

**Study on the identification and characterization of virulence
determinants of *Francisella novicida***

フランシセラ・ノヴィシダの病原性決定因子の同定と特徴付けに関する
研究

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March 2026

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General Introduction

Zoonotic diseases are infections that spread from animals to humans. They may be caused by bacteria, viruses, parasites, or fungi, and they pose risks to people of all ages and backgrounds. Humans can contract these illnesses through direct contact with infected animals [1], by consuming contaminated food or water [2], or from bites of infected vectors such as ticks and mosquitoes [3]. Gaining a clear understanding of how zoonotic diseases arise and spread is a pressing concern for public health. In this study, I focused on uncovering the transmission routes and pathogenic mechanisms of *Francisella tularensis*, the bacterium responsible for tularemia.

Tularemia is a rare bacterial zoonotic disease caused by *Francisella tularensis*. No vaccine is currently available to prevent tularemia. Human infection can occur naturally through an arthropod bite (e.g., deer flies and ticks), improper handling of infected animals, inhaling contaminated aerosols, or drinking contaminated water. Clinical manifestations typically include fever and localized symptoms that vary by route of infection, such as skin ulcers, regional lymphadenopathy, and pneumonia [4].

Francisella tularensis is a highly infectious Gram-negative bacterium that causes tularemia, a zoonosis affecting humans and animals. The genus *Francisella* comprises several species including *F. tularensis*, *F. philomiragia*, *F. noatunensis*, *F. hispaniensis* and *F. novicida* [5, 6]. This is due to recent outbreaks of tularemia around the world and the fact that *F. tularensis* is classified as a Category A biological threat agent. *F. tularensis* is further divided into four subspecies: *tularensis* (type A), *holarctica* (type B), *mediasiatica*, and *novicida*. Among them, *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* exhibit the highest virulence in humans and are responsible for the majority of tularemia cases [5].

The less virulent environmental strain *Francisella novicida* is frequently employed as a model organism for *Francisella* research. This is largely due to its high genetic similarity to *F. tularensis* (>97%), its ease of genetic manipulation, the availability of a comprehensive transposon mutant library, and its classification as a biosafety level 2 organism given its limited pathogenicity in humans. While *F. tularensis* subsp. *novicida* (commonly referred to as *F. novicida*) rarely causes disease in humans; it is a facultative intracellular pathogen that can replicate within macrophages and is pathogenic in mouse models. For this reason, *F. novicida* serves as a useful surrogate for studying *Francisella* biology [5,7].

In this Ph.D. thesis, I attempted to identify the important factors contributing to the characteristic phenotypes of *Francisella* by transposon mutant strain library screening. I screened 2,232 transposon mutants of *F. novicida* by infecting the mutants to the newly developed epithelial infection models, HeLa–FcγRII, to search for mutants that have less cytotoxicity. Among the 13 mutants isolated, particular focus was placed on the *FTN_0096* and *wbtF* genes. Compared with phagocytic cell models, HeLa–FcγRII cells may improve the efficiency of cell-based infection assays, including large-scale genetic screens, and allow for a more comprehensive analysis of *Francisella*'s behavior within epithelial cells. This system allows for the investigation of early host–pathogen interactions and provides a platform to dissect bacterial determinants of epithelial infection [8].

In Chapter 1, I focus on the identification and functional analysis of *FTN_0096*, a gene identified through the screening of 2,232 transposon mutants of *F. novicida*. This research aims to elucidate the role of *FTN_0096* in intracellular replication and host responses. I identify *FTN_0096* by infecting HeLa–FcγRII and THP-1 cells with the *FTN_0096* mutant strain and subsequently assessing for intracellular replication and its association with phagolysosomes, mitochondria, and the Golgi complex. The *FTN_0096* protein product is a conserved hypothetical membrane protein. *FTN_0096* is a member of the DUF1275 superfamily of

proteins. Members of this family are found across all three domains of life, making them highly widespread. Although previous comprehensive analyses had implicated *FTN_0096* as a potentially important factor for intracellular replication within macrophages [9], its detailed function remains to be elucidated. The results showed that *F. novicida* wild-type strain proliferated in HeLa–FcγRII and THP-1 cells, but the number of intracellular *FTN_0096* mutants decreased. The *FTN_0096* mutant strain was unable to escape from phagolysosomes during the early stages of infection. It was also found near host organelles such as mitochondria and the Golgi complex, indicating a potential spatial association with these organelles. While the precise mechanisms remain unclear, this association suggests that *FTN_0096* may influence interactions with host organelle-mediated defenses.

Chapter 2 of this thesis addresses the identification and characterization of the *wbtF* gene, which is part of the O-antigen biosynthetic gene cluster. The study explores its role in cytotoxicity, intracellular replication, interactions with host organelles, and induction of cytokine responses. *wbtF* is located in the O-antigen biosynthetic gene cluster and has been implicated as a potential virulence determinant, but its role in epithelial or monocytic infection models had remained unclear [10]. The O-antigen capsule in *Francisella* comprises a polysaccharide that is structurally identical to the O-antigen found in lipopolysaccharide (LPS). This LPS O antigen is a critical pathogenic factor. O-antigen deficiency results in increased sensitivity to serum and a loss of virulence in Type A and B strains of *F. tularensis* [11]. A previous study by Thomas et al. [12] demonstrated that O-antigen knockouts in both *F. novicida* and *F. tularensis* SchuS4, leading to the complete loss of O-antigen production. Notably, the SchuS4 mutant lacking the O-antigen was fully attenuated in mice. By comparing the *wbtF* mutant with the wild type in both HeLa–FcγRII and THP-1 cells, I could directly compare the ability of *wbtF* mutant with the *F. novicida* Wild type.

The studies presented in Chapters 1 and 2 describe two investigations into the specific genetic determinants *FTN_0096* and *wbtF*, thereby expanding our understanding of the pathogenesis of *F. novicida*. By linking gene identification with functional testing, these studies share a common goal: to identify and characterize the genetic factors that enable *F. novicida* to survive, replicate, and exert pathogenic effects within host cells. Furthermore, revealing how *Francisella* manipulates host immune pathways provides broader insights into host-pathogen interactions, particularly those involving intracellular bacteria. Findings from the *FTN_0096* and *wbtF* studies not only expand our understanding of *Francisella novicida* pathogenesis but may also provide a framework for developing new strategies to prevent or mitigate tularemia and other intracellular bacterial infections

Chapter 1

Identification of the *Francisella novicida* FTN_0096 as a factor involved in intracellular replication and host response

1.1 Abstract

Francisella tularensis is the causative agent of the zoonotic disease tularemia. I investigated a pathogenic factor of *F. tularensis* subsp. *novicida* (*F. novicida*). Accordingly, I established a novel infection model using HeLa cells. *F. novicida* usually infects macrophage lineage cells and less frequently epithelial cells. I successfully infected HeLa cells expressing the Fc receptor (HeLa–FcγRII cells) using *F. novicida* supplemented with mouse serum containing *F. novicida* antibodies. A total of 2,232 transposon mutants of *F. novicida* were screened to determine the relatively fewer cytotoxic strains of the HeLa–FcγRII cells, and 13 strains were thus isolated. Sequencing analysis of transposon insertion sites identified 13 genes, including *FTN_0096*. I focused on *FTN_0096*. Although the *F. novicida* wild-type strain proliferated in HeLa–FcγRII and THP-1 cells, the number of intracellular *FTN_0096* mutant decreased. *FTN_0096* mutant cannot escape from phagolysosomes in the initial phases of infection. Moreover, *FTN_0096* mutant was detected in the mitochondria and Golgi complex. These findings indicate the importance of *FTN_0096* of *F. novicida* for intracellular replication in the cells.

1.2 Introduction

Francisella tularensis is a gram-negative coccobacillus that infects animals and humans through contact with infected arthropods or animals, consumption of contaminated food or water, or inhalation of bacterial aerosols [13, 14]. Taxonomy of *F. tularensis* consists of some sub-species, including *tularensis* (type A), *holartica* (type B), *mediaasiatica*, and *novicida*. Cottontail rabbits (*Sylvilagus* spp.) and ticks are the primary reservoirs of type A [15]. *F. tularensis* is a highly pathogenic facultative intracellular bacteria that can cause tularemia—a zoonosis disease [11]. *F. tularensis* is a dangerous pathogen that can incur a high risk of morbidity and mortality. *F. tularensis* causes typhoidal and pneumonic systemic tularemia, which has a fatality rate of up to 60% [16]. *F. tularensis* has been classified as a Tier 1 select agent by the Centers for Disease Control and Prevention (CDC) due to its extremely low infectious dose, potential for broad dissemination, and documented history of use as a biological weapon [17].

F. tularensis subsp. *novicida* is similar to *F. tularensis* but is a less virulent subspecies, as it can cause severe illness in mice but does not, usually, have much impact on the immunocompetence of humans. *F. novicida* is characterized as an uncommon opportunistic human infecting agent, capable of inducing morbidity and fatality in case of severe pathologies in either immunocompromised or medically weakened patients. *F. novicida*, which is regarded as a more environmentally adapted species, also shares several similarities to more virulent subspecies, including the LVS and Schu S4, a human virulent type A strain. *F. novicida* has a similar intracellular replication cycle within macrophages, characterized by swift phagosome escape and vigorous cytosolic replication, making it a commonly employed mouse model for studying the pathogenic mechanisms of tularemia [5, 18].

For *F. tularensis* to effectively develop an infection and cause disease, it needs to replicate within the host cells. The intracellular lifecycle of *F. tularensis* has been observed in

vitro with fixed and live-cell microscopy. Phagosome escape is necessary for intracellular replication, after which the organism must adapt to and replicate inside the host cytoplasm. When *F. tularensis* is internalized by a host cell, it enters macrophages via the phagocytosis process and evades lysosomal fusion, then the bacterium degrades the phagosome within 30 min and proliferates in the cytosol [19-21]. Phagosome escape is a crucial phase in *F. tularensis* lifecycle, and mutations in the *Francisella* pathogenicity island (FPI), which is responsible for encoding an alternate secretion pathway, lead to failed phagosome escape [22]. A characteristic of *F. tularensis* pathogenicity is its ability to remain immunologically silent during infection in both types of hosts (i.e., mice and people) and at the cellular level [23]. *F. tularensis* virulence factors, intracellular lifestyle, and interaction with different cell organelles are poorly known [24].

F. novicida infections rarely involve them due to their significantly lower infection efficiency in epithelial cell compared to macrophage-like cells. Research involving epithelial cell lines is markedly limited when compared to that of macrophage cell lines [8]. The efficiency of *F. novicida* uptake by macrophages is strongly influenced by opsonization. Opsonized bacteria, whether by serum or antibodies, are internalized by macrophages at levels that are 10-fold greater than their unopsonized counterparts [25]. Macrophages derived from the bone marrow of mice lacking Fc-gamma receptors (Fc γ R) show a 90% decrease in their ability to take up immunoglobulin G (IgG)-opsonized bacteria. Consequently, Fc γ R-mediated uptake could be a promising strategy for facilitating *F. novicida* infection in epithelial cells. Based on a previous research, HeLa–Fc γ RII cells were used as a new model to study how *F. novicida* infects epithelial cells [8].

The *FTN_0096* protein product is a conserved hypothetical membrane protein. *FTN_0096* is a member of the DUF1275 superfamily of proteins [9]. Members of this family are found across all three domains of life, making them highly widespread. DUF1275 belongs

to a large family characterized by a consistent structure of six—and occasionally seven—transmembrane segments (TMSs), which aligns with what's typically seen in half-sized proteins of the Major Facilitator Superfamily (MFS). Some of these proteins are encoded by genes located near what appears to be a YtcJ-like metallo–amido–hydrolase, suggesting a possible role in transporting peptides or other amido compounds, or exporting their breakdown products. However, none of these proteins have been functionally studied in detail, and their transport mechanisms remain unclassified [26].

In this study, I generated a transposon mutant library of *F. novicida* and screened it by infecting the mutant to the epithelial cells, HeLa cells expressing the Fc-gamma receptor (HeLa–FcγRII). This cell has the potential to enhance the efficiency of cell-based infection assays, such as large-scale genetic screening, and to offer novel insights into *Francisella* infection in epithelial cells, which has been difficult to analyze in phagocytic cells [8]. I focused on *FTN_0096* and discovered that the *FTN_0096* gene is a pathogenic factor in *F. novicida*.

1.3 Materials and Methods

Bacterial strains and culture conditions

F. novicida U112 was sourced from the Pathogenic Microorganism Genetic Resource Stock Center (Gifu University). The bacterium was cultured aerobically at 37°C using either a chemically defined medium (CDM) [27] or a brain–heart infusion broth (Becton, Dickinson and Company, Franklin Lakes, NJ) enriched with cysteine (BH1c) [28] which included 1.5% agar (Wako Laboratory Chemicals, Osaka, Japan).

Cell culture

HeLa–FcγRII cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) enriched with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, MA, USA) and incubated at 37°C in a humidified atmosphere with 5% CO₂. THP-1 cells, a human monocytic cell line, were cultured in RPMI 1640 medium (Sigma-Aldrich) enriched 10% heat-inactivated FBS under the same temperature and CO₂ conditions.

Construction of a transposon mutant library

The transposon mutant library was developed using the Ez-Tn5 transposon system (Epicentre, Madison, WI). To construct pMOD3-FtKm, the multiple cloning sites of pMOD3 were digested with *Hind*III and *Eco*RI, and the kanamycin-resistance cassette of pKEK1140 [29] was inserted into these sites to generate pMOD3-FtKm. The transposon region of pMOD3-FtKm was then amplified by PCR, purified, and mixed with transposase following the protocol. This mixture was used to transform *F. novicida* via cryotransformation. The resulting transformants were plated on BH1c agar supplemented with 50 µg/mL of kanamycin.

Screening of transposon mutant library

Microscopic observations were used to screen a mutant library of *Francisella novicida* to identify mutants with less cytotoxicity toward HeLa-FcγRII cells. To validate the cytotoxicity, lactate dehydrogenase (LDH) release assays were performed. HeLa-FcγRII cells (5×10^4 cells/well) were seeded into 48-well tissue culture plate for 24 h. *F. novicida* strains preincubated with mouse serum containing specific antibodies were used at a multiplicity of infection (MOI) of 1.0. The cultured plates were centrifuged at $300 \times g$ for 10 minutes at room temperature, followed by incubation at 37°C for a designated period. After incubation, the cells were washed thrice with DMEM medium, and extracellular bacteria were eliminated by treating the cultures with gentamicin (50 µg/mL) for 1 hour. To measure LDH release as a marker of cytotoxicity, cells were incubated in the DMEM medium at 37°C for the specified time. The release of LDH into the culture supernatant was quantified using the LDH Cytotoxicity Detection Kit (Takara Bio, Shiga, Japan).

Sequence analysis of transposon mutants

The plasmid pMOD3 contains the R6Kγ origin of replication from *Escherichia coli*. Genomic DNA from *F. novicida* transposon mutants was extracted using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific) and digested with a combination of restriction enzymes *Xho*I, *Bgl*II, *Eco*RI, *Sal*I, *Not*I, *Bam*HI, *Pst*I, and *Sph*I. The resulting DNA fragments were blunt-ended using the DNA-Blunting Kit (Takara Bio) and subsequently ligated using Ligation High Ver. 2 (Toyobo). The ligation products were used to transform One-Shot PIR1 Chemically Competent *E. coli* (Thermo Fisher Scientific). Transformants were selected on kanamycin-containing plates, and plasmid DNA was purified from the resistant colonies. Sequence analysis was performed using a primer described in the Ez-Tn5 transposon system manual.

GFP-expression of *F. novicida* and complimentary strains

The GFP-expressing plasmid pOM5-GFP was constructed in accordance with the procedures published elsewhere [30]. This plasmid was introduced into the wild-type and *FTN_0096* strains of *F. novicida* via electroporation. To create pOM5-0096 or its complementary, *FTN_0096* gene along with its native promoter region (300-bp upstream) was cloned into the pOM5 vector. pOM5-0096 was then used to transform into the *FTN_0096* mutant of *F. novicida* via electroporation.

Intracellular growth assay

THP-1 cells (4×10^5 cells/well) were seeded into 24-well tissue culture plate and treated with 200 nM phorbol 12-myristate 13-acetate (PMA) for 48 h to induce differentiation. *F. novicida* strains were added at an MOI of 1. HeLa-FcγRII cells (1×10^5 cells/well) were incubated overnight in 24-well tissue culture plates. *F. novicida* strains were incubated with mouse serum containing *F. novicida* antibodies and infected at an MOI of 1 to the HeLa-FcγRII cells. The THP-1 cells were washed thrice with RPMI1640 medium, HeLa-FcγRII cells were washed thrice with DMEM medium, and extracellular bacteria were eliminated by treating the cultures with gentamicin at the concentration of 50 µg/mL for 1 h. The cells were incubated in a fresh medium at 37°C for the desired time points. The cells were washed with phosphate-buffered saline (PBS) and then lysed with 0.1% Triton X-100 in CDM to determine the intracellular growth. Colony-forming units (CFUs) were determined by plating serial dilutions of the lysates on BHIc agar plates.

Fluorescence microscopy

THP-1 cells (4×10^5 cells per well) were seeded onto 12-mm glass coverslips placed in 48-well tissue culture plates and treated with 100 nM of PMA for 48 h. HeLa-FcγRII cells ($1 \times$

10⁵ cells/well) were plated onto 12-mm glass coverslips in 24-well tissue culture plates and incubated overnight. THP-1 cells were infected with GFP-expressing *F. novicida* strains and incubated for the specified times. Separately, GFP-expressing *F. novicida* strains were opsonized with mouse serum and used to infect HeLa-FcγRII cells at a multiplicity of infection (MOI) of 1.0, followed by incubation at the indicated time points. The cells were stained with LysoTracker Red DND-99, MitoTracker[®] Deep Red FM, and BODIPY TR (Thermo Fisher Scientific) to visualize lysosomes, mitochondria, and Golgi complex as per the source's instruction manual. To detect lysosomal-associated membrane protein 1 (LAMP-1), the cells were fixed using the PLP Solution Set (Wako Laboratory Chemicals) containing 5% sucrose at 37°C for 1 h, followed by permeabilized with cold methanol for 10 s. The cells were treated with an anti-LAMP-1 antibody (ab25245, 1:100, Abcam) and subsequently stained with FITC-conjugated anti-rat IgG (1:1000, Abcam). The percentage of colocalizing bacteria was calculated manually by counting the total colocalized and non-colocalized bacteria in at least 100 infected cells. Confocal images were acquired using a FluoView FV100 confocal laser scanning microscope (Olympus, Tokyo, Japan).

Statistical analysis

Statistical significance between groups was analyzed with the Bonferroni/Dunnett or the Student's *t*-test, $P < 0.01$ was considered statistically significant.

1.4 Results

Identification of *F. novicida* genes with reduced cytotoxicity to HeLa–FcγRII cells

I previously developed new infection model of *F. novicida* using HeLa cells expressing mouse FcγRII (HeLa–FcγRII). HeLa–FcγRII cells enhanced *F. novicida* infection efficiency in epithelial cells and enabled more detailed study of *Francisella*'s intracellular behavior [8]. Using this new infection model, I screened the *F. novicida* transposon mutant library to identify the new virulence factor of *F. novicida*. Considering that intracellular invasion of bacteria was mediated by antibodies and FcγRII in this model, I expected to elucidate new intracellular growth factors that could not be detected via conventional analysis using macrophages. I demonstrated in a previous study that *F. novicida* infection was cytotoxic to HeLa–FcγRII and induced cell death. Therefore, I made microscopic observations to screen the mutant library of *F. novicida* that lacked cytotoxicity (Figs 1A and 1B). Among 2,232 transposon mutants examined, 13 were identified as being less cytotoxic. LDH assays were conducted to validate these findings. When compared to the wild-type strain, these mutants reduced the extent of LDH release from HeLa–FcγRII cells (Fig 1C). The transposon-insertion sites in the mutant strains were identified through sequencing to identify the genes. I focused on mutants with the code number E12-3, where the sequencing results encoded *FTN_0096* and the function of *FTN_0096* were analyzed.

Effect of *FTN_0096* mutation on intracellular growth and cytotoxicity in HeLa–FcγRII cells

To analyze the function of *FTN_0096* on intracellular growth and cytotoxicity, I infected the *FTN_0096* transposon mutant of *F. novicida* to HeLa–FcγRII cells. The *FTN_0096* mutant (E12-3) induced a decreased LDH-release in comparison with the wild-type strain in HeLa–FcγRII cells (Fig 1C). To investigate the mechanism underlying cytotoxicity, I assessed

the intracellular growth of the *FTN_0096* mutant in HeLa–FcγRII cells. While the wild-type *F. novicida* strain exhibited robust intracellular proliferation, the *FTN_0096* mutant showed significantly reduced intracellular growth in HeLa–FcγRII cells (Fig 2B). Replication was reinstated to the wild-type level in the complemented strains (Fig 2B). I also detected intracellular bacteria using GFP-expressing *F. novicida* strains. I examined the intracellular growth of the wild type and *FTN_0096* mutant at 12 and 24 h after infection in HeLa–FcγRII cells (Fig 2A). These results indicated that *FTN_0096* was essential for the cytotoxicity and intracellular growth of *F. novicida* using HeLa–FcγRII cells.

Escape of the bacterial cells from phagolysosomes in HeLa–FcγRII cells

To investigate the function of *FTN_0096* in intracellular growth, the ability of *F. novicida* strains to escape from phagolysosomes was measured. HeLa–FcγRII cells were infected with both the wild-type and *FTN_0096* mutant, and lysosomes were identified using an antibody against the lysosome marker LAMP-1 (Fig 3A). The wild-type strain exhibited continuous intracellular replication from 2 to 24 h post-infection, in line with the number of intracellular bacteria. In contrast, the number of *FTN_0096* mutant escalated until 12 h after the infection. However, the bacterial count diminished 24 h after the infection. In the case of infection with the wild-type strain, only a few numbers of bacteria showed colocalization with LAMP-1 in 27–42% of the cells (Fig 3B). On the other hand, the large numbers of *FTN_0096* mutant were observed with LAMP-1 in 70–79% cells at 2, 6, 12, and 24-h post-infection.

In order to further investigate the role of *FTN_0096* in the escape from phagolysosomes in HeLa–FcγRII cells, I observed the acidic organelles using LysoTracker. HeLa–FcγRII cells were infected with GFP-expressing *F. novicida* strains to examine their ability to escape from the phagolysosome during the early stages of infection (2 and 6 h post-infection). The cells were stained with LysoTracker and analyzed by confocal microscopy to visualize lysosomal

localization. The co-localization of the Lysotracker of the wild type was observed in only 4-5% of infected cells, but the colocalization of *FTN_0096* mutant and Lysotracker was observed in 49-50% of the cells (Fig 3C). Few wild-type bacteria colocalized with lysosome, and several from the *FTN_0096* mutant colocalized with lysosome in HeLa-FcγRII cells (Fig 3D). These observations indicated that the wild-type bacteria evaded the phagolysosomes, whereas *FTN_0096* mutant failed to escape from them in HeLa-FcγRII cells.

Effect of *FTN_0096* mutant on the mitochondria of HeLa-FcγRII cells

To further investigate the functions of *FTN_0096* in the mitochondria, HeLa-FcγRII were infected with the wild-type strains and the *FTN_0096* mutant. The mitochondria were visualized by using MitoTracker® Deep Red FM (Fig 4A) to observe whether the mitochondria can colocalize wild-type and *FTN_0096* mutant. The wild-type strain infected the HeLa-FcγRII cells, after which the intracellular bacterial cells were examined at 2 to 6 h post-infection. Nonetheless, a few cells colocalized with the mitochondria (Fig 4B). However, *FTN_0096* mutant was observed from 2 and 6 h after the infection, and several of the bacteria colocalized with the mitochondria, at approximately 75%–81% in HeLa-FcγRII cells (Fig 4B). These results suggest that *FTN_0096* mutant cannot modulate the mitochondria, rather *F. novicida* wild-type can modulate the mitochondria function and maintain their replication.

Effect of *FTN_0096* mutant infection on the Golgi complex of HeLa-FcγRII cells

To analyze the function of *FTN_0096* on the Golgi complex, I infected HeLa-FcγRII cells with the wild-type strains and *FTN_0096* mutant. The Golgi complex was stained using BODIPY TR (Fig 4D) to observe whether the Golgi complex can colocalize with the wild-type strain and *FTN_0096* mutant. The wild-type strain infected the HeLa-FcγRII cells, and the intracellular bacterial cells were then examined at 2, 6, and 12 h after infection. Few bacteria

colocalized with the Golgi complex (Fig 4C). Nevertheless, the *FTN_0096* mutant colocalized with the Golgi complex at all time points after the infection. Approximately 69%–84% of the bacteria from *FTN_0096* mutant colocalized with the Golgi complex in HeLa–FcγRII cells (Fig 4C).

Intracellular growth of *FTN_0096* mutant in THP-1 cells

To explore the function of *FTN_0096* in THP-1 cells, I measured the intracellular growth of the *FTN_0096* mutant in THP-1 cells. While the wild-type strain exhibited robust intracellular proliferation, in contrast to the *FTN_0096* mutant, for which the numbers of intracellular growths reduced in THP-1 cells (Fig 5B). Replication was reinstated up to the wild-type level in the complemented strains (Fig 5B). I also detected intracellular bacteria using GFP-expressing *F. novicida* strains. Next, I observed the intracellular growth of the wild-type strain and *FTN_0096* mutant at 12, 24, and 48 h post-infection in THP-1 cells, respectively (Fig 5A). These results indicate the importance of *FTN_0096* for the intracellular growth of *F. novicida* in THP-1 cells.

Escape of the bacteria from phagolysosomes in THP-1 cells

To assess ability of *F. novicida* to escape from the phagolysosome in THP-1 cells, I infected the wild-type and *FTN_0096* mutant into THP-1 cells and detection of lysosomes was performed using a LAMP-1–specific antibody (Fig 6A). The wild-type strain showed robust intracellular proliferation from 2 to 24 h post-infection, in line with the number of intracellular bacteria. *FTN_0096* mutant proliferated until 12 h after infection; however, the bacterial count diminished 24 h post-infection. In the case of wild-type infection, only a few numbers of bacteria showed colocalization with LAMP-1 in 27–33% of infected cells (Fig 6B). However, a large number of colocalization of *FTN_0096* mutant with LAMP-1 was observed in 70–79%

of the cells at 2, 6, 12, and 24 h post-infection, indicating significant colocalization of the mutant with LAMP-1 (Fig 6B).

To further investigate the role of *FTN_0096* in the escape from phagolysosomes in THP-1 cells, the cells were infected with GFP-expressing wild-type strain and *FTN_0096* mutant and acidic organelles were stained using Lysotracker. The wild-type strain showed colocalization with Lysotracker only in 5-6% of the cells, whereas the *FTN_0096* mutant was colocalized with Lysotracker in 50-57% of the cells (Fig 6C). A few wild-type bacteria colocalized with lysotracker and several the *FTN_0096* mutant had colocalized with lysotracker in THP-1 cells (Fig 6D). These observations indicated that the wild-type bacteria evaded the phagolysosomes, whereas *FTN_0096* mutant failed to escape from them in THP-1 cells.

Effect of *FTN_0096* mutant on the mitochondria of THP-1 cells

To further investigate the functions of *FTN_0096* in the mitochondria of THP-1 cells, THP-1 cells were infected with the wild-type strains and *FTN_0096* mutant. The mitochondria were visualized using MitoTracker[®] Deep Red FM (Fig 7A) to observe whether the mitochondria colocalized the wild-type strain and *FTN_0096* mutant. The wild-type strain infected the THP-1 cells, and intracellular bacterial cells were examined 2 to 6 h post-infection. Nonetheless, few cells colocalized with the mitochondria (Fig 7B). However, the *FTN_0096* mutant was observed from 2 to 6 h post-infection, and several cells (57%–77%) had colocalized with the mitochondria in THP-1 cells (Fig 7B).

Effect of *FTN_0096* mutant infection on the Golgi complex of THP-1 cells

To analyze the functions of *FTN_0096* in the Golgi complex, I infected THP-1 cells with wild-type strains and *FTN_0096* mutant. The Golgi complex was stained with the

BODIPY TR (Fig 7D) to observe whether the Golgi complex can colocalize with the wild-type strain and *FTN_0096* mutant. The wild-type strain infected the THP-1 cells, and intracellular bacterial cells were examined at 2, 6, and 12 h post-infection. Few bacterial cells colocalized with the Golgi complex (Fig 7C). Nevertheless, the *FTN_0096* mutant colocalized with the Golgi complex from 2, 6, and 12 h post-infection. Around 63%–82% of the bacterial cells from *FTN_0096* mutant colocalized with the Golgi complex in THP-1 cells (Fig 7C).

1.5 Discussion

Intracellular bacteria, such as *Francisella*, possess mechanisms that help evade the host immune system and aid survival in the host cells. Macrophage-based cell lines, such as bone marrow-derived macrophage cells, phorbol myristate acetate (PMA)-differentiated U937, THP-1, and J774 cells have been widely used to study *Francisella* infection [18, 31-33]. I previously developed an infection model of *F. novicida* using HeLa cells expressing mouse FcγRII (HeLa–FcγRII). When using the HeLa–FcγRII cells with antibody-treated bacteria, the efficiency of infection will be 100-fold greater compared to the normal HeLa cells [8]. This model promoted the discovery of novel factors vital for intracellular proliferation that could not be found through conventional analysis using macrophages.

Accordingly, I constructed a transposon mutant library of *F. novicida* and screened it to identify mutants that were relatively less cytotoxic to the HeLa–FcγRII. As such, 13 mutants that showed decreased LDH release in HeLa–FcγRII cells were compared to the wild-type strain, and transposon-inserted genes were determined. Contrary to our initial expectations of finding novel virulence factors, the identified gene set largely consisted of previously characterized virulence genes such as *Slt*, *IglC*, *pdpA*, and *pdpB*. Although previous comprehensive analyses had implicated *FTN_0096* as a potentially important factor for intracellular proliferation within macrophages [9], its detailed function remains to be elucidated. Consequently, I selected *FTN_0096* for further investigation.

In this research, I had difficulty making homologous recombination, which is one of the steps to make deletion mutants for this gene. It was suggested that complete gene deletion of *FTN_0096* might result in lethal. Therefore, I attempted to directly analyze *FTN_0096* from the transposon mutant library of *F. novicida*. A detailed examination of the dynamics of the *FTN_0096* transposon mutant was accordingly performed. The observed dynamics of the mutant were consistent in both HeLa–FcγRII cells and the macrophage-derived THP-1 cell line.

These findings implied that the infection model using HeLa–FcγRII accurately reflected the behavior of macrophage-derived cells, making it a valuable platform for the study of virulence factors of *F. novicida*.

FTN_0096 encodes a conserved hypothetical membrane protein and is a member of the DUF1275 superfamily, which includes proteins found in a wide range of bacterial species. While the function of DUF1275 remains unidentified, some of its members are encoded near genes for a predicted metallo–amido–hydrolase, suggesting a role related to the transport of peptides or other amido compounds, or in exporting of their hydrolysis products [26]. Nonetheless, the pathogenicity mechanism of *FTN_0096* of *F. novicida* remains unidentified.

Francisella enters the macrophages through phagocytosis and avoids lysosomal fusion by escaping from the resultant phagosome in 1–4 h [20]. Upon phagosomal escape, *Francisella* localizes to the host cytosol, an environment rich in nutrients and innate immune signaling components. *Francisella* manipulates the host cell metabolism to promote massive replication within the host cytosol [34]. The bacteria then re-enter the autophagosomes in the latter phase of infection [35].

F. novicida wild-type strain proliferated intracellularly well 12–48 h post-infection in HeLa–FcγRII and THP-1 cells. Nakamura et al. [36] demonstrated that the *F. novicida* wild-type strain proliferated intracellularly within THP-1 cells. In contrast, the *FTN_0096* mutant proliferated intracellularly until 12 h post-infection in HeLa–FcγRII and THP-1 cells but decreased 24 and 48 h post-infection. The rate of intracellular growth of the wild-type strain differed significantly from that in the *FTN_0096* mutant ($P < 0.01$).

The *F. novicida* wild-type strain colocalized with acidic organelles (LysoTracker) 4%–5% and LAMP-1 at 27%–42% in HeLa–FcγRII. Similarly, in THP-1 cells, LysoTracker colocalization ranged from 5%–6%, while LAMP-1 levels were between 27%–33%. *F. novicida* wild-type strain can escape from phagosomes and replicate within the cytosol of

HeLa-FcγRII and THP-1 cells. *FTN_0096* mutant was found within acidic compartments marked by LysoTracker during the initial phases of infection (2–6 h), and the mutant also localized with the lysosomal marker LAMP-1 during the later stages of the infection (12–48 h post-infection). The *FTN_0096* mutant colocalized with LysoTracker at 49%–50% and LAMP-1 at 80%–86% in HeLa-FcγRII cells, while in THP-1 cells colocalized by LysoTracker 50%–57% and LAMP-1 70%–79%. These results demonstrate that *FTN_0096* plays a critical role in the intracellular lifecycle of *F. novicida*, particularly in the ability to escape from the phagosome into the host cytosol. As successful escape into the host cytosol is essential for intracellular replication and the induction of cytotoxicity, the impaired ability of the *FTN_0096* mutant to escape from the phagosome likely accounts for its reduced intracellular proliferation and attenuated cytotoxic effects.

Lysosomes are membrane-bound organelles containing hydrolytic enzymes that facilitate the breakdown of diverse biological macromolecules. They exhibit considerable dynamic behavior and are capable of fusing with vesicles formed through endocytosis, autophagocytosis, and phagocytosis [37, 38]. Lysosomes are essential for cellular waste disposal, digestion of foreign substances, recycling of cellular components, and the regulation of various signaling pathways. Dysfunction in lysosomal activity is associated with lysosomal storage disorders and can significantly impair cellular homeostasis [39].

Previous data have revealed that *F. novicida* can evade the phagosome and proliferate within the cytosol after the infection of mammalian cells. Intracellular pathogens have developed several methods to survive and evade the phagosome–lysosome process [40, 41]. The role of host intracellular acidity in facilitating *F. novicida* reproduction remains unclear. However, past studies have demonstrated that some bacteria become sequestered within lysosomes where the alkalized pH of the intracellular environment can inhibit their replication

[42-44]. Perhaps, *FTN_0096* may related to the mechanisms to survive in various pH of intercellular environment.

The *FTN_0096* mutant colocalized with mitochondria stained with MitoTracker[®] during the initial stages of infection (2–6 h), whereas the wild-type strains were not colocalized with the mitochondria. The *FTN_0096* mutant colocalized with the mitochondria (MitoTracker[®]) in HeLa–FcγRII cells at 75%–81% proportion and in THP-1 cells at 60%–77% proportion. These results indicate that the *FTN_0096* of *F. novicida* plays an important role in escape from the mitochondria and survive intracellularly.

The mitochondrion is a vital organelle that facilitates several functions of the immune system, particularly via metabolic regulation, calcium homeostasis, and mitochondrial ROS (mtROS) production and degradation [45]. A distinct metabolic signature is mitochondrial clearance via mitophagy, which is important for activating the macrophage. Mitophagy, a form of autophagy specific to mitochondria, is designed to remove damaged mitochondria, either in response to stress or as a part of targeted mitochondrial clearing [46-49].

Previous results have shown the capability of *F. tularensis* SchuS4 to modulate mitochondrial processes early in infection by increasing mitochondrial activity and disrupting the metabolic transition from oxidative phosphorylation to glycolysis. Virulent *F. tularensis* manipulates host mitochondrial metabolism as a central strategy to restrict inflammation and regulate cell death for optimal bacterial replication [50]. *F. tularensis* also acts at different levels to maintain mitochondrial integrity and modulate cytosolic regulatory factors that alter the mitochondria to suppress caspase activity [51].

Caspases are essential components in apoptosis and are responsible for activating dismantling of the cell. Apoptosis is one of the host defense mechanisms that restricts the spread of pathogens. Apoptotic cells maintain their contents inside and are cleared by phagocytes. Some intracellular pathogens, such as *Rickettsia rickettsii*, *Legionella*

pneumophila, *Salmonella enterica*, and *Chlamydia* sp., prevent host cell apoptosis by acting on the mitochondria. Thus, the mitochondria are important targets for the regulation of apoptosis by intracellular pathogens [52, 53]. Since *FTN_0096* mutant captured in mitochondria in this study, these findings suggested that *FTN_0096* may play an important role in intracellular growth by affecting the function of mitochondria.

To observe the activity of the *F. novicida* wild-type strain and *FTN_0096* mutant in the Golgi complex, the infected HeLa-FcγRII and THP-1 cells were stained using Bodipy TR. The result revealed that the colocalization of the Golgi complex to *F. novicida* wild-type strain was not observed, and *FTN_0096* mutant was observed. The percentage of wild-type strains that colocalized with the Golgi complex was 1%–5% in HeLa-FcγRII cells and 4%–12% in THP-1 cells. The *FTN_0096* mutant colocalized with the Golgi complex (Bodipy TR) in HeLa-FcγRII cells at 69%–84% and in THP-1 cells at 63%–82%. These results suggest that the *F. novicida* wild-type strain can escape from the intracellular events related to Golgi complex, but the *FTN_0096* mutant was detected within the Golgi complex, indicating that this mutant cannot escape from the events related to Golgi complex.

The Golgi complex, a vital cytoplasmic organelle, is the primary site for the post-translational modification, transport, and sorting of lipids and proteins synthesized by the endoplasmic reticulum. This complex ensures their transport to designated cellular locations via cytophagy and cytotoxicity. The typically compact structure of the Golgi complex can be disrupted, leading to various levels of disorganization and unstacking because of the cytotoxic agents. These changes in Golgi structure are often linked to problems with the transport and secretion of several essential hepatic glycoproteins. Furthermore, it engages in other biological processes, such as cell signaling, division, and apoptosis. Because of this primary function of the Golgi complex, pathogens try to develop strategies to disrupt and utilize these processes to facilitate the proliferation of pathogens [54-56]. The Golgi complex is essential for the

induction of type I interferon (IFN-I) in response to several stimuli, such as *Francisella* infection, Toll-like receptors activation, and viral infection [57].

IFN-I is a class of cytokines that are deemed essential for viral and bacterial infections in regulating innate and adaptive immunity [58]. Previous research has suggested that IFN-I can impair the host's ability to clear intracellular bacteria, such as *Listeria monocytogenes* [59], *Mycobacterium tuberculosis* [60], and *F. tularensis* [61]. Intracellular bacteria, which are similar to viruses, proliferate within the cytoplasm and can trigger IFN-I products through intracellular pathways. Intracellular bacterial infections can create a cellular environment that mimics viral infection, potentially helping pathogens evade immune surveillance. IFN-I helps limit neutrophil swarming as a mechanism to mitigate excessive tissue injury, whereas intracellular bacteria may utilize this process to elude immunological detection by neutrophils. Disruption of neutrophil swarming, caused by IFN- α , has been recognized as a significant contributor to increased susceptibility to bacterial infections [58]. These findings may suggest that *FTN_0096* may affect the function of Golgi complex to survive intracellularly.

Although the detailed function of *FTN_0096* in the mitochondria and the Golgi complex is not yet fully understood. The *FTN_0096* mutant showed increased colocalization with these organelles, indicating a failure to escape host cell defenses. *FTN_0096* may modulate host intracellular trafficking or stress response pathways that are regulated by these organelles, thereby promoting bacterial survival by interfering with host defense mechanisms. Comprehensive research is needed to determine whether *FTN_0096* directly modulates mitochondrial dynamics or Golgi-associated pathways during infection.

In conclusion, I screened a transposon mutant library using HeLa-Fc γ RII cells and identified *FTN_0096* as a pathogenic factor of *F. novicida*. *FTN_0096* is required for intracellular replication in the cells. *FTN_0096* contributes to bacterial survival by promoting escape from the phagosome and avoiding host organelle-mediated defenses, including those

involving the mitochondria and Golgi complex. Further studies are however warranted to establish the detailed pathogenicity mechanisms of *F. novicida*, which may provide a basis for comprehending how *Francisella* expresses its pathogenicity. Moreover, the detailed mechanisms of *FTN_0096* can contribute to developing vaccines that can prevent diseases caused by *Francisella*.

1.6 Figures

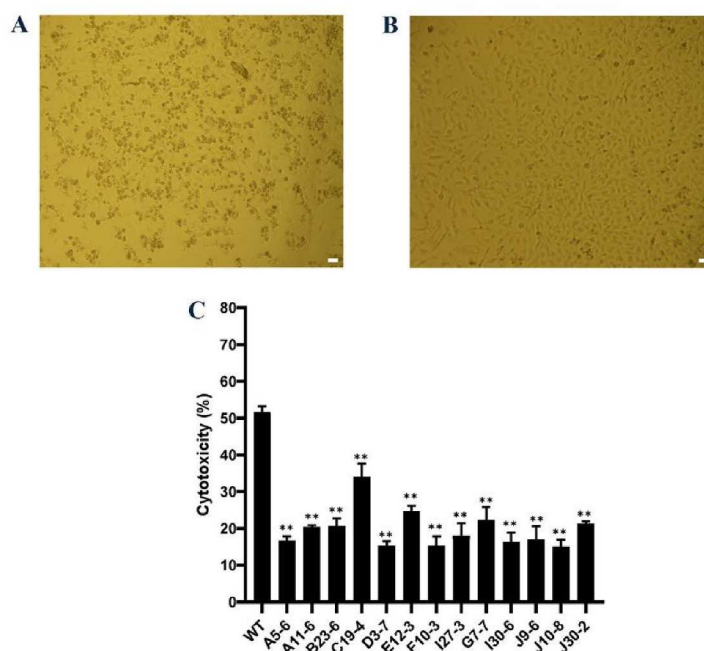


Figure 1. Screening of the Transposon Mutant Library. (A) HeLa-FcγRII cells were infected with *F. novicida* wild-type strain, preincubated with mouse serum containing *F. novicida* antibodies and incubated at 37°C for 24 h. Scale bar = 20 μm. (B) HeLa-FcγRII cells were infected with E12-3 transposon mutant of *F. novicida*, preincubated with mouse serum containing *F. novicida* antibodies, and incubated at 37°C for 24 h. Scale bar = 20 μm. (C) The transposon mutant library was screened using LDH cytotoxicity assay. HeLa-FcγRII cells were infected with the wild-type strain of *F. novicida* and transposon mutants of *F. novicida* preincubated with mouse serum containing *F. novicida* antibodies and incubated at 37°C for 24 h. LDH release was measured as an indicator of cytotoxicity. Data represent the mean and standard deviations of three identical experiments. Significant differences were evaluated in comparison to the wild-type strain using multiple comparison analyses as indicated by asterisks, ** $P < 0.01$.

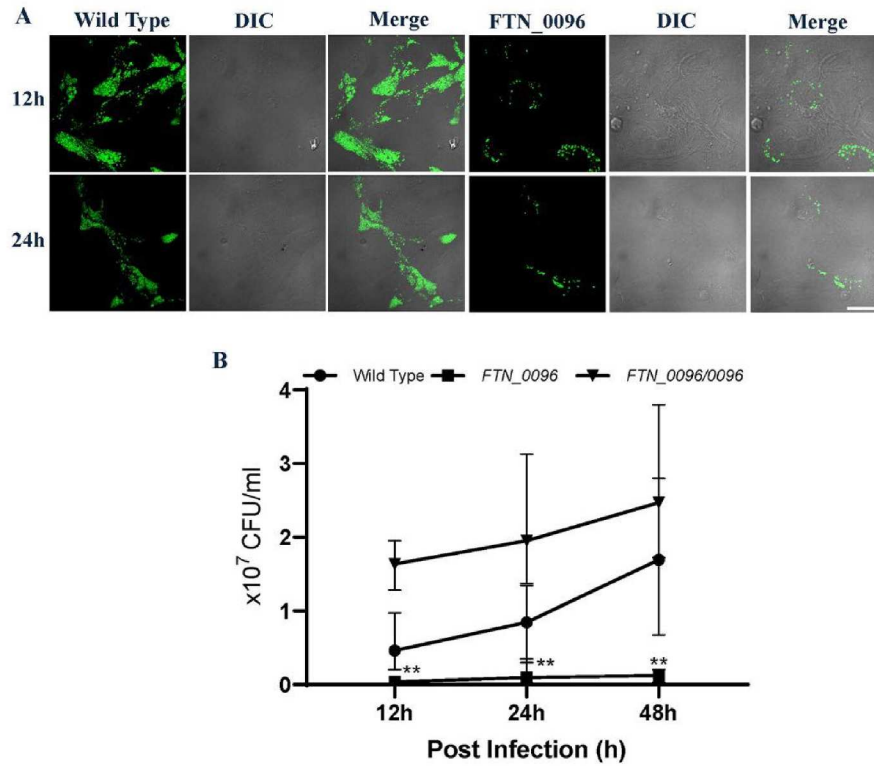


Figure 2. Intracellular growth and cytotoxicity of the wild-type strain and *FTN_0096* mutant in HeLa-FcγRII cells. (A) HeLa-FcγRII cells were infected with *F. novicida* wild-type strain and *FTN_0096* mutant, preincubated with mouse serum containing *F. novicida* antibodies at MOI = 1, and following treatment with gentamicin (50 μg/mL) for 1 h. The cells were fixed and examined at 12 and 24 h post-infection. Scale bar = 20 μm. (B) HeLa-FcγRII cells were infected with *F. novicida* wild-type, *FTN_0096* mutant, and complementary strain (*FTN_0096/0096*), preincubated with mouse serum containing *F. novicida* antibodies at MOI = 1, and following treatment with 50 μg/mL of gentamicin for 1 h. At 12, 24, and 48 h post-infection, cells were lysed with 0.1% Triton X-100, and plating serial dilutions on BHIc agar. Data represent the mean and standard deviations of three identical experiments. Significant differences were evaluated in comparison to the wild-type strain using multiple comparison analyses as indicated by asterisks, ** $P < 0.01$.

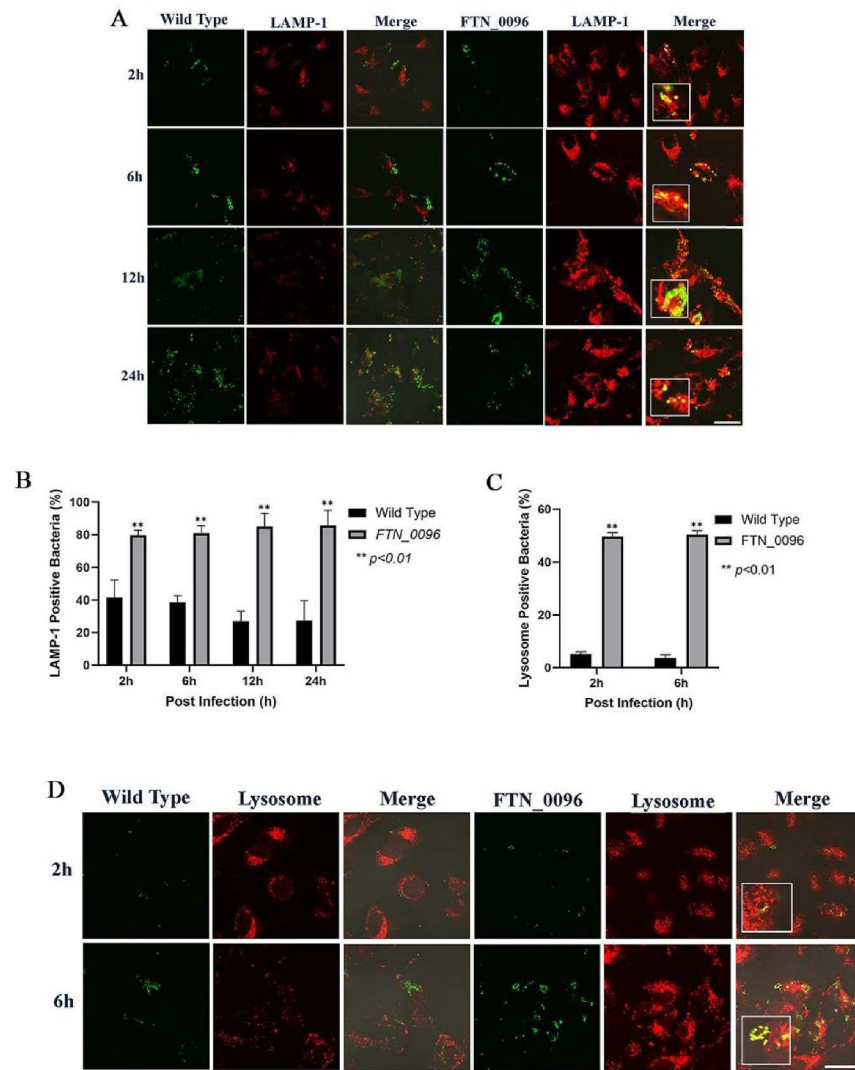


Figure 3. Recognition of *F. novicida* strains by lysophagosomes and lysosomes in HeLa-Fc γ RII cells. (A) HeLa-Fc γ RII cells were infected with *F. novicida* wild-type strain and *FTN_0096* mutant preincubated with mouse serum containing *F. novicida* antibodies at MOI = 1, and following treatment with gentamicin (50 μ g/mL) for 1 h. At 2 to 24 h post-infection, cells were treated with an anti-LAMP-1 antibody and visualized using a TRITC-conjugated anti-rat IgG. Scale bar = 20 μ m. (B) The percentage of *F. novicida* wild-type strain and *FTN_0096* mutant colocalized with LAMP-1. The data represent the mean and standard deviation from three identical experiments. Significant differences were evaluated in

comparison to the wild-type strain using multiple comparison analyses as indicated by asterisks, $**P < 0.01$. (C) The percentage of *F. novicida* wild-type strain and *FTN_0096* mutant colocalized with lysosomes. The data represent the mean and standard deviation from three identical experiments. Significant differences were evaluated in comparison to the wild-type strain using multiple comparison analyses as indicated by asterisks, $**P < 0.01$. (D) HeLa–FcγRII cells were infected with *F. novicida* wild-type strain and *FTN_0096* mutant preincubated with mouse serum containing *F. novicida* antibodies at MOI = 1, and following treatment with gentamicin (50 µg/mL) for 1 h. At 2 to 6 h post-infection, cells were stained with LysoTracker™ Red DND-99 to visualize lysosomes. Scale bar = 20 µm.

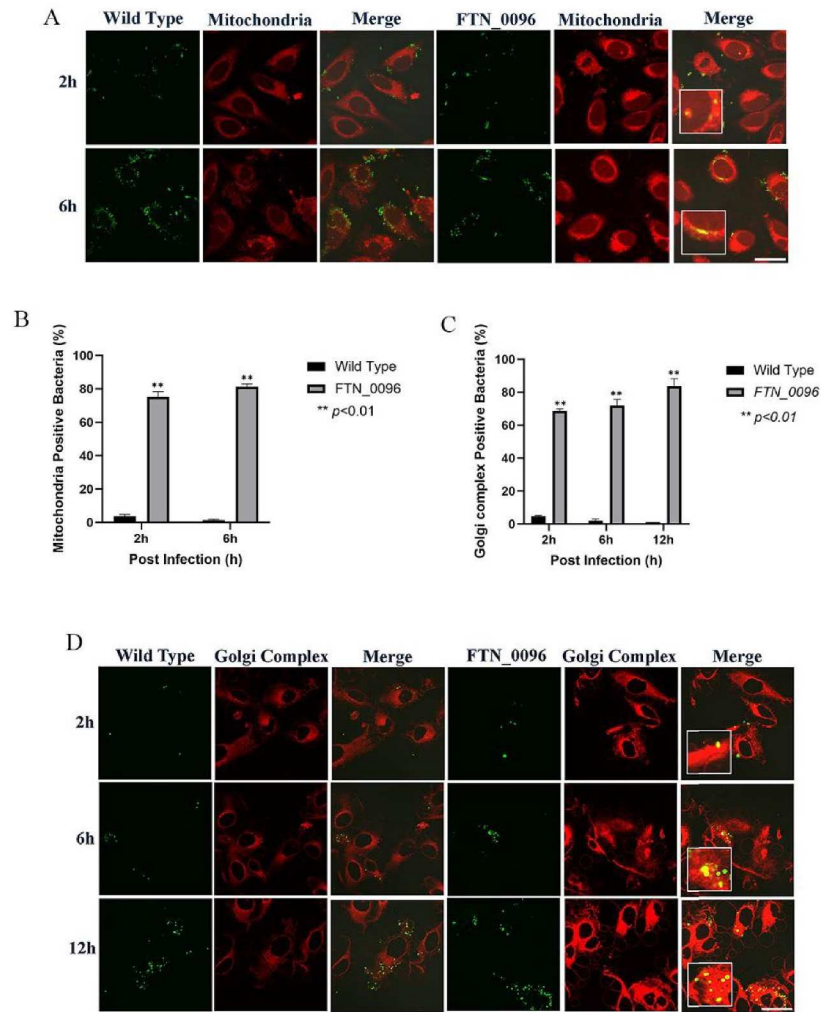


Figure 4. Recognition of *F. novicida* strains by the mitochondria and Golgi complex in HeLa–FcγRII cells. (A) HeLa–FcγRII cells were infected with *F. novicida* wild-type strain and *FTN_0096* mutant preincubated with mouse serum containing *F. novicida* antibodies at MOI = 1, and following treatment with gentamicin (50 µg/mL) for 1 h. Cells were stained with Mitotracker® Deep Red FM at 2 and 6 h post-infection. Scale bar = 20 µm. (B) Percentage of *F. novicida* wild-type strain and *FTN_0096* mutant that colocalized with the mitochondria. The data represent the mean and standard deviation from three identical experiments. Significant differences were evaluated in comparison to the wild-type strain using multiple comparison analyses as indicated by asterisks, ** $P < 0.01$. (C) The percentage of *F. novicida* wild-type strain and *FTN_0096* mutant that colocalized with the Golgi complex. The data represent the

mean and standard deviation from three identical experiments. Significant differences were evaluated in comparison to the wild-type strain using multiple comparison analyses as indicated by asterisks, $**P < 0.01$. (D) HeLa–FcγRII cells were infected with *F. novicida* wild-type strain and *FTN_0096* mutant preincubated with mouse serum containing *F. novicida* antibodies at MOI = 1, and treated with 50 µg/mL gentamicin. At 2, 6, and 12 h post-infection, cells were stained with BODIPY TR. Scale bar = 20 µm.

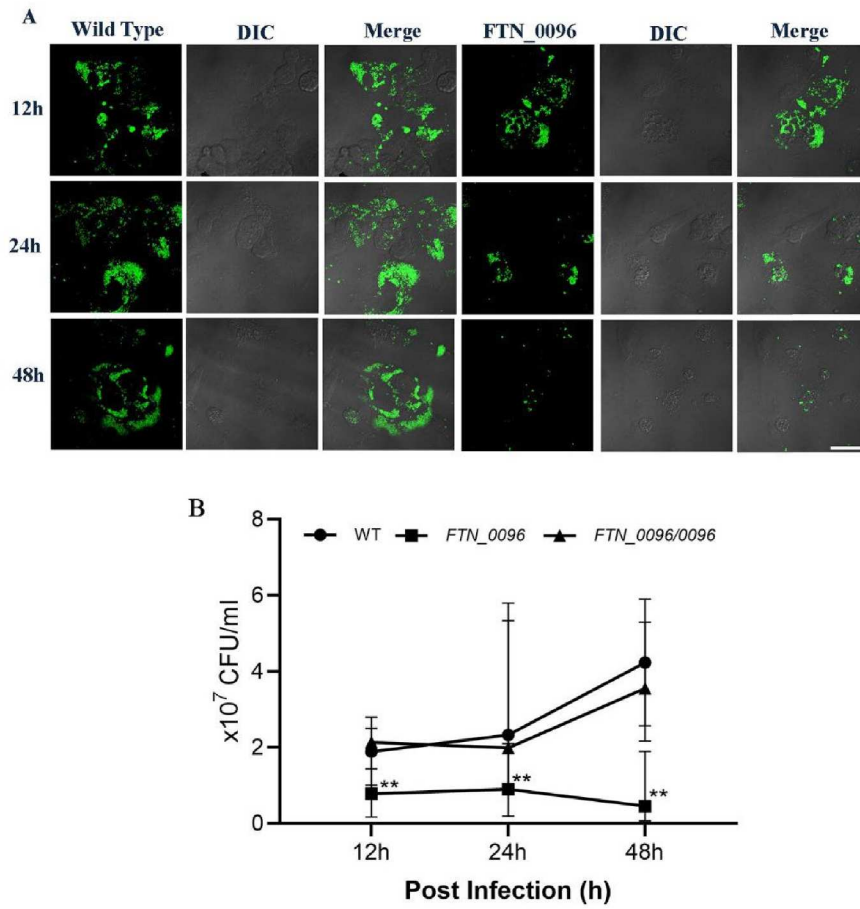


Figure 5. Intracellular growth of the wild-type strain and *FTN_0096* mutant in THP-1 cells. (A) THP-1 cells were infected with *F. novicida* wild-type strain and *FTN_0096* mutant at MOI = 1, following treatment with gentamicin (50 µg/mL) for 1 h. Cells were fixed and examined at 12, 24, and 48 h post-infection. Scale bar = 20 µm. (B) THP-1 cells were infected with *F. novicida* wild-type, *FTN_0096* mutant, and *FTN_0096/0096* at MOI = 1, and following treatment with gentamicin (50 µg/mL) for 1 h. At 12, 24, and 48 h post-infection, cells were lysed with 0.1% Triton X-100 and plating serial dilutions on BHIc agar. The data represent the mean and standard deviation from three identical experiments. Significant differences were evaluated in comparison to the wild-type strain using multiple comparison analyses as indicated by asterisks, ** $P < 0.01$.

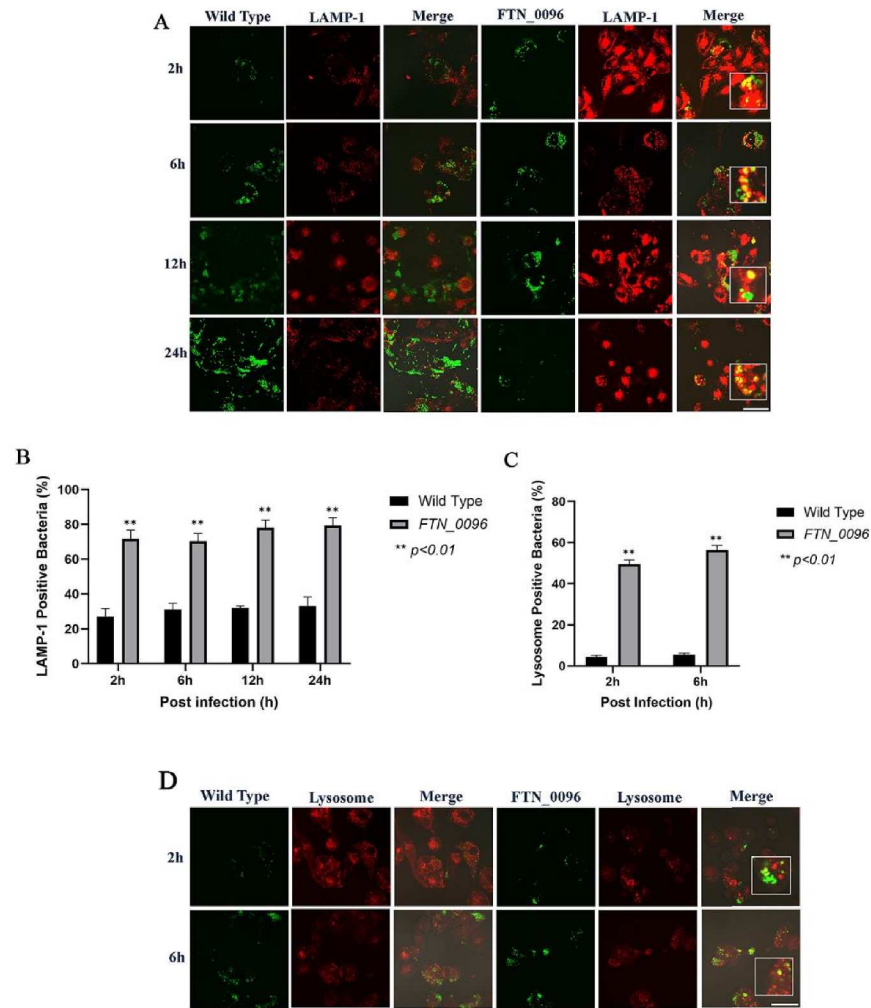


Figure 6. Recognition of *F. novicida* strains by lysophagosomes and lysosomes in THP-1 cells. (A) THP-1 cells were infected with *F. novicida* wild-type strain and *FTN_0096* mutant at MOI = 1, following treatment with gentamicin (50 µg/mL) for 1 h. Cells were treated with an anti-LAMP-1 antibody and stained with a TRITC-conjugated anti-rat IgG at 2–24 h after the infection. Scale bar = 20 µm. (B) The percentage of *F. novicida* wild-type strain and *FTN_0096* mutant colocalized with LAMP-1. The data represent the mean and standard deviation from three identical experiments. Significant differences were evaluated in comparison to the wild-type strain using multiple comparison analyses as indicated by asterisks, ** $P < 0.01$. (C) The percentage of *F. novicida* wild-type strain and *FTN_0096* mutant colocalized with lysosomes.

The data represent the mean and standard deviation from three identical experiments. Significant differences were evaluated in comparison to the wild-type strain using multiple comparison analyses as indicated by asterisks, $**P < 0.01$. (D) THP-1 cells were infected with *F. novicida* wild-type strain and *FTN_0096* mutant MOI = 1, following treatment with gentamicin (50 µg/mL) for 1 h. Cells were treated with LysoTracker™ Red DND-99 from 2 to 6 h after the infection. Scale bar = 20 µm.

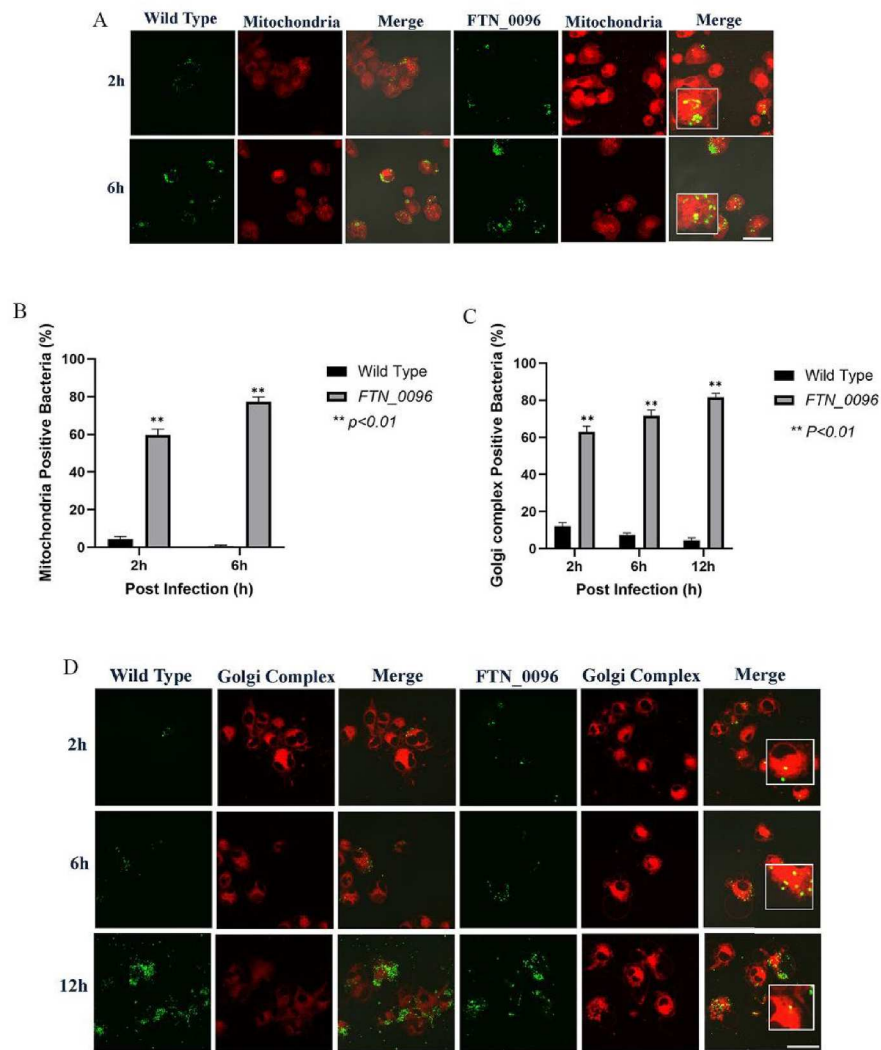


Figure 7. Recognition of *F. novicida* strains by the mitochondria and Golgi complex in THP-1 cells. (A) THP-1 cells were infected with *F. novicida* wild-type strain and *FTN_0096* mutant, MOI = 1, and following treatment with gentamicin (50 μ g/mL) for 1 h. Cells were stained with the Mitotracker[®] Deep Red FM at 2 and 6 h post-infection. Scale bar = 20 μ m. (B) The percentage of *F. novicida* wild-type strain and *FTN_0096* mutant that colocalized with mitochondria. The data represent the mean and standard deviation from three identical experiments. Significant differences were evaluated in comparison to the wild-type strain using multiple comparison analyses as indicated by asterisks, ** $P < 0.01$. (C) The percentage of *F. novicida* wild-type strain and *FTN_0096* mutant that colocalized with the Golgi complex. The

data represent the mean and standard deviation from three identical experiments. Significant differences were evaluated in comparison to the wild-type strain using multiple comparison analyses as indicated by asterisks, $**P < 0.01$. (D) THP-1 cells were infected with *F. novicida* wild-type strain and *FTN_0096*, MOI = 1, and following treatment with gentamicin (50 $\mu\text{g/mL}$) for 1 h. Cells were stained with BODIPYTR at 2, 6, and 12 h post-infection. Scale bar = 20 μm .

Chapter 2

Identification of the *wbtF* gene as a cytotoxicity-associated factor in *Francisella novicida* infection

2.1 Abstract

Francisella tularensis is a highly infectious Gram-negative bacterium that causes tularemia in humans and animals. It has a remarkable ability to survive and replicate within a wide range of host cells. *F. novicida* shares many characteristics with *F. tularensis*. However, it is rarely pathogenic in humans, and its reduced virulence makes it a suitable model organism for studying *F. tularensis* infection. This study aimed to identify the pathogenic factors of *F. novicida*. Using a novel infection model with HeLa cells expressing FcγRII (HeLa-FcγRII cells), I screened 2,232 transposon mutants of *F. novicida* pre-treated with antiserum containing *F. novicida* antibodies to find less cytotoxicity strains. The transposon insertion site was identified by sequencing, leading to the determination of the genes responsible for the attenuated cytotoxicity. Additionally, the intracellular behavior of the mutant was investigated within both HeLa-FcγRII and THP-1 cells. A total of thirteen mutants with attenuated cytotoxicity were isolated, and their responsible genes were identified. They are *figE*, *slt*, *fopA*, *iglC*, *igID*, *iglF*, *iglI*, *pdpB*, *pdpA*, *ampG*, *wbtF*, and one unnamed gene (*FTN_0096*). I focused on the *wbtF* gene. The *F. novicida* wild-type (WT) strain showed intracellular replication in HeLa-FcγRII and THP-1 cells, but the number of intracellular *wbtF* mutants decreased. The *wbtF* mutant could not escape from phagolysosomes in the initial phases of infection and was digested within the lysosome. The *wbtF* mutant was also detected in the mitochondria and the Golgi complex. The cytokine response induced by *wbtF* mutant was comparable to that of the WT strain. These findings indicate that *wbtF* is important for the intracellular replication of *F. novicida*.

Keywords: *F. novicida*, *wbtF*, cytotoxicity, intracellular replication, tularemia.

2.2 Introduction

Francisella tularensis is a highly infectious Gram-negative bacterium that causes tularemia, a zoonosis affecting humans and animals. The genus *Francisella* comprises several species including *F. tularensis*, *F. philomiragia*, *F. noatunensis*, *F. hispaniensis* and *F. novicida* [5, 8]. *F. tularensis* is divided into two main subspecies: Type A (*F. tularensis* subsp. *tularensis* [*F. tularensis*]) and Type B (*F. tularensis* subsp. *holarctica* [*F. holarctica*]). Type A is considerably more virulent than Type B and is commonly associated with severe disease in humans, whereas Type B typically causes milder illness. Pneumonic tularemia, the most dangerous form, is transmitted through aerosols or inhalation. Remarkably, inhaling as few as 25 colony-forming units (CFUs) may result in pulmonary infection, making *F. tularensis* one of the most infectious bacteria transmitted via the aerosol route [62]. *Francisella* is a significant pathogen associated with high morbidity and mortality rates. The most virulent strain of *Francisella* induces systemic forms of tularemia (pneumonic, typhoidal), with fatality rates reaching 60% [16, 63]. Due to its high infectivity and potential for airborne spread, *F. tularensis* has been developed and stockpiled as a biological warfare agent by several nations [64].

F. novicida is classified as a separate species from *F. tularensis*, but due to the high genomic homology, it is sometimes classified as a subspecies of *F. tularensis* [65]. Because *Francisella novicida* (*F. novicida*) is considerably less virulent than *F. tularensis*, it is commonly used as a surrogate strain for virulent *F. tularensis*. In contrast to *F. tularensis*, *F. novicida* is an opportunistic pathogen that causes illness and even death in debilitated or immunocompromised patients but not in healthy individuals. Due to the rarity of *F. novicida* infections, effective detection is challenging [5, 66]. *F. novicida* shares many characteristics with Type A strains of *F. tularensis*, showing similarities in the genome sequence, intracellular life cycle, and mechanisms of infectivity, including rapid phagosomal escape followed by

vigorous cytosolic replication. It also infects macrophages, causing disease in mice. However, due to its lower virulence in humans, it can be safely handled under Biosafety Level 2 laboratory conditions [5, 67]. *F. novicida* strain U112 possesses a potent tool in the form of a specific transposon mutant, which has been used to investigate the intracellular life cycle of *Francisella* and elucidate its virulence mechanisms [7, 68].

Typically, nonpathogenic bacteria taken up by host cells are enclosed in vacuoles or phagosomes, which mature through the endocytic pathway and ultimately fuse with lysosomes, leading to bacterial degradation. Intracellular pathogens have evolved strategies to evade this process within phagocytic cells and resist phagosome–lysosome fusion [41]. In particular, *Francisella* species exhibit a complex intracellular life cycle and notable environmental persistence [69]. Their survival and replication rely heavily on escaping the initial phagosomal compartment and replicating within the host cell cytosol, making *Francisella* a model cytosolic pathogen. The ability to persist and multiply inside phagocytes and other host cells is fundamental to its pathogenicity. Disruptions in key mechanisms, such as receptor engagement, resistance to reactive oxygen species (ROS), phagosomal escape, cytosolic replication, and evasion of innate immune recognition, significantly impair *F. tularensis* survival and reduce its virulence in both in vitro and in vivo models [70].

wbtF is assigned as a potential NAD-dependent epimerase and UDP-glucose 4 epimerase (UGE) [12]. It shows sequence similarity with established UGEs, including *wbpP* from *Pseudomonas aeruginosa* [71]. *wbpP* is necessary for producing UDP-N-acetyl-D-galacturonic acid, a precursor to the galactosaminuronic acid-derived components of the O-antigen [72]. UGE, part of the short-chain dehydrogenase/reductase enzyme superfamily, catalyzes the final step in the interconversion of UDP-glucose and UDP-galactose during galactose metabolism in bacteria and mammals [73]. Thomas et al. [12] identified *wbtF* genes as a virulence determinant of *F. tularensis*, but its function remains unknown.

In this study, I created a transposon mutant library of *F. novicida* and screened for infection using epithelial cells, HeLa cells expressing the FcγRII (HeLa-FcγRII cells) [8]. I focused my investigation on *wbtF* and analyzed that the *wbtF* was associated with the intracellular replication of *F. novicida*.

2.3 Materials and Methods

Bacterial strains and culture conditions

F. novicida U112 was obtained from the Pathogenic Microorganism Genetic Resource Stock Center (Gifu University) and cultured aerobically at 37°C in a chemically defined medium (CDM) [27] or on brain–heart infusion broth enriched with cysteine (BHlc) [28] and solidified with 1.5% agar (Wako Laboratory Chemicals, Osaka, Japan).

Cell culture conditions

HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher, Waltham, MA, USA). The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Similarly, the human monocytic cell line THP-1 was cultured under the same incubation conditions in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FBS.

Transposon mutant library construction

A transposon mutant library was constructed using the Ez-Tn5 Transposome system (Epicentre, Madison, WI, USA). The multiple cloning sites of plasmid pMOD3 were digested with *Hind*III and *Eco*RI to construct the transposon. A kanamycin resistance cassette from pKEK1140 [29] was inserted into these sites, creating the plasmid pMOD3-FtKm. The transposon region of pMOD3-FtKm was amplified by polymerase chain reaction, purified, and combined with transposase according to the manufacturer's instructions. This transposon mixture was introduced into *F. novicida* by cryotransformation. Following transformation, the bacteria were plated on BHlc agar supplemented with 50 µg/mL kanamycin to select for mutants.

Cytotoxicity assay

HeLa-FcγRII cells (5×10^4 cells/well) were seeded in 48-well tissue culture plates and incubated for 24 h. *F. novicida* strains preincubated with mouse serum containing anti-*F. novicida* antibodies were added at a multiplicity of infection (MOI) of 1. The plates were centrifuged at $300 \times g$ at room temperature for 10 min to promote bacterial contact with the cells and then incubated at 37°C for the designated times. Following incubation, the cells were washed three times with DMEM to remove nonadherent bacteria and treated with gentamicin (50 µg/mL) for 1 h to eliminate extracellular bacteria. Infected cells were monitored microscopically at designated time intervals to assess the relative cytotoxicity of the transposon mutant library. To evaluate cytotoxicity, lactate dehydrogenase (LDH) release was measured after incubating cells in DMEM at 37°C for a defined period. LDH levels in the culture supernatant were then quantified using the LDH Cytotoxicity Detection Kit (Takara Bio, Shiga, Japan). In brief, cell culture supernatants were collected and incubated with the catalyst and dye solutions for 30 min at room temperature in the dark. Absorbance was measured at 490 nm with a reference wavelength of 600 nm using a microplate reader. LDH activity was expressed as a percentage of total LDH release, determined from lysed cell controls.

Sequence analysis of transposon mutants

The plasmid pMOD3 carries the R6Kγ origin of replication, facilitating propagation in *Escherichia coli*. Genomic DNA from *F. novicida* transposon mutants was extracted using a PureLink Genomic DNA Mini Kit (Thermo Fisher) and digested with restriction enzymes (*Xho*I, *Bgl*II, *Eco*RI, *Sal*I, *Not*I, *Bam*HI, *Pst*I, and *Sph*I). The resultant DNA fragments were blunted using a DNA Blunting Kit (Takara Bio) and then ligated using Ligation High Ver. 2 (Toyobo). The ligation products were introduced into One Shot PIR1 Chemically Competent *E. coli* (Thermo Fisher) via transformation. Kanamycin-resistant colonies were selected, and

plasmid DNA was isolated. Sequencing was performed using the primers specified in the Ez-Tn5 Transposome system manual to determine transposon insertion sites.

Green fluorescent protein (GFP)-expression by *F. novicida* and complementary strains

GFP-expressing plasmids (pOM5-GFP) were generated as described by Shimizu et al. [22]. Briefly, the plasmids were introduced into the wild-type (WT) and *wbtF* mutant strains of *F. novicida* via electroporation. To create pOM5-*wbtF* or complementary strains, *wbtF* of *F. novicida*, along with its native promoter region (300 bp upstream), was cloned into the pOM5 vector. The resultant plasmid (pOM5-*wbtF*) was used to transform the *wbtF* mutant strains of *F. novicida* via electroporation for complementation studies.

Intracellular replication assay

THP-1 cells (4×10^5 cells/well) were seeded in 24-well tissue culture plates and differentiated by treatment with 200 nM phorbol 12-myristate 13-acetate (PMA) for 48 h. HeLa-FcγRII cells (1×10^5 cells/well) were cultured overnight on 12-mm glass coverslips placed in 24-well plates. *F. novicida* strains were added to the THP-1 cells and *F. novicida* strains were opsonized with mouse serum added to the HeLa-FcγRII cells at an MOI of 1. After infection, the THP-1 and HeLa-FcγRII cells were washed three times with RPMI 1640 medium and DMEM, respectively. To remove extracellular bacteria, all wells were treated with gentamicin (50 µg/mL) for 1 h. Then, the cells were incubated in fresh medium at 37°C for the designated times. To analyze intracellular replication, infected cells were washed with phosphate-buffered saline and lysed using 0.1% Triton X-100 in chemically defined medium (CDM). The lysates underwent serial dilution and were plated on BHIc agar to determine the number of CFUs.

Fluorescence microscopy

THP-1 cells (4×10^5 cells/well) were seeded on 12-mm glass coverslips in 24-well tissue culture plates and differentiated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 48 h. HeLa-FcγRII cells (1×10^5 cells/well) were cultured overnight on 12-mm glass coverslips placed in 24-well plates. The THP-1 cells were infected with GFP-expressing *F. novicida* strains and incubated for the designated times. For HeLa-FcγRII cells, GFP-expressing *F. novicida* strains were preincubated with mouse serum and added at an MOI of 1, followed by incubation for the designated times. The cells were stained with LysoTracker Red DND-99, MitoTracker Deep Red FM, and BODIPY TR Ceramide (Thermo Fisher) to visualize lysosomes, mitochondria, and the Golgi complex, respectively. To detect lysosomal-associated membrane protein 1 (LAMP-1), cells were fixed using a PLP Solution Set (FUJIFILM Wako Chemicals) containing 5% sucrose for 1 h at 37°C and then permeabilized using ice-cold methanol for 10 s. The fixed cells were incubated with anti-LAMP-1 antibody (ab25245, 1:100; Abcam), followed by staining with TRITC-conjugated anti-rat IgG secondary antibody (ab150158, 1:1000; Abcam). Confocal images were captured using a FluoView FV1000 laser scanning microscope (Olympus, Tokyo, Japan).

ELISA

THP-1 cells (4×10^5 cells per well) were seeded into a 48-well culture plate and treated with 100 nM PMA for 48 hours. Following this, they were infected with *F. novicida* strains, including the *wbtF* mutant and its complementary strains. After 2, 6, 12, and 24 h of incubation, the levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in the culture supernatants were quantified using the ELISA MAX Standard Kit (Biolegend, San Diego, CA), following the manufacturer's protocol.

Statistical analysis

Differences between groups were evaluated using Welch's *t*-test or multiple comparison tests, including the Bonferroni and Dunnett methods, as appropriate. Before these analyses, data normality was assessed using the Shapiro–Wilk test. $P < 0.01$ was considered statistically significant.

2.4 Results

Thirteen genes in the *F. novicida* transposon mutant library showed less cytotoxicity to HeLa-FcγRII cells

I constructed an *F. novicida* transposon mutant library to identify the virulence factors of *F. novicida*. I previously developed a novel infection model of *Francisella* using HeLa cells expressing mouse FcγRII (HeLa-FcγRII) cells [8]. In this cell line, cellular uptake occurs via antibodies and FcγRII. Therefore, it was expected that screening with this cell line would allow us to identify novel virulence factors crucial for pathogenesis after cellular uptake. I screened the *F. novicida* transposon mutant library using HeLa-FcγRII cells. *F. novicida* exhibits cytotoxicity toward epithelial cells, including HeLa-FcγRII cells, leading to cell death. I microscopically screened the transposon mutant library for strains lacking cytotoxic activity to identify genes associated with this cytotoxic effect. Of the 2,232 transposon mutants screened, 13 were identified as less cytotoxic. Significant differences in host cell condition were evident, while wild-type infection caused pronounced disruption and cell loss, monolayers exposed to the 13 mutants retained their structure and remained largely intact. To confirm these results, LDH assays were performed. The mutant strains caused less LDH release from HeLa-FcγRII cells than the WT strain, indicating reduced cytotoxicity (Fig 1). The insertion sites in these mutant strains were sequenced to determine the disrupted genes (Table 1). I focused on mutants with the code number G7-7, which encode *wbtF*, and analyzed the gene function.

Effect of *wbtF* on intracellular replication in HeLa-FcγRII cells

To determine the effect of *wbtF* on intracellular replication in HeLa-FcγRII cells in detail, I infected them with *F. novicida* WT and the *wbtF* mutant. Through a fluorescence microscope, we discovered intracellular bacteria using GFP-expressing *F. novicida* strains. I analyzed the intracellular of the WT and *wbtF* mutant 12h and 24h after infection in HeLa-FcγRII cells (Fig

2A). From 12 to 48 h postinfection, the rate of intracellular replication of the WT strain increased, while that of the *wbtF* mutant decreased (Fig 2B). Complementation of the *wbtF* mutant restored replication to the WT level (Fig 2B). These results suggested *wbtF* involvement in the intracellular replication of *F. novicida* in HeLa-FcγRII cells.

Role of *wbtF* in avoiding phagolysosomes in HeLa-FcγRII cells

I studied how the *wbtF* mutant replicates intracellularly in HeLa-FcγRII cells to clarify the role of *wbtF* in the intracellular replication of *Francisella* and its mechanism of escape from phagolysosomal digestion during the early stages of infection. HeLa-FcγRII cells were infected with GFP-expressing *F. novicida* strains. At 2 and 6 h postinfection, the cells were stained with LysoTracker and examined using confocal microscopy. The *F. novicida* WT bacteria did not exhibit LysoTracker colocalization. In contrast, the *wbtF* mutant strain exhibited noticeable colocalization with LysoTracker-positive compartments, suggesting defective phagolysosomal evasion (Fig 3A). A low percentage of WT bacteria showed lysosomal colocalization in HeLa-FcγRII cells. In contrast, a significantly higher percentage of *wbtF* mutant bacteria showed lysosomal colocalization (Fig 3B). This suggested that the WT bacteria, but not the *wbtF* mutant bacteria, escaped from the phagolysosomes in HeLa-FcγRII cells.

***F. novicida* escape from lysosomes in HeLa-FcγRII cells**

HeLa-FcγRII cells were infected with GFP-expressing *F. novicida* WT and *wbtF* mutant strains to determine whether they underwent lysosomal digestion. Lysosomes were visualized using an anti-LAMP-1 antibody (Fig 3D). At 2–24 h postinfection, the WT strain replicated intracellularly, with bacterial numbers increasing over time. The *wbtF* mutant showed intracellular replication for up to 12 h postinfection. However, replication was decreased at 24 h postinfection, suggesting impaired long-term survival or replication within the host cells (Fig.

3C). Compared with the *wbtF* mutant, a significantly lower percentage of WT strains that infected the HeLa-FcγRII cells colocalized with LAMP-1 at 2, 6, 12, and 24 h postinfection (Fig 3C). These results indicated that *F. novicida* WT escaped lysosomal degradation in the HeLa-FcγRII cells compared to the *wbtF* mutant, which exhibits reduced escape capacity.

Impact of *wbtF* in the mitochondria of HeLa-FcγRII cells

I infected HeLa-FcγRII cells with WT and *wbtF* mutant strains to determine the role of *wbtF* in the mitochondria. I visualized the mitochondria using MitoTracker Deep Red FM (Fig 4A) to observe whether the WT and the *wbtF* mutant strains colocalized with the mitochondria. At 2 and 6 h postinfection, a low percentage of the HeLa-FcγRII cells infected with the WT strain colocalized with the mitochondria (Fig 4B). In contrast, HeLa-FcγRII cells infected with the *wbtF* mutant showed 61%–76% colocalization with the mitochondria (Fig 4B). These results demonstrated that *F. novicida* WT effectively modified mitochondrial function and sustained replication, while the *wbtF* mutant could not.

Effect of *wbtF* on the Golgi complex of infected HeLa-FcγRII cells

I infected HeLa-FcγRII cells with WT and *wbtF* mutant strains to investigate the function of *wbtF* in the Golgi complex of infected cells. The Golgi complex was stained using BODIPY TR ceramide at 2, 6, and 12 h postinfection. A low percentage of WT bacteria colocalized with the Golgi complex (Fig 4C). In contrast, 57%–75% of the *wbtF* mutant bacteria colocalized with the Golgi complex at 2, 6, and 12 h postinfection (Fig 4C). Visualization of intracellular colocalization of the bacteria with the Golgi complex 2, 6, and 12 h postinfection can be seen in Fig 4D.

Effect of *wbtF* on intracellular replication in THP-1 cells

THP-1 cells were infected with the WT and *wbtF* mutant of *F. novicida* to evaluate the role of *wbtF* in intracellular replication. Fluorescence microscopy using GFP-expressing strains confirmed the presence and localization of intracellular bacteria. My analysis of the intracellular levels of WT and *wbtF* mutant bacteria at 12, 24, and 48 h postinfection in THP-1 cells further validated the reduced replication capacity of the *wbtF* mutant compared with the WT strain (Fig 5A). The WT strain showed a steady increase in intracellular replication, while the number of *wbtF* mutants was significantly decreased between 12 and 48 h postinfection (Fig. 5B). Complementation of the *wbtF* mutant restored replication to the WT level, confirming the role of *wbtF* in intracellular survival (Fig. 5B). These findings suggested *wbtF* involvement in *F. novicida* intracellular replication in THP-1 cells.

Role of *wbtF* in avoiding phagolysosomes in THP-1 cells

I infected THP-1 cells with GFP-expressing *F. novicida* strains to investigate the role of *wbtF* in intracellular survival, specifically its involvement in phagolysosomal escape. I assessed the ability of *F. novicida* to evade phagolysosomal fusion during the initial stage of infection (2 and 6 h postinfection) using LysoTracker staining visualized under confocal microscopy. *F. novicida* WT showed low levels of colocalization with LysoTracker, indicating efficient evasion of phagolysosomal fusion. In contrast, the *wbtF* mutant colocalized with LysoTracker-positive lysosomes (Fig 6A). Only a limited number of WT bacteria were colocalized with the lysosomes, while a significantly higher number of *wbtF* mutant bacteria were detected within the lysosomal compartments (Fig 6B). This suggested that the WT strain escaped from the phagolysosomes in THP-1 cells, but the *wbtF* mutant could not.

***F. novicida* escape from lysosomes in THP-1 cells**

THP-1 cells were infected with the GFP-expressing *F. novicida* WT and *wbtF* mutant strains to investigate whether lysosomes contributed to *F. novicida* degradation. Lysosomes were visualized using an anti-LAMP-1 antibody (Fig 6D). The WT strain exhibited consistent intracellular replication at 2–24 h postinfection, as indicated by the increasing number of bacteria. In contrast, the *wbtF* mutant replicated up to 12 h postinfection, but the intracellular bacterial load declined markedly by 24 h postinfection. Compared with the *wbtF* mutant, a significantly lower percentage of WT strains colocalized with LAMP-1 at 2, 6, 12, and 24 h postinfection (Fig 6C). These results indicated that *F. novicida* WT escaped lysosomal degradation in the THP-1 cells, whereas the *wbtF* mutant could not.

Impact of *wbtF* in the mitochondria of THP-1 cells

THP-1 cells were infected with WT and *wbtF* mutant strains to determine the role of *wbtF* in the mitochondria. I visualized the mitochondria using MitoTracker Deep Red FM (Fig 7A) to observe whether the WT and the *wbtF* mutant bacteria colocalized with the mitochondria. A low percentage of the WT bacteria colocalized with the mitochondria (Fig 7B). In contrast, 69%–78% of the *wbtF* mutant bacteria colocalized with the mitochondria at 2 and 6 h postinfection (Fig 7B). These results indicated that *F. novicida* WT modified mitochondrial function and sustained replication in the THP-1 cells, while the *wbtF* mutant could not.

Effect of *wbtF* on the Golgi complex of infected THP-1 cells

I infected THP-1 cells with the WT and *wbtF* mutant strains to analyze the function of *wbtF* in the Golgi complex. The Golgi complex was stained using BODIPY TR ceramide to determine if the Golgi complex could colocalize the WT strain and the *wbtF* mutant. The intracellular bacteria were examined at 2, 6, and 12 h after infection. A low percentage of the WT strain

colocalized with the Golgi complex (Fig 7C). In contrast, 61%–75% of the *wbtF* mutant bacteria were colocalized with the Golgi complex at 2, 6, and 12 h after infection (Fig 7C). Visualization of intracellular colocalization of the bacteria with the Golgi complex 2, 6, and 12 h postinfection can be seen in Fig 7D.

Effect of *wbtF* on cytokine production

To evaluate the effect of *wbtF* in cytokine responses, I measured the induction of cytokines produced by THP-1 cells that were infected with WT, *wbtF* mutant and complementary strains. The induction of TNF- α (Fig 8A) and IL-6 (Fig 8B) did not differ significantly among the WT, *wbtF* mutant, and complementary strains.

2.5 Discussion

Francisella is a Gram-negative bacterium, and its highly pathogenic species, *F. tularensis*, is a well-known cause of tularemia in humans and other mammals. *F. tularensis* outmaneuvers the host's immune defenses by escaping from the phagosome—a cellular structure meant to destroy invaders. Instead of being degraded, the bacteria multiply inside the host cell's cytoplasm, where they are better protected from immune responses [74]. Scientists are still piecing together the mechanism of phagosomal escape, and a gene cluster called the *Francisella* pathogenicity island (FPI) has emerged as a key player. The FPI encodes component proteins of a unique type VI secretion system thought to be critical to the bacterium's survival and virulence [75-77].

I constructed a transposon mutant library of *F. novicida* to isolate mutants that were less cytotoxic. I screened the transposon mutants using a new infection model of *Francisella*, HeLa cells expressing mouse FcγRII (HeLa-FcγRII). HeLa-FcγRII cells are a cell line that expresses the FcγRII receptor, enabling efficient uptake of IgG-opsonized bacteria [15]. Although epithelial cells, like HeLa, are readily amenable to genetic modification, infection with *Francisella* is rarely effective and significantly lower than the infection efficiency of macrophage-like cells. *Francisella* infection of epithelial cell lines, such as like HeLa, A549, and HEK293, has been performed at a high MOI (100–10,000) and requires a relatively long time (approximately 8–24 h) [78-80]. The ability of *Francisella* to infect macrophages largely depends on whether the bacteria are opsonized. The use of unopsonized bacteria leads to rapid disruption of the phagosome. In contrast, opsonizing *Francisella* with fresh serum facilitates directing the bacteria to various phagocytic receptors, resulting in delayed phagosomal escape [25]. Notably, when HeLa cells engineered to express FcγRII were infected with antibody-coated bacteria, the infection efficiency was approximately 100 times higher compared with normal HeLa cells [8].

Among the 2,232 transposons mutants library, 13 showed less cytotoxicity and were successfully sequenced. Two mutants involved the same gene: FTN_0496 (*slt*). FTN_1686 (*figE*), FTN_0496 (*slt*), FTN_1322 (*IglC*), FTN_1321 (*IglD*), FTN_1309 (*pdpA*), FTN_1310 (*pdpB*), FTN_1317 (*IglI*), FTN_1313 (*IglF*), and FTN_0096 were previously reported as pathogenic factors of *Francisella* [9, 36, 81-84]. FTN_0756 (*fopA*) and FTN_1641 (*ampG*) were previously reported as immunosuppressive factors involved in dampening host immune responses [31]. However, this was the first report of FTN_1425 (*wbtF*). Therefore, I focused on *wbtF*. Although *wbtF* has been reported as a non-essential gene that can be disrupted [12], I were unable remove the gene using a suicide vector [36] in this study. Therefore, I attempted to directly investigate *wbtF* gene from a transposon mutant library of *F. novicida*.

wbtF is one of the O-antigen biosynthetic gene clusters of *F. tularensis* [10]. The O-antigen capsule in *Francisella* comprises a polysaccharide that is structurally identical to the O-antigen found in lipopolysaccharide (LPS). This LPS O-antigen is a critical pathogenic factor. O-antigen deficiency results in increased sensitivity to serum and a loss of virulence in Type A and B strains of *F. tularensis* [11]. Thomas et al. [12] created knock-out mutants targeting the O-antigen gene cluster in *F. novicida* and *F. tularensis* SchuS4, leading to the complete loss of O-antigen production. Notably, the SchuS4 mutant lacking the O-antigen was fully attenuated in mice.

Francisella species are highly pathogenic, largely due to their ability to replicate within the cytosol of phagocytic cells, such as macrophages and dendritic cells. After entering these cells through phagocytosis, the bacteria are initially enclosed within a membrane-bound compartment called the *Francisella*-containing phagosome. However, they rapidly disrupt the phagosomal membrane and escape into the host cell cytosol, where they replicate. The bacteria adapt their metabolism to the cytosolic environment by relying on gluconeogenesis and amino acid catabolism as carbon and energy sources. Thus, phagosomal escape is critical for

Francisella's ability to replicate inside host cells, leading to disease in vivo. Phagosomal escape also triggers the host's antimicrobial and innate immune defenses. In many cases, once the infected cell becomes overloaded with bacteria and dies, the bacteria exit and potentially spread to neighboring cells [34, 85].

In this research, intracellular replication of the *F. novicida* WT strain in HeLa-FcγRII cells and THP-1 cells increased at 12–48 h after infection. This correlated with previous findings of increased intracellular replication of *Francisella* WT in HeLa-FcγRII and THP-1 cells [8, 86]. Compared with the WT strain, the *wbtF* mutant underwent intracellular replication for up to 12 h after infection in HeLa-FcγRII and THP-1 cells, but then decreased at 24 and 48 h after infection. The rate of intracellular replication of the *wbtF* mutant was significantly lower than that of the WT strain ($P < 0.01$). Thomas et al. [12] demonstrated that the O-antigen of *F. tularensis* was essential for the survival and replication inside host cells.

The acidic organelles were stained with LysoTracker. The *wbtF* mutant bacteria showed colocalization with LysoTracker in the early stages of infection (2–6 h) of HeLa-FcγRII cells (60%–71%) and THP-1 cells (72%–74%). Furthermore, the *wbtF* mutant also colocalized with the lysosome marker LAMP-1 at 2, 6, 12, and 48 h postinfection in 78%–88% of HeLa-FcγRII cells and 71%–82% of THP-1 cells.

F. tularensis virulence is closely associated with its ability to survive inside phagocytic cells and escape into the cytosol. Upon host cell entry, the bacterium is initially enclosed in a phagosome that typically matures into a phagolysosome. However, *F. tularensis* disrupts this maturation by blocking fusion between the phagosome and lysosome. Then, it breaks down the phagosomal membrane and escapes into the cytoplasm, where it replicates. The molecular mechanism underlying the phagosomal escape of *Francisella* remains poorly understood. However, it has been shown that disrupting genes within the FPI prevents the bacterium from phagosomal escape [77]. Thus, my findings suggest that the *wbtF* mutant could not escape

from the phagosomes, indicating that *wbtF* is required for intracellular replication of *F. novicida*.

Mitochondria are double-membraned organelles vital for various cellular processes and cell survival. Their metabolic functions are crucial for maintaining tissue balance across different cell types and organisms. Unsurprisingly, these metabolic pathways often shift in response to stressors, like nutrient deprivation. Beyond their role in energy production, mitochondria are also highly involved in regulating cell death during infection and have been increasingly recognized as important hubs for immune signaling [87,88]. I observed that the *F. novicida* WT strain associates with mitochondria in HeLa-FcγRII cells and THP-1 cells, and underwent intracellular replication. Furthermore, it did not colocalize with mitochondria (MitoTracker) at 2–6 h after infection. In contrast, the *wbtF* mutant colocalized with the mitochondria during the initial stages of infection (2–6 h) in HeLa-FcγRII cells (61%–76%) and THP-1 cells (69%–78%).

Mitochondria are highly susceptible to damage, and their dysfunction disrupts cellular homeostasis. Such disturbances are closely linked to the development of various diseases, including neurodegenerative and cardiovascular conditions, cancer, metabolic disorders, and infections. Thus, the impaired mitochondria must be promptly isolated and selectively eliminated. Mitophagy (mitochondrial autophagy) is a critical cellular process that eliminates damaged mitochondria, thereby preserving mitochondrial and overall cellular homeostasis. When cells are exposed to stressors such as ROS, nutrient scarcity, or aging, mitochondria become depolarized and dysfunctional, triggering their selective removal through mitophagy [89-92].

Pathogens stimulate mitophagy to suppress the host defense mechanism. They likely employ a common mechanism that inhibits innate immune responses and promotes persistent infection by directly or indirectly facilitating mitophagy [93]. The initiation of mitophagy to

eradicate intracellular mitochondrial ROS (mtROS) is an essential survival tactic for intracellular infections. mtROS are essential for innate host defense, functioning as effectors by harming pathogens [94,95]. Alqahtani et al. [96] showed that infection with *F. tularensis* live vaccine strain induced mitophagy. Generally, mitophagy is considered to limit the accumulation of mtROS, a significant source of cellular ROS. Microbial pathogens often evade or suppress the host's protective mtROS pathway and neutralize bactericidal free radicals [95, 97]. Other intracellular bacteria, such as *Staphylococcus aureus*, promote mitophagy to clear mtROS. *S. aureus* not only survives within macrophages but also stimulates mitophagy in host cells to clear mtROS. It uses HDAC11 to augment interleukin 10 (IL-10) transcription, promoting mitophagy to clear mtROS, reduce mitochondrial damage, and maintain an ecological environment favorable for bacterial survival. *Listeria monocytogenes* diminishes mtROS and inhibits cell death by promoting mitophagy through the secretion of its virulence factor, listeriolysin O [94,98]. These findings suggest that the intracellular survival of *Francisella* WT depends on mitochondrial modulation and escape. Indeed, the effector protein *IglJ* has been reported to interact directly with mitochondrial targets [99]. In contrast, the *wbtF* mutant cannot modulate mitochondria and is therefore detected in association with mitochondria. The *wbtF* gene might be involved in this kind of mitochondrial modulation.

The Golgi complex is a crucial membrane-bound organelle in the perinuclear region that consists of flattened disk-shaped cisternae connected in a ribbon-like structure. It comprises three distinct subcompartments: the *cis*-Golgi, medial-Golgi, and *trans*-Golgi network [100]. The Golgi complex is the modification and sorting hub for proteins and lipids. Newly synthesized proteins and lipids are delivered to the Golgi complex, where they undergo posttranslational modifications and are sorted into vesicles for transport to other organelles or extracellular secretion [101].

To explore the activities of the *F. novicida* WT and *wbtF* mutant strains within the Golgi complex, infected HeLa-FcγRII and THP-1 cells were stained with BODIPY TR ceramide. The *Francisella* WT strain only showed colocalization with the Golgi complex in 2%–5% of HeLa-FcγRII cells and 4%–12% of THP-1 cells. However, the *wbtF* mutant was colocalized with the Golgi complex in 57%–75% of HeLa-FcγRII cells and 61%–75% of THP-1 cells.

The Golgi complex is a crucial organelle in the host defense systems against intracellular infections. Additionally, recent research indicates that the Golgi complex plays a critical role in immunological signaling [102]. Disruption of the structure of the Golgi complex catalyzes the pathogenesis of infectious diseases. Various intracellular bacterial, viral, and protozoan infections trigger Golgi complex destabilization. This evolutionarily conserved strategy enables pathogens to access nutrients, hijack organelle membranes for replication, and subvert host immune defenses [103]. Type I interferons (IFNs) are induced by the Golgi complex in response to various stimuli, including *Francisella* infection [57]. Type I IFNs are a group of cytokines crucial for shaping innate and adaptive immune responses during viral and bacterial infections. However, they also suppress antibacterial immunity by promoting IL-10 production, inhibiting proinflammatory cytokines and chemokines, and promoting immune cell apoptosis, thereby exacerbating *Francisella* infections [58,61]. The translocation of the signaling intermediate stimulator of IFN genes (STING, TMEM173) from the endoplasmic reticulum to the *cis*-Golgi is initiated by cyclic GMP-AMP synthase or interferon-γ inducible protein 16 following the identification of cytosolic exogenous DNA [104-106]. STING activates TANK-binding kinase 1 (TBK1) at the Golgi complex by inducing its autophosphorylation at serine 172. Following activation, TBK1 phosphorylates interferon regulatory factor 3, facilitating dimerization and translocation to the nucleus, which drives the expression of immune-related genes, including type I IFNs [107]. Interestingly, structural disruption of the Golgi complex using golgicide A blocks TBK1 phosphorylation in response to *F. tularensis* infection and LPS

[57]. These results suggest that *F. novicida* WT escapes from the Golgi complex, but *wbtF*, which was detected in the Golgi complex, cannot escape from the immune response.

Since *wbtF* is a gene involved in O-antigen synthesis, I investigated the cytokine production of the *wbtF* deletion mutant to confirm whether the attenuated cytotoxicity and intracellular proliferation of the mutant were due to the LPS-mediated activation of immune responses. Infection of THP-1 cells with *F. novicida* WT, *wbtF* mutant, and the complemented strain produced similar TNF- α and IL-6 responses, indicating that *wbtF* does not significantly affect cytokine production. The *wbtF* gene, a consecutive gene located within the O-antigen-biosynthetic gene clusters spanning a 17-kb chromosomal region of *F. tularensis*, is directly implicated in LPS biosynthesis. The O-antigen constitutes one of the three components of LPS, along with the lipid A and the oligosaccharide core [10,108]. *Francisella* LPS does not function as an antagonist of TLR4-mediated innate immune signalling in either humans or mice. *Francisella* LPS is tetraacylated, and it elicits only weak activation of TLR4 and demonstrates a markedly reduced ability to induce the secretion of inflammatory cytokines like TNF- α and IL-6 by murine and human innate immune cells [109-111]. It seems that *Francisella* LPS represents, at most, a minor contributor to the induction of proinflammatory responses within the innate immune system. Although deletion of LPS synthesis genes, such as *kdsA*, have been reported to enhance cytokine production [31], the absence of *wbtF* did not impact cytokine production in this study. Taken together, these results imply that the attenuated cytotoxicity and intracellular growth ability observed in *wbtF* deletion mutant are likely due to mechanisms distinct from LPS-mediated immune activation. Although comprehensive understanding of the detailed mechanisms necessitates further investigation, my results may suggest that *wbtF* is important for modulating LPS structure, potentially facilitating the evasion of recognition and uptake by cellular organelles, including phagosomes, mitochondria, and the Golgi apparatus.

In summary, my research focused on identifying a virulence factor of *F. novicida* by screening a transposon mutant library. I used a new infection model of *Francisella* using HeLa cells expressing mouse FcγRII (HeLa-FcγRII) [8]. I identified *wbtF* as a pathogenic factor of *F. novicida*. *wbtF* is required for escaping from organelles such as phagosomes, mitochondria, and the Golgi apparatus, followed by intracellular replication in HeLa-FcγRII and THP-1 cells. Thus, it may be a potential target for controlling *Francisella* infection. However, further research is necessary to elucidate the detailed mechanism of *wbtF* function in *Francisella* infection.

2.6 Table

Table 1. Sequence Analysis of Transposon Mutant Library

strain	locus_tag	gene name	product
A5-6	FTN_0496	Slr	Soluble lytic transglycosylase
A11-6	FTN_0756	fopA	Outer membrane protein A
B23-6	FTN_1641	ampG	peptide-acetyl-coenzyme A transporter family protein
C19-4	FTN_0496	Slr	Soluble lytic transglycosylase
D3-7	FTN_1321	IglD	Intracellular growth locus protein D
E12-3	FTN_0096	-	Conserved hypothetical membrane protein
F10-3	FTN_1309	pdpA	Protein of unknown function
I27-3	FTN_1686	figE	Hypothetical membrane protein
G7-7	FTN_1425	wbtF	NAD dependent epimerase
I30-6	FTN_1322	IglC	Intracellular growth locus protein C
J9-6	FTN_1313	IglF	Hypothetical protein
J10-8	FTN_1317	IglI	Protein of unknown function
J30-2	FTN_1310	pdpB	Protein of unknown function

2.7 Figures

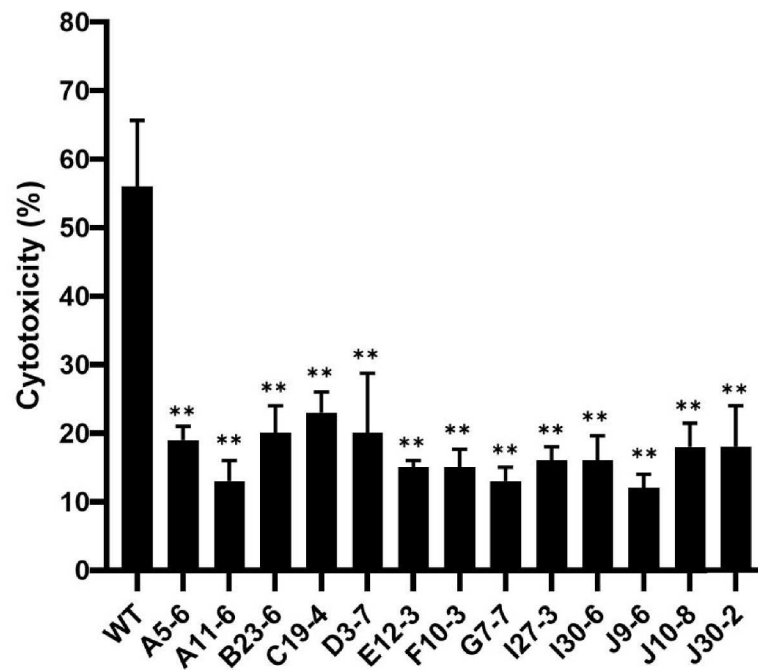


Figure 1. HeLa-Fc γ RII cells were infected with transposon mutants of *F. novicida* preincubated with mouse serum containing anti-*F. novicida* antibodies at an MOI of 1 and incubated at 37°C for 24 h. LDH release was measured as an indicator of cytotoxicity. The graph presents the mean and standard deviations (SD) of the LDH release at 24 h from three identical experiments. Statistical significance relative to the WT strain was determined using multiple comparisons and is indicated by asterisks. ** $P < 0.01$.

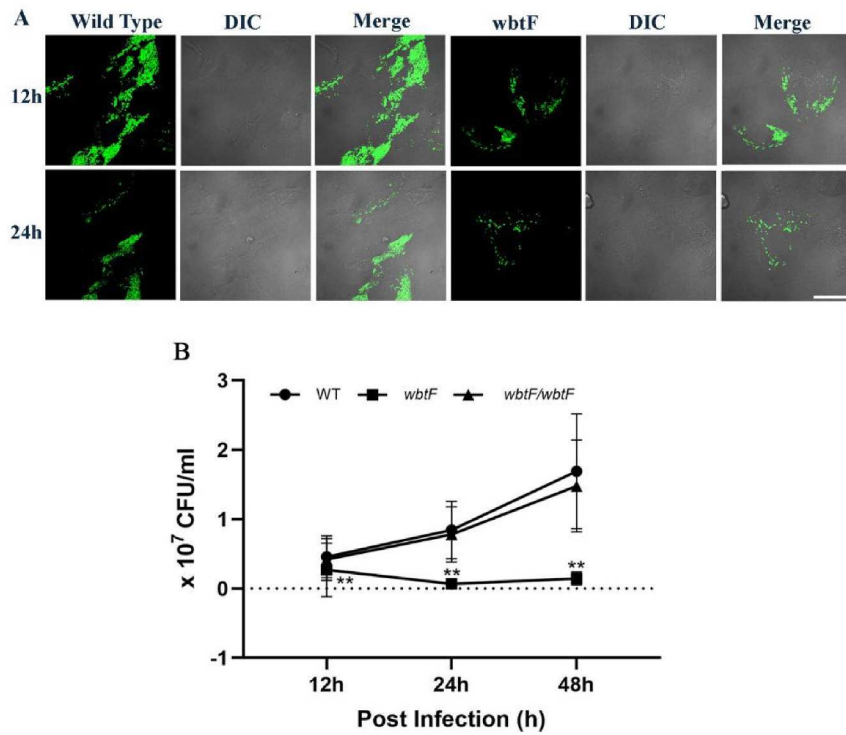


Figure 2. HeLa-Fc γ RII cells were infected with *F. novicida* strains preincubated with mouse serum containing anti-*F. novicida* antibodies at an MOI of 1, followed by gentamicin treatment (50 μ g/mL) for 1 h. (A) The infected HeLa-Fc γ RII cells were fixed and observed at 12 and 24 h postinfection. Scale bar = 20 μ m. (B) At 12, 24, and 48 h postinfection, the infected HeLa-Fc γ RII cells were lysed with 0.1% Triton X-100, and the lysates were plated on BHIc agar. The graph presents the mean and SD of the number of CFUs at the designated time points from three identical experiments. Statistical significance relative to the WT strain at each designated time point was determined using multiple comparisons and is indicated by asterisks. ** $P < 0.01$.

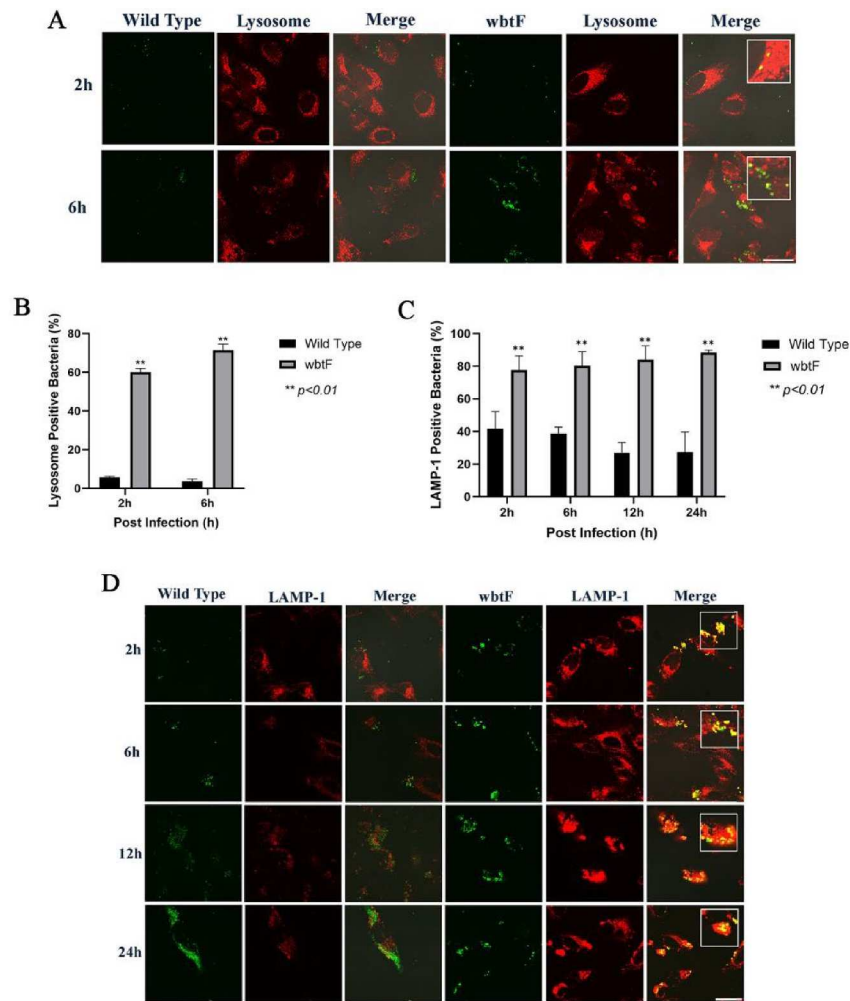


Figure 3. (A) HeLa-FcγRII cells were infected with *F. novicida* strains preincubated with mouse serum containing anti-*F. novicida* antibodies at an MOI of 1, followed by gentamicin treatment (50 μg/mL) for 1 h to eliminate extracellular bacteria. The infected HeLa-FcγRII cells were treated with LysoTracker Red DND-99 to visualize the acidic compartments at 2 and 6 h postinfection. Scale bar = 20 μm. (B) Percentage of *F. novicida* colocalization with LysoTracker-labeled lysosomes. Data represent the mean and SD from three identical experiments. Statistical significance relative to the WT strain at each designated time point was determined using Welch's *t*-test and is indicated by asterisks. ** $P < 0.01$. (C) Percentage of colocalization between *F. novicida* and LAMP-1. Data represent the mean and SD from

three identical experiments. Statistical significance relative to the WT strain at each designated time point was determined using Welch's *t*-test and is indicated by asterisks. ** $P < 0.01$. (D) HeLa-Fc γ RII cells were similarly infected then treated with an anti-LAMP-1 antibody and stained with a TRITC-conjugated anti-rat IgG for 2–24 h postinfection. Scale bar = 20 μ m.

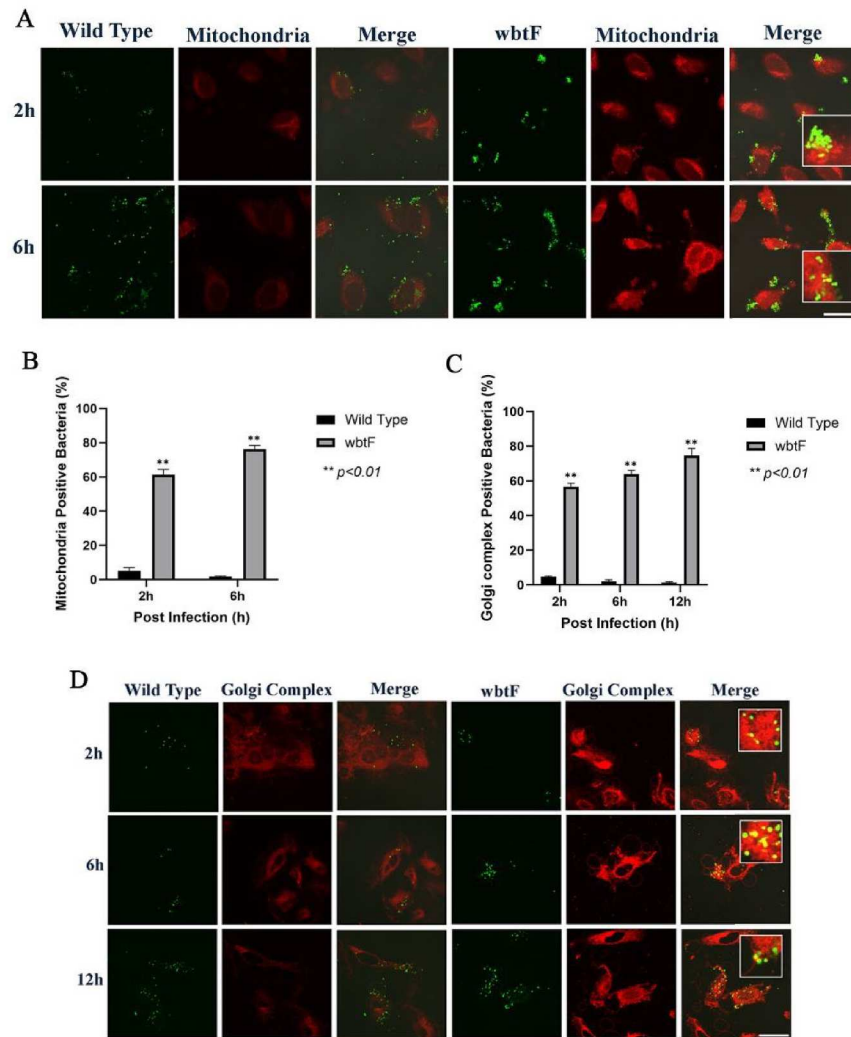


Figure 4. HeLa-Fc γ R11 cells were infected with *F. novicida* strains preincubated with mouse serum containing anti-*F. novicida* antibodies at an MOI of 1, followed by gentamicin treatment (50 μ g/mL) for 1 h to eliminate extracellular bacteria. (A) The infected HeLa-Fc γ R11 cells were stained with MitoTracker Deep Red FM at 2 and 6 h postinfection. Scale bar = 20 μ m. (B) Percentage of *F. novicida* colocalized with the mitochondria. Data represent the mean and SD from three identical experiments. Statistical significance relative to the WT strain at each designated time point was determined using Welch's *t*-test and is indicated by asterisks. ***P* < 0.01. (C) Percentage of *F. novicida* colocalized with the Golgi complex. Data represent the mean and SD from three identical experiments. Statistical significance

relative to the WT strain at each designated time point was determined using Welch's *t*-test and is indicated by asterisks. $**P < 0.01$. (D) The infected HeLa-FcγRII cells were stained with BODIPY TR ceramide at 2, 6, and 12 h postinfection. Scale bar = 20 μm.

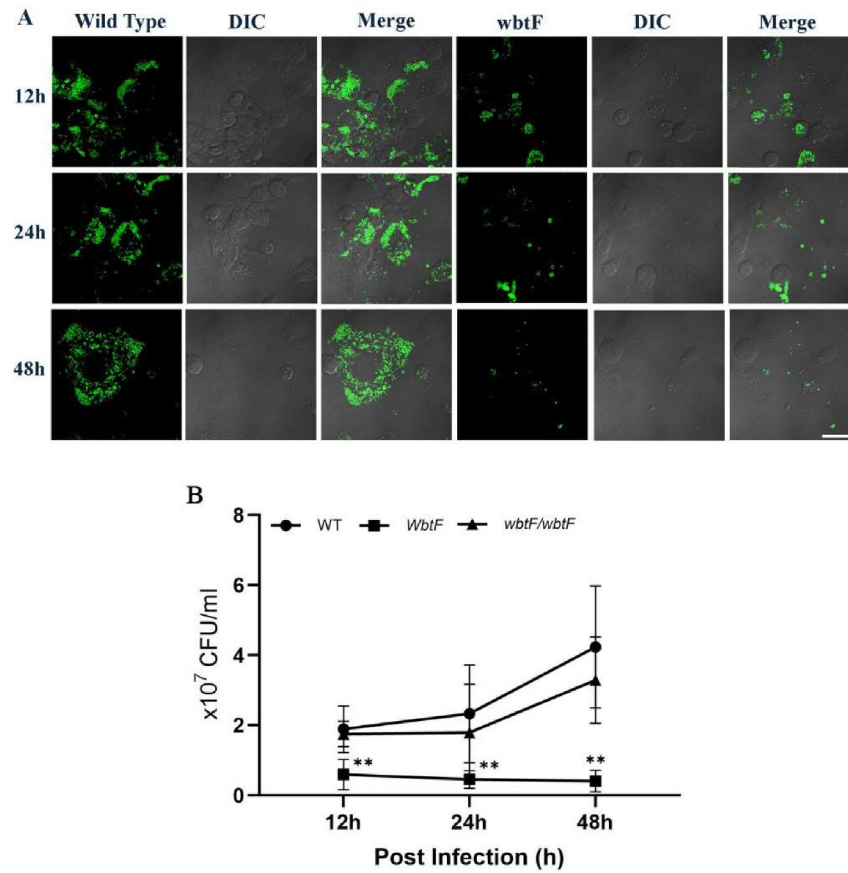


Figure 5. THP-1 cells were infected with *F. novicida* at an MOI of 1, followed by gentamicin treatment (50 μ g/mL) for 1 h to eliminate extracellular bacteria. (A) The infected THP-1 cells were fixed and observed at 12, 24, and 48 h after infection. Scale bar = 20 μ m. (B) The infected THP-1 cells were lysed with 0.1% Triton X-100 at 12, 24, and 48 h postinfection, and the lysates were plated on BHIc agar. Data represent the mean and SD from three identical experiments. Statistical significance relative to the WT strain at each designated time point was determined using multiple comparisons and is indicated by asterisks. ** $P < 0.01$.

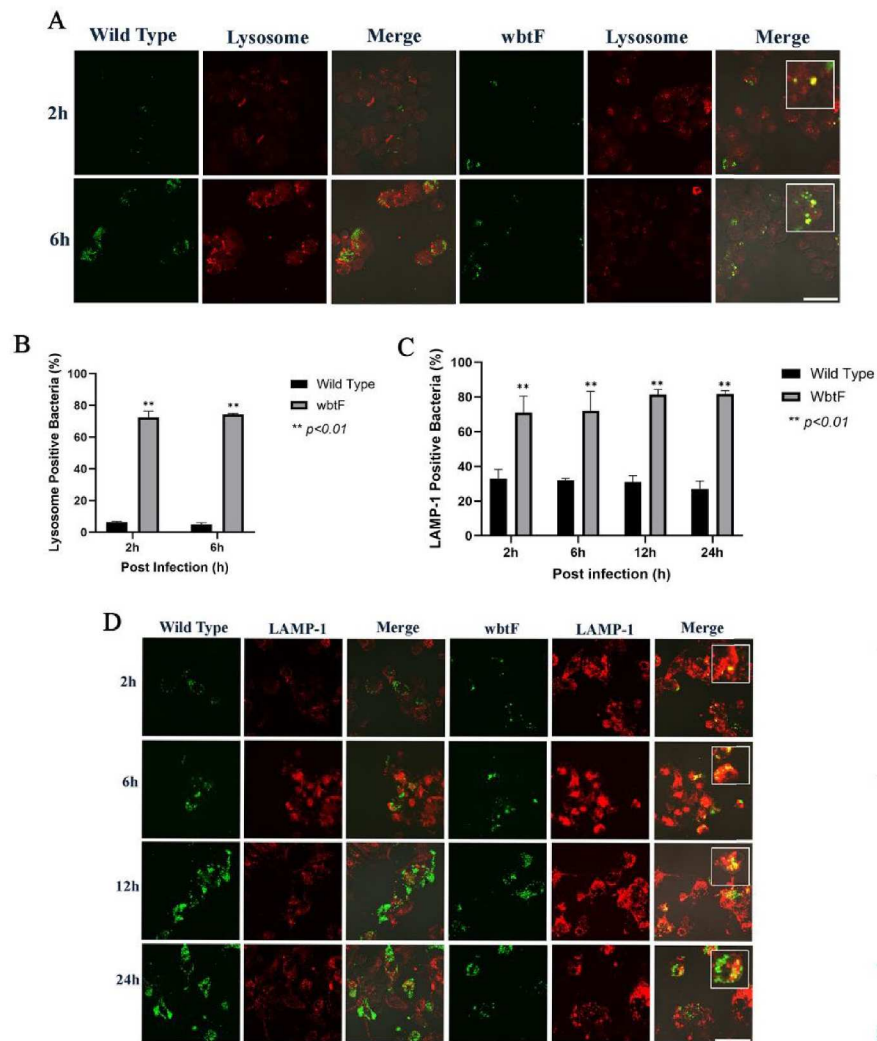


Figure 6. THP-1 cells were infected with *F. novicida* strains at an MOI of 1, followed by gentamicin treatment (50 $\mu\text{g/mL}$) for 1 h to eliminate extracellular bacteria. (A) The infected THP-1 cells were treated with LysoTracker Red DND-99 for 2–6 h postinfection. Scale bar = 20 μm . (B) Percentage of *F. novicida* colocalized with lysosomes. Data represent the mean and SD from three identical experiments. Statistical significance relative to the WT strain at each designated time point was determined using Welch's *t*-test and is indicated by asterisks. ** $P < 0.01$. (C) Percentage of *F. novicida* colocalized with LAMP-1. Data represent the mean and SD from three identical experiments. Statistical significance relative to the WT strain at each designated time point was determined using Welch's *t*-test and is indicated by

asterisks. $**P < 0.01$. (D) The infected THP-1 cells were treated with anti-LAMP-1 antibody and stained with TRITC-conjugated anti-rat IgG for 2–24 h postinfection. Scale bar = 20 μm .

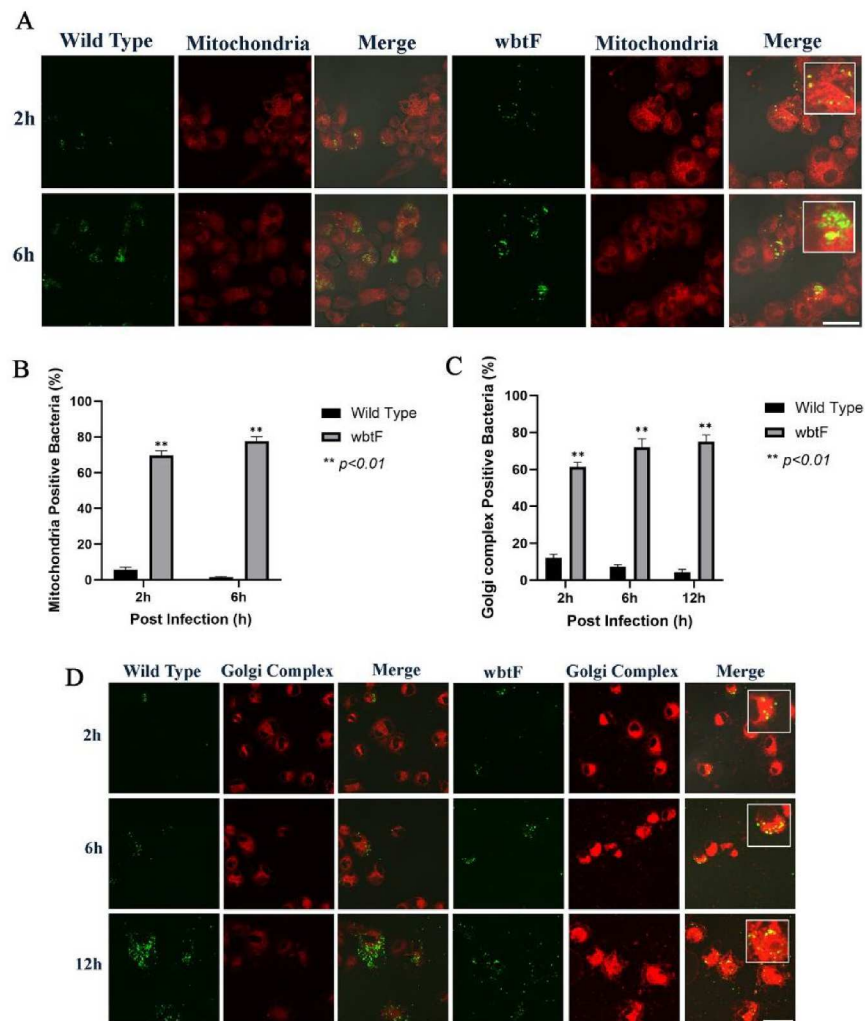


Figure 7. THP-1 cells were infected with *F. novicida* at an MOI of 1, followed by gentamicin treatment (50 $\mu\text{g/mL}$) for 1 h to eliminate extracellular bacteria. (A) The infected THP-1 cells were treated with MitoTracker Deep Red FM at 2 and 6 h postinfection. Scale bar = 20 μm . (B) Percentage of *F. novicida* colocalized with the mitochondria. Data represent the mean and SD from three identical experiments. Statistical significance relative to the WT strain at each designated time point was determined using Welch's *t*-test and is indicated by asterisks. ** $P < 0.01$. (C) Percentage of *F. novicida* colocalized with the Golgi complex. Data represent the mean and SD from three identical experiments. Statistical significance relative to the WT strain at each designated time point was determined using Welch's *t*-test and is

indicated by asterisks. $**P < 0.01$. (D) The infected THP-1 cells were treated with BODIPY TR ceramide at 2, 6, and 12 h after infection. Scale bar = 20 μm .

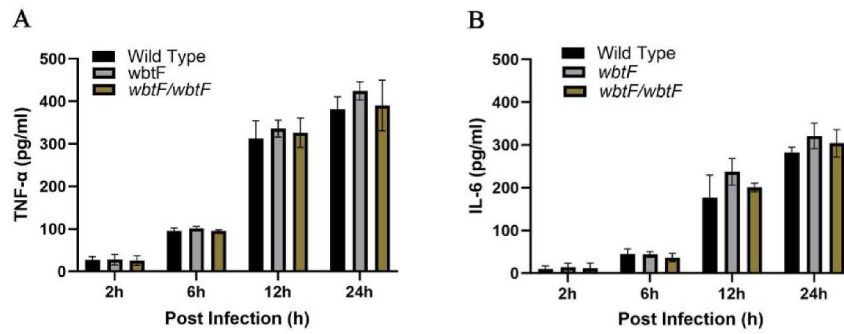


Figure 8. THP-1 cells were infected with *F. novicida* at an MOI of 1, followed by gentamicin treatment (50 µg/mL) for 1 h to eliminate extracellular bacteria. After 2 h, 6 h, 12 h, and 24 h of incubation, cell supernatants were collected, and the levels of TNF-α (A) and IL-6 (B) were determined using ELISA. The data represent the averages and standard deviations of three identical experiments. Statistical significance relative to the WT strain at each designated time point was determined using multiple comparisons and no significant difference was observed.

General Conclusion

This thesis elucidated the genetic determinants underlying the cytotoxicity, intracellular survival, and host–pathogen interactions of *Francisella novicida*. Using a screening of a transposon mutant library and a newly established epithelial cell infection model (HeLa–FcγRII cells), two genes, *FTN_0096* and *wbtF*, were identified and characterized as virulence determinants.

In chapter 1, I identified *FTN_0096* as a pathogenic factor of *F. novicida*. *FTN_0096* is required for intracellular replication in the cells. Functional analyses revealed that *FTN_0096* plays a central role in promoting bacterial survival by facilitating escape from phagosomal compartments, thereby allowing the pathogen to avoid degradation within lysosomes. In its absence, the mutant strain failed to efficiently exit the phagosome and instead colocalized extensively with lysosomal markers, leading to progressive bacterial clearance. *FTN_0096* also associates with host organelle-mediated defenses in HeLa–FcγRII cells and THP-1 cells, including those involving the mitochondria and Golgi complex. Detailed investigations into the function of *FTN_0096* are required to clarify its role in the pathogenicity of *F. novicida*. Moreover, the detailed mechanisms of *FTN_0096* can contribute to developing vaccines that can prevent diseases caused by *Francisella*.

In chapter 2, my research focused on identifying *wbtF* as a pathogenic factor of *F. novicida*. The *wbtF* mutant exhibited reduced intracellular replication and was required for escaping from phagolysosomes. *wbtF* associates with mitochondria and the Golgi complex in HeLa–FcγRII cells and THP-1 cells, although it did not significantly affect cytokine production. The *wbtF* gene located within the O-antigen-biosynthetic gene clusters. The O-antigen constitutes one of the three components of LPS, along with the lipid A and the oligosaccharide core. *Francisella* LPS demonstrates a markedly reduced ability to induce the secretion of inflammatory cytokines. These results highlight *wbtF* as an important virulence determinant,

deepening our understanding of *Francisella* pathogenesis and providing new insights into potential targets for therapeutic intervention and controlling *Francisella* infection. However, further research is necessary to elucidate the detailed mechanism of *wbtF* function in *Francisella* infection.

Both *FTN_0096* and *wbtF* mutants exhibited significantly reduced intracellular replication in epithelial (HeLa–FcγRII) and monocytic (THP-1) cells compared with the wild-type strain. These mutants also exhibited impaired escape from phagolysosomes during the early stages of infection, resulting in their retention and degradation within lysosomal compartments. In addition, altered localization to mitochondria and the Golgi complex was observed, indicating that these genes are involved in modulating host cell organelles to favor bacterial survival.

Across the two studies, I identify important factors contributing to the pathogenicity of *Francisella* and conduct detailed analyses on two of these genes. Although my analyses were limited to only two genes, this study advances our understanding of *Francisella* pathogenesis by identifying and functionally characterizing the virulence factors that contribute to intracellular replication and host–pathogen interactions. These insights not only improve our knowledge of *Francisella* biology but may also guide the development of future therapeutic or preventive strategies against tularemia.

Acknowledgement

All praise goes to Almighty God for His gracious guidance, which enabled me to complete my studies through all the difficulties over the past four years. I am so grateful to have had my student life at the Laboratory of Veterinary Public Health, Joint Graduate School of Veterinary Medicine, Yamaguchi University, from 2022 to 2026.

I am deeply grateful to have Prof. Masahisa Watarai, DVM, PhD, as the main supervisor, Takashi Shimizu, PhD and Kenta Watanabe, DVM, Ph.D, during my PhD studies in the Laboratory of Veterinary Public Health, Yamaguchi University for his wholehearted guidance, advice, and inspiration. Dr. Akihiko Uda (Department of Veterinary Science, National Institute of Infectious Diseases) provided the materials necessary for *Francisella* research. I would like to gratefully acknowledge other members of the supervisory committee, Hidekazu Niwa, DVM, PhD, and Yusuke Matsumoto, DVM, PhD, for their kindness during my study.

I also want to express my deep gratitude to all current and former members Laboratory of Veterinary Public Health for their assistance, technical support, and scientific collaboration during my research. I am thankful to the staff members of the United Graduate School of Veterinary Science's office and the International Support Center of Yamaguchi University for their cooperation in preparing the necessary documents.

Special thanks appreciated to the Ministry of Education, Culture, Sports, Science and Technology (Monbukagakusho) of Japan for awarding me the scholarship that allowed me to pursue my studies and research at Yamaguchi University.

I would like to thank the members of Division Veterinary Public Health, Department of Veterinary Sciences, Universitas Airlangga, and all my colleagues in the Faculty of Veterinary Medicine, Universitas Airlangga, whom I could not mention individually, for supporting me and helping me to achieve the highest level of success in my studies.

Finally, I would like to express my sincere gratitude to my beloved parents, my brother, my sister, my family, and especially to my wife, who kept me in their prayers for my success. Their never-ending support has been crucial in helping me achieve the highest level of success in my studies. I am also deeply grateful to all my friends who have stood by me throughout this journey. I hope the result of this study will be helpful in the veterinary field.

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