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RtxA like protein contributes to infection of *Francisella novicida* in silkworm and human macrophage THP-1

Shib Shankar Saha[a,b], Akihiko Uda[c], Kenta Watanabe[a], Takashi Shimizu[a], Masahisa Watarai[a,*]

[a] The United Graduate School of Veterinary Science, Yamaguchi University, 1677-1, Yoshida, Yamaguchi, 753-8515, Japan
[b] Department of Pathology and Parasitology, Patuakhali Science and Technology University, Babugonj, Barisal, 8210, Bangladesh
[c] Department of Veterinary Science, National Institute of Infectious Diseases, Shinjuku, Tokyo, 162-8640, Japan

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ABSTRACT

Tularemia is a zoonosis caused by CDC-declared Tier 1 threat agent *Francisella tularensis*. *F. tularensis* subsp. *novicida* (*F. novicida*) is virulent in mice but non-pathogenic in immunocompetent humans and serves as a potential surrogate organism. In a recent study, we established a silkworm (*Bombyx mori*) model of infection for *F. novicida*. *Francisella* secretes its virulence factors through various mechanisms that modify the intracellular environment to ensure its replication and survival. To identify new pathogenic factors, we focused on the type I secretory system (T1SS) of *Francisella*. In silico analysis revealed a RtxA (Repeats-in-toxin) like protein in the *Francisella* genome. The characteristics of RtxA like protein were investigated using mutant analysis. Firstly, the role of *rtxA* in silkworms was investigated by infecting them with *F. novicida* strains into the hemocoel. The *rtxA* mutant failed to kill the silkworms, whereas *F. novicida* wild-type (WT) strain killed silkworms within 3–7 days post infection. The arrested growth of the mutant strain in silkworms was observed using a whole-body CFU count assay. We also investigated the growth characteristics of the *rtxA* mutant in hemocytes, one of the primary multiplication sites of *Francisella* within silkworms. Interrupted growth of the *rtxA* mutant with significantly reduced cytotoxicity was observed in hemocytes via confocal microscopy. Next, we analyzed the effect of *rtxA* in human monocyte cell line THP-1. The mutant strain showed significantly decreased growth and reduced cytotoxicity compared with its parental strain in THP-1 cells. This study newly identified RtxA like protein of *F. novicida* as an important lethal pathogenic factor in silkworm and mammalian cells.

1. Introduction

Tularemia is a disease of both man and animals, caused by *Francisella tularensis,* a facultative and Gram-negative intracellular bacterium [1]. *Francisella* transmission into hosts such as rabbits, hares, and small rodents occurs by various routes, for example, arthropod bites, direct contact with an infected animal, ingestion of contaminated food and water, or through aerosols [2]. Since there are no reports of person-to-person transmission, multiplication within arthropods is considered to enhance *Francisella* virulence. The genus *Francisella* comprises two species: *F. tularensis* and *F. philomiragia*. *F. philomiragia* is a fish pathogen, occasionally causing disease in humans [3]. *F. tularensis* has four subspecies: *F. tularensis* subsp. *tularensis* (type A), *F. tularensis* subsp. *holarctica* (type B), *F. tularensis* subsp. *mediasiatica*, and *F. tularensis* subsp. *novicida.* The subspecies are markedly different in their virulence but are closely related phylogenetically [2,4]. *F. tularensis* subsp. *novicida* (referred to here as *F. novicida*) shares a high percentage of homology with *F. tularensis* subsp. *tularensis* and is thus widely used as a model organism for *Francisella* research [5].

For successful parasitism, *Francisella* survive and replicate in a number of mammalian cells but multiply mainly within macrophages [6,7]. A large number of identified potential virulence factors affect the ability of *Francisella* to survive in macrophages. The virulence factors are secreted through various secretion systems, classified into nine systems (Type I–IX secretion systems) [8,9]. The gene product of the *Francisella* pathogenicity island (FPI) is secreted through the type VI secretion system. However, the secretion system of other virulence factors such as MsrB, AcpA, KatG, and SodB remain unknown [10,11]. Type I secretion systems (T1SS) are widespread in pathogenic Gram-negative bacteria such as *Escherichia coli*, *Vibrio cholerae*, *Bordetella pertussis*, and *Legionella pneumophila* [12,13]. This secretion system comprises three specific proteins that transport substrates to the cell exterior in one step across the cell wall. TolC, the outer membrane protein, is composed of a short β-barrel (outer membrane) and a long α-helical (periplasmic) structure, which is deeply embedded into the periplasm forming a long channel [14]. TolC, in association with inner

* Corresponding author.
E-mail address: watarai@yamaguchi-u.ac.jp (M. Watarai).

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membrane ABC exporter and a membrane fusion protein, forms the T1SS. The interaction of TolC with diversified inner membrane and periplasmic proteins forms efflux systems such as the AcrAB RND efflux pump that transports various molecules including virulence-associated proteins, antibiotics, and detergents [12,15–17]. TolC of *Francisella* is an important virulence determinant factor, modulating host adaptive and innate immune responses. However, the exact mechanism is yet to be elucidated. TolC neither aids phagosomal escape nor initiates replication within the host cytosol. Instead, it continually secretes a number of uncharacterized effector proteins that delay host cell apoptosis induction by suppressing proinflammatory cytokines, resulting in the preservation of the replicative niche and boosting bacterial growth [18,19].

There is no report of the existence of a complete T1SS in *Francisella*. Repeats-in-toxin (RTX) proteins are generated by various Gram-negative bacteria presenting two main features. Firstly, repetitions of glycine- and aspartate-rich sequences, typically nanopeptides, are present, which are located in the carboxy terminal of the protein. Secondly, RTX protein is secreted through the T1SS [20]. HlyA is the firstly reported as RTX toxin (repeats-in-toxin) secreted by T1SS in *E. coli* strains [21]. *Francisella* genome possesses the nucleotide having amino acid sequence similarity (named here as RtxA like protein) with FrpC of *Neisseria meningitides* which is homolog to HlyA of *E. coli*. The contributory role of RTX toxin to virulence has been widely studied in many Gram-negative bacteria [20]. However, the role of RtxA like protein in the pathogenesis of *Francisella* has not been reported.

In this present study, we constructed *F. novicida rtxA* mutant by disrupting the nucleotide sequence of RtxA like protein to characterize the phenotype of *rtxA* in both silkworm and human macrophages (THP-1). The resulting mutant strain revealed significantly reduced intracellular growth and cellular cytotoxicity in silkworms and THP-1 cells compared with its parental strain of *F. novicida*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

F. novicida U112 was obtained from the Pathogenic Microorganism Genetic Resource Stock Center, Gifu University, Japan. *F. novicida* was cultured aerobically at 37 °C in brain heart infusion broth (BHI; BD, Franklin Lakes, NJ, USA) supplemented with cysteine (BHIc) [22], or Brucella broth (BD) containing 1.5% agar (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Bacterial strains were maintained as glycerol stock and stored at −80 °C.

2.2. In vitro growth assay of Francisella novicida strains in BHIc broth

Overnight cultures of *Francisella* strains were diluted to an OD_{660} of 0.05 and then incubated under shaking in BHIc broth at 37 °C. Mid-exponential phase of bacterial growth of each strain were diluted to an OD_{660} of 0.05 in BHIc broth. The OD_{660} was measured every hour up to 12 h. Experiments were performed in triplicates.

2.3. Expression analysis of rtxA mRNA in Francisella novicida

Expression of RtxA like protein was analyzed by isolating total RNA from an exponential phase culture of *Francisella* using a NucleoSpin® RNA isolation kit (Macherey-Nagel, Düren, Germany). The RNA was quantified by measuring $A_{260\ nm}$ on a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription used ReverTra Ace® qPCR RT Master Mix (Toyobo Co. Ltd, Osaka, Japan), and the cDNA samples were stored at −30 °C prior to use. The StepOne™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with KOD SYBR® qPCR Mix (Toyobo) was used for quantitative PCR (qPCR). The primer sets utilized in this experiment are listed in Table 1. The *tul4* gene was employed as an internal control to normalize the data. The expression level was calculated by ΔC_t quantification method.

2.4. Construction of F. novicida rtxA mutant

The *rtxA* mutant of *F. novicida* (Δ*rtxA*) was generated by group II intron insertion using the TargeTron® Gene Knockout System (Sigma-Aldrich, St. Louis, MO, USA) modified for *Francisella* species [23], as described previously [24]. pKEK-RtxA was constructed using primers rtxA-IBSXhoI, rtxA-EBS1d, and rtxA-EBS2. Cryotransformation of 2 μg of pKEK-RtxA was performed [25], and the cells were precultured in a chemically defined medium (CDM) [26] at 30 °C for 6 h. The bacterial cells were then collected and cultured at 30 °C on BHIc plates containing 50 μg/mL of kanamycin. Mutagenesis was confirmed using PCR to detect a 915-bp insertion. For plasmid removal, the mutants were further cultured at 37 °C on BHIc plates without antibiotics. Primers used for the construction of the *rtxA* mutant are shown in Table 1.

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

The *gfp* (green fluorescent protein) gene with a Shine-Dalgarno sequence was cloned from pGreenTIR [27] and ligated with pOM5 plasmid as described previously [28].

2.6. Silkworm infection assay

Fourth instar *B. mori* larvae (Hu•Yo × Tukuba•Ne) were obtained from Ehime Sanshu (Ehime, Japan), raised at room temperature (25 °C), and fed Silkmate 2 M (Nosan Corporation, Kanagawa, Japan). For the silkworm survival assay, day 2 fifth instar larvae underwent hemocoel inoculation with 50 μL of bacterial suspension in PBS (1×10^8 CFU/mL) using a 1-mL syringe equipped with a 30-gauge needle (Terumo Inc., Tokyo, Japan). Following inoculation, the silkworms were incubated at room temperature with food. The mortality rate was determined by observing the infected silkworms daily for up to ten days post inoculation. The bacterial load (as CFU/ml) was monitored at 1, 24, 48, 60, 72, and 96 h post inoculation. Briefly, the infected silkworm larvae were weighed and placed in disposable 15-mL centrifuge tubes before homogenizing with a Biomasher SP (Funakoshi Co., Ltd, Tokyo, Japan) and suspending in 3 mL of PBS. The mixture was centrifuged at 300 × *g* for 30 s and the concentrated suspension was separated from the solid tissues. Appropriate dilutions were used to spread the suspension samples onto Brucella agar plates, incubated at 37 °C incubator, and the colonies were counted. The summed volumes of the hemolymph and tissues were estimated together (1 g = 1 mL) to calculate the CFUs.

2.7. Fluorescence microscopy for observing the growth of Francisella in silkworm hemocytes

The growth pattern of *F. novicida* strains within silkworm hemocytes was investigated by inoculating day 2 fifth instar larvae with GFP-expressing bacteria. At 1, 14, and 24 h post inoculation, hemolymph was collected and examined under a FluoView FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) as before [28].

2.8. Detection of live or dead hemocytes

The cytotoxicity of *F. novicida* to silkworm hemocytes was examined using a LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes™, Invitrogen, Carlsbad, CA, USA). For this assay, day 2 fifth instar larvae were infected with 50 μL of bacterial suspension in PBS (1×10^8 CFU/mL) as per the bacterial CFU count assay. At 1, 14, 24, and 48 h post inoculation, hemolymph was collected and plated on 24-well tissue culture plates covered with 12-mm glass coverslips (Thermo Fisher Scientific). For attachment of the silkworm hemocytes to the coverslips,