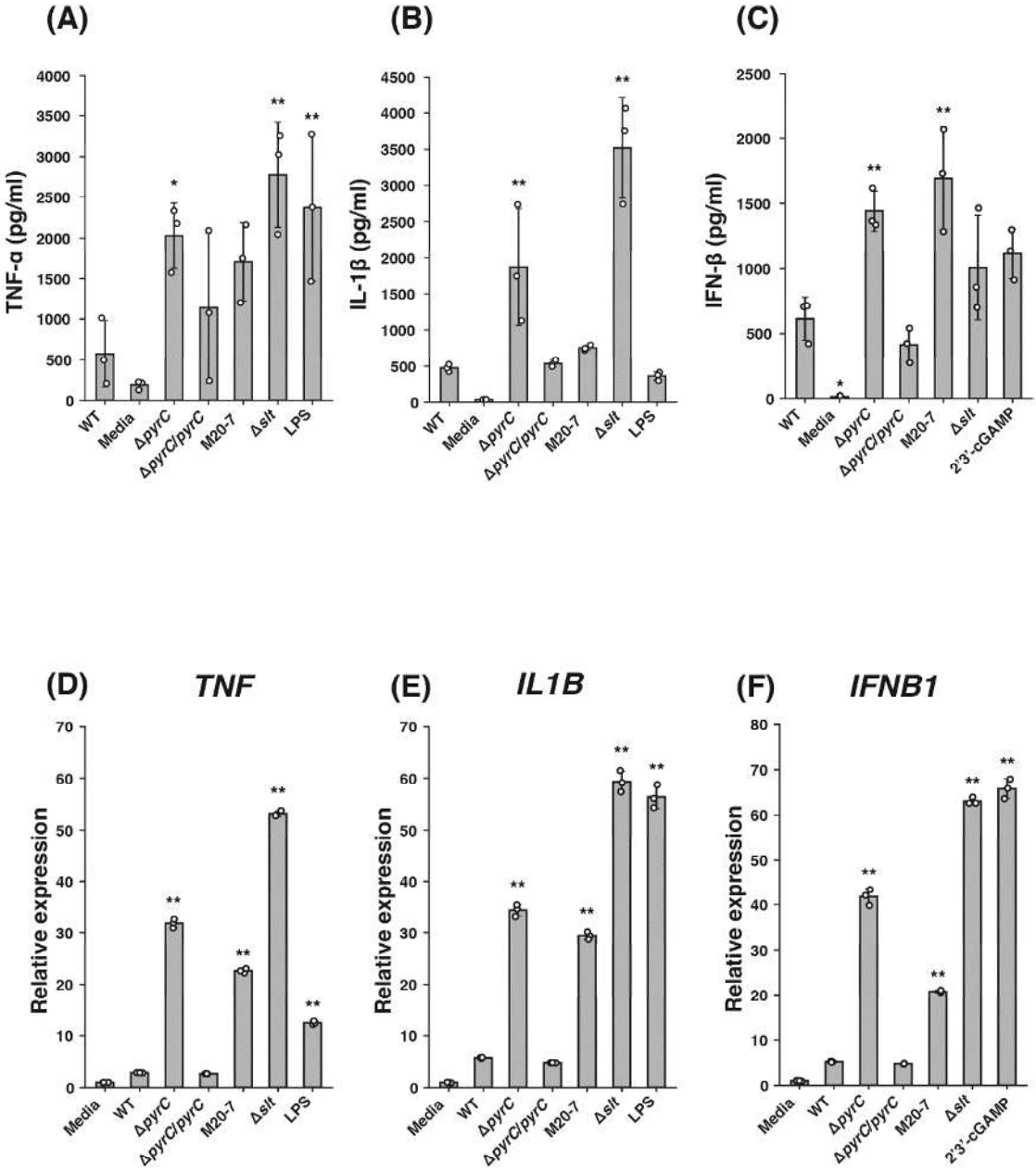

(A)
TNF-α (pg/ml)
(B)
IL-1β (pg/ml)
(C)
IFN-β (pg/ml)
WT
Media
ΔpyrC
ΔpyrC/pyrC
M20-7
Δslt
LPS
2'3'-cGAMP
(D)
TNF
(E)
IL1B
(F)
IFNB1
Relative expression

Figure 3

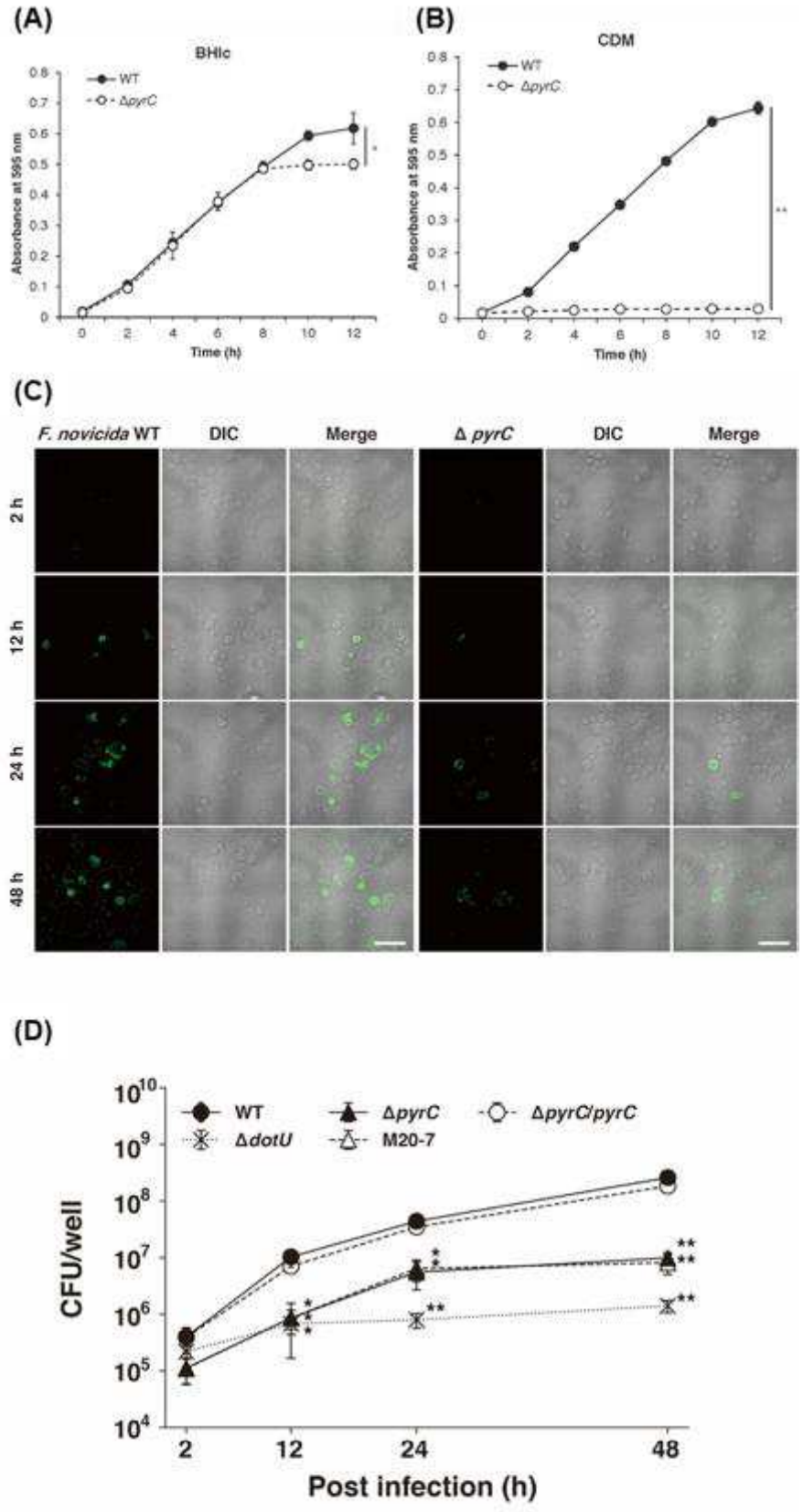

(A)
BHIc
WT
ΔpyrC
Absorbance at 595 nm
Time (h)
(B)
CDM
WT
ΔpyrC
Absorbance at 595 nm
Time (h)
(C)
F. novicida WT
DIC
Merge
Δ pyrC
DIC
Merge
2 h
12 h
24 h
48 h
(D)
WT
ΔpyrC
ΔpyrC/pyrC
ΔdotU
M20-7
CFU/well
Post infection (h)

Figure 4

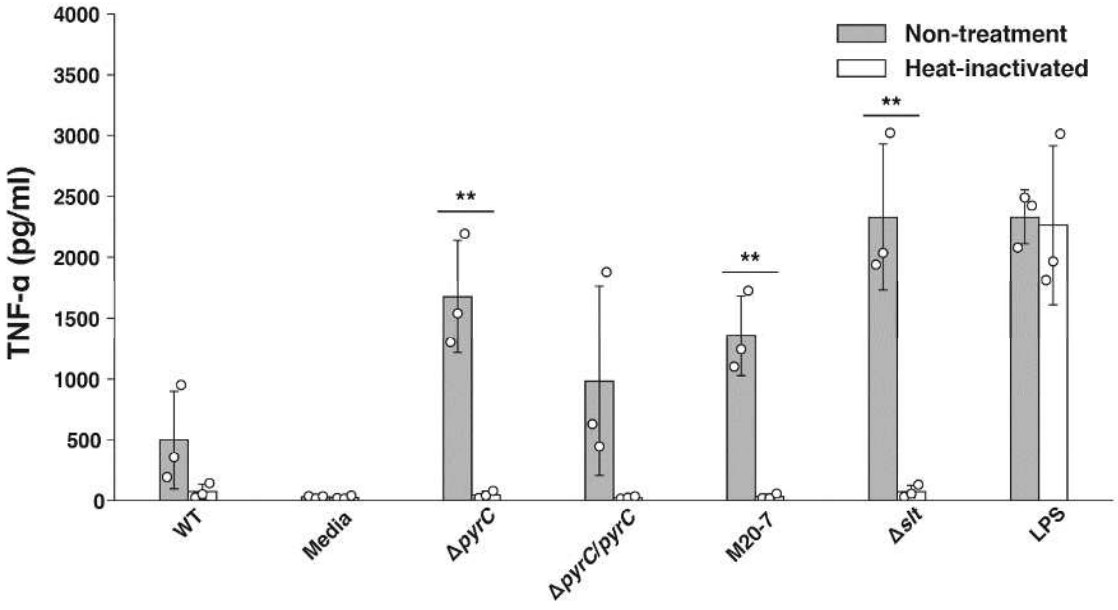
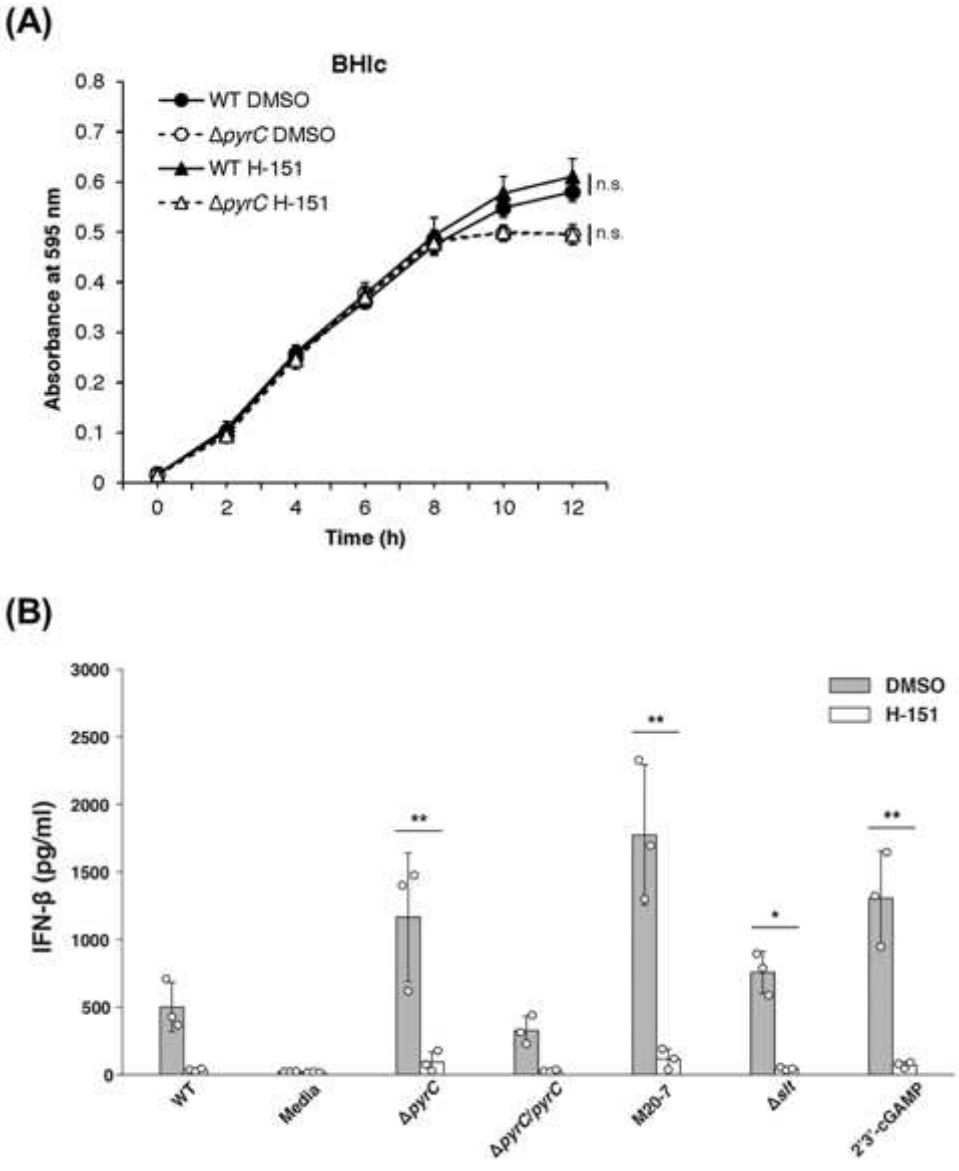

Non-treatment
Heat-inactivated
TNF-α (pg/ml)
4000
3500
3000
2500
2000
1500
1000
500
0
**
**
**
WT
Media
ΔpyrC
ΔpyrC/pyrC
M20-7
Δslt
LPS

Figure 5


(A)
BHIc
WT DMSO
ΔpyrC DMSO
WT H-151
ΔpyrC H-151
n.s.
n.s.
Absorbance at 595 nm
Time (h)
(B)
DMSO
H-151
IFN-β (pg/ml)
WT
Media
ΔpyrC
ΔpyrC/pyrC
M20-7
Δsit
2'3'-cGAMP

General conclusion

Since the discovery of antibiotics by Fleming in 1929 [115], bacterial infections are changed controllable diseases. However, there are still numerous infectious diseases for which the mechanism of transmission is unclear. Recently, drug-resistant bacteria have been causing an estimated 700,000 deaths each year worldwide [116], which has begun to raise questions about the infection control through antibiotics. Additionally, recent climate changes affecting the distribution of arthropods and the rapid movement of people have made the spread of infectious diseases more global, increasing the risk of emerging and re-emerging infectious diseases [117]. These facts highlight the urgent need for new methods of infection control. In such times, the importance of understanding each bacterial infection mechanisms are growing. In this Ph. D. theses, we focused on elucidating the infection mechanisms of bacteria, specifically *Francisella tularensis*, a bacterium that transmission and pathogenicity are not yet fully understood.

In Chapter 1, we identified genes crucial for the maintenance of *F. novicida* within arthropod vectors using a silkworm infection model. The *F. novicida* mutant strain deficient in the gene *mtlA*, which is involved in the degradation of peptidoglycan, showed a significant decrease in proliferation ability within the silkworm body and intracellularly. The *mtlA* was also found to be important for proliferation in human cells. The *Francisella* genus has a diverse host range that extends from mammals to insects and fish. Although there are genetic differences resulted from adaptation to each host among different strains [118], pathogenic factors crucial for survival inside host cells, such as the type VI secretion system, are conserved between subspecies [119]. Experiments conducted using human cells in this study demonstrated that a system utilized to identify pathogenicity in silkworms can also be effective to search for pathogenic

factors for humans. This result suggests that the *mltA* is important for resisting mechanisms common to various hosts to eliminate *Francisella*. These results highlighted the need for a thorough investigation of the differences and similarities among *Francisella* subspecies. A comparative approach that elucidates the relationships between *Francisella* subspecies and multiple hosts is crucial for understanding this bacterium.

In Chapter 2, we attempted to identify factors important for the evasion/suppression of innate immunity in infected human macrophage cells by measuring the amount of the pro-inflammatory cytokine in the cell culture supernatant using the ELISA. As a result, the deficiency of the gene *pyrC,* involved in the synthesis of pyrimidine bases, decreased the immune evasion ability of *F. novicida* and enhanced the production of major cytokines in infected cells. The deficiency in *pyrC* affecting the production of various cytokine productions suggests that it is a key factor broadly involved in the immune evasion system of *F. novicida*. In particular, the data indicated the involvement of *pyrC* gene in the recognition by the inflammasome and STING pathway. Recent studies highlighted the activation of the STING pathway and inflammasome by *Francisella* DNA within infected cells shows different reactivities among subspecies, suggesting an important role in the host's immune response to *Francisella* infection [102,120,121]. Further detailed analysis of the function of *pyrC* using different subspecies may provide insights that could lead to vaccine development.

Across the two studies, we used a transposon mutant library to identify important factors contributing to the pathogenicity of the *Francisella* and conducted detailed analyses on two of these genes. Although our analyses were limited to only two genes, the results of both screenings indicate that there is an involvement of multiple genes in

transmission and immunosuppression of *Francisella*. These findings emphasized that many functional unknown factors related to the pathogenicity and transmission of the *Francisella* still exist. Though this study was performed using the low-virulent strain *F. novicida*, genes homologous to those identified are conserved in the virulent strains. Further investigation is required to discuss their association with the pathogenicity of highly virulent strains. Nonetheless, the insights gained from this Ph. D. thesis will contribute to comprehensive understanding of the *Francisella*.

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