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Impact of *Francisella tularensis* pilin homologs on pilus formation and virulence

Nicole M. Ark and Barbara J. Mann*

Department of Internal Medicine, Division of Infectious Diseases and International Health, University of Virginia, Charlottesville, VA 22908

Abstract

Francisella tularensis is a facultative intracellular bacterium and the causative agent of tularemia. Virulence factors for this bacterium, particularly those that facilitate host cell interaction, remain largely uncharacterized. However, genes homologous to those involved in type IV pilus structure and assembly, including six genes encoding putative major pilin subunit proteins, are present in the genome of the highly virulent Schu S4 strain. To analyze the roles of three putative pilin genes in pili structure and function we constructed individual *pilE4*, *pilE5*, and *pilE6* deletion mutants in both the *F. tularensis tularensis* strain Schu S4 and the Live Vaccine Strain (LVS), an attenuated derivative strain of *F. tularensis holarctica*. Transmission electron microscopy (TEM) of Schu S4 and LVS wild-type and deletion strains confirmed that *pilE4* was essential for the expression of type IV pilus-like fibers by both subspecies. By the same method, *pilE5* and *pilE6* were dispensable for pilus production. *In vitro* adherence assays with J774A.1 cells revealed that LVS *pilE4*, *pilE5*, and *pilE6* deletion mutants displayed increased attachment compared to wild-type LVS. However, in the Schu S4 background, similar deletion mutants displayed adherence levels similar to wild-type. *In vivo*, LVS *pilE5* and *pilE6* deletion mutants were significantly attenuated compared to wild-type LVS by intradermal and subcutaneous murine infection, while no Schu S4 deletion mutant was significantly attenuated compared to wild-type Schu S4. While *pilE4* was essential for fiber expression on both Schu S4 and LVS, neither its protein product nor the assembled fibers contributed significantly to virulence in mice. Absent a role in pilus formation, we speculate *PilE5* and *PilE6* are pseudopilin homologs that comprise, or are associated with, a novel type II-related secretion system in Schu S4 and LVS.

1. Introduction

Francisella tularensis is a Gram-negative, facultative intracellular bacterium and the causative agent of tularemia [1]. This zoonotic organism infects hundreds of animal species including humans. Signs and symptoms of illness are dependent upon the route of infection and the extent of systemic involvement [2]. Pneumonic tularemia has the most severe presentation and greatest potential for fatality, whereby inhalation of as few as 10 bacteria can elicit illness in humans [3]. This low lethal dose, ease of dissemination, and the anticipated public health consequences resulting from an aerosol release have led to the classification of *F. tularensis tularensis* as a select agent by the Centers for Disease Control

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*Corresponding author. Tel.: +1 434-924-9666; fax: +1 434-924-0075, bjm2r@virginia.edu.

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and Prevention (CDC), the US Department of Health and Human Services, and the Department of Agriculture [4, 5].

F. tularensis consists of three subspecies that differ in virulence capacities and global distribution [1]. *F. tularensis tularensis* (*F. tularensis*) and *F. tularensis holarctica* (*F. holarctica*) are the most prevalent and responsible for the majority of infections; *F. tularensis* is the most virulent among the subspecies, while *F. holarctica* causes a milder form of the disease that is rarely fatal. *F. tularensis mediasiatica* exhibits even lower virulence, and is found within a geographically limited niche. *F. novicida* is a closely related species that has low virulence in human, but is highly virulent in mice [1, 6]. The Live Vaccine Strain (LVS) is an attenuated strain derived from serial passage of an *F. holarctica* strain. This strain retains virulence for mice (albeit attenuated compared to the more virulent *Francisella* spp.), but affords protection to laboratory workers [3, 7]. However, this strain is not licensed for use as a vaccine in the United States. Compared to a wild-type *F. holarctica* genome, the LVS genome has a number of polymorphisms within predicted coding regions. Some of these disrupted genes have been implicated in the pathogenicity of more virulent *Francisella* subspecies [7, 8]; the reintroduction of FTT0918, encoding a putative outer membrane protein, is able to restore wild-type levels of virulence in a mouse model of infection [3, 9].

The genomes of *F. tularensis* spp. contain 14 genes with similarity to those with characterized involvement in the structure and assembly of type IV pili [10, 11]. Type IV pili are associated with virulence in many bacterial species by promoting attachment to host cells as a first step in pathogenicity [12, 13]. The genetic organization of the type IV pilin-related genes in *Francisella* is similar to that in other type IV pili-bearing organisms, with some genes clustered together and others scattered throughout the genome [11]. *In silico* analyses of other members within the *Francisella* species, *F. holarctica* (NCBI Accession Number NC_008369), LVS (NC_007880), and *F. novicida* (NC_008601) revealed similar organizations [14–16].

Type II secretion systems use components homologous to those of the type IV pilus system to mediate substrate release. Certain proteins (e.g. PilT which controls filament disassembly) are generally specifically indicative of type IV pilus systems; others are restricted to type II secretion systems (e.g. GspC, GspL, and GspM, for which no homologues exist in *Francisella* subspecies [17]). Type II secretion systems can produce surface-associated fibers termed “pseudopili” upon overexpression of certain pilin homologs [18, 19], and some type IV pilus systems also secrete virulence factors into the extracellular environment, even in an organism possessing a functional type II secretion system [20].

In *Francisella* both surface fiber expression (LVS and *F. novicida* [11, 21, 22]) and a novel type II protein secretion system (*F. novicida*, [17, 21]) have been documented. However, a functional type II secretion system has not been confirmed in LVS or highly virulent *F. tularensis* [17]. *In vitro* studies indicate overlap among the putative type IV pilus and type II secretion systems in *F. novicida*; certain proteins are essential to only one system, while others are crucial for the activities of both [21, 23]. Six genes in the *Francisella* spp. are predicted to encode major type IV pilin subunit proteins, and have been designated *pilE1*–*pilE6* (FTT0890c, FTT0889c, FTT0888c, FTT0861c, FTT0230c, and FTT1314c, respectively, according to Schu S4 nomenclature, see suppl. Table 1 for gene names and orthologous loci) [11]. Full open reading frames for *pilE1*–*pilE6* are present in both *F. tularensis* and *F. novicida*. However, *F. holarctica* strains possess truncations in one or more of *pilE1*, *pilE2*, or *pilE3*, while its derivative LVS completely lacks *pilE1*, and has truncation mutations in both *pilE2* and *pilE3*. Despite these deficiencies, fibers have been

documented on the surface of LVS [11], implicating the remaining three pilin subunit homologs in fiber assembly and/or expression.

Notably, Pile4 has been previously implicated as the major subunit pilin protein because its absence abrogates pilus expression in *F. novicida* [21]. In this study, we aimed to determine the role(s) of the proteins designated Pile4, Pile5, and Pile6 in type IV pilus formation in the virulent Schu S4- and the attenuated LVS. We found that Pile4 is required for pilus formation in both subspecies, but that neither this protein nor the pilus structure itself were essential virulence factors for *Francisella* subspecies in mice. Pile5 and Pile6 were required for full virulence, but did not noticeably influence pilus formation.

2. Methods

2.1 Bacterial strains, plasmids, and culture

All bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar supplemented with 100 µg/ml ampicillin. Schu S4, LVS, and their derivatives were grown on Muller-Hinton (MH) agar supplemented with 0.01% glucose (Sigma, St. Louis, MI), 0.0025% ferric pyrophosphate (Sigma), 0.1% cysteine (Sigma), and 2.5% horse serum (Invitrogen); liquid cultures were grown in trypticase soy broth supplemented with 0.1% cysteine (TSBc), and 15 µg/ml kanamycin when appropriate. All studies involving *F. tularensis* Schu S4 and mutant derivative strains were carried out in an approved biosafety level 3 laboratory; manipulation of the *F. holarctica* derivative LVS was carried out in an approved biosafety level 2 laboratory.

2.2 DNA manipulation

Oligonucleotides were synthesized by Integrated DNA Technologies Incorporated (Coralville, IA). The FastStart High-fidelity PCR system (Roche, Indianapolis, IN) and Sahara DNA polymerase (Bioline, Taunton, MA) were used to amplify genomic DNA regions for suicide and complementation vector construction. DNA sequencing was performed by the University of Virginia Biomolecular Research Facility (Charlottesville, VA) and by Genewiz (South Plainfield, NJ). PCR products and plasmid DNA were purified using commercial kits (Qiagen, Valencia, CA). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). DNA and protein sequences were aligned and compared with CLUSTAL W [24]. All reagents were used according to manufacturers' protocols.

2.3 RNA manipulation

Bacterial cultures were grown overnight at 37°C in a shaking incubator. Bacteria were pelleted from approximately 1 OD₆₀₀ of culture, resuspended in 1 ml Trizol reagent (Invitrogen, Carlsbad, CA), and stored overnight at -80°C. RNA was precipitated the following day according to manufacturer's protocol, and purified using the RNeasy Mini Kit (Qiagen). cDNA was synthesized from approximately 300 ng RNA using random hexamers (Invitrogen) and Superscript II Reverse Transcriptase (Invitrogen), and used in PCR reactions to identify regions of transcription. Resulting amplicons were analyzed by agarose gel electrophoresis; DNA was stained with ethidium bromide and visualized using an ultraviolet light source.

2.4 Expression of tagged proteins for recombinant antibody production and screening

We used the cloning vector pCRT7NT-TOPO (Invitrogen) to generate recombinant N-terminally His₆-tagged fusion proteins of Pile5 and Pile6 that could be stably expressed in *E. coli*. Between Schu S4 and LVS these proteins differ by only two (Pile5) or three (Pile6) amino acids; antibodies were raised against the LVS proteins with the assumption they

would recognize their protein counterparts from Schu S4. Amplification of *pilE5* and *pilE6* from 100 ng LVS genomic DNA yielded PCR products that could be directly ligated into the TOPO vector. Ligation reactions were performed as per manufacturer's protocol and transformed into TOP10 *E. coli* cells (Invitrogen). Transformants were screened via sequencing to assess insert orientation and reading frame prior to protein expression and purification. Confirmed plasmids were transformed into *E. coli* BL21 cells (Invitrogen) to facilitate IPTG-induced protein expression. Propagated bacteria bearing expressed protein were lysed in Cell Lytic 2x lysis reagent (Sigma), and cleared lysates added to TALON Metal Affinity Resin (Becton-Dickinson) to facilitate protein purification. Mixtures were transferred to gravity flow columns and washed with buffer containing 20 mM imidazole (Sigma) to remove unbound proteins and reduce nonspecific binding. Polyhistidine-tagged proteins were eluted in 1 M imidazole, pH 7.0, and those fractions containing protein (determined via the Bradford Assay [25] (Pierce)) were dialyzed and concentrated in Centricon Plus-20 centrifugal filter devices (Millipore, Billerica, MA). A Bradford Assay determined the final concentrations of purified proteins.

We used the cloning vector pGST-Parallel1 [26] to generate N-terminally GST-tagged fusion proteins of PilE1-PilE6 that could be stably expressed in *E. coli*. Amplification from 100 ng Schu S4 (and LVS where designated, Table 1) genomic DNA yielded PCR products, which were enzymatically, digested, ligated into the parent vector, and transformed into subcloning efficiency DH5 α . Transformant colonies were screened for sequence and proper reading frame prior to protein expression and purification. Confirmed plasmids were transformed into *E. coli* BL21 cells (Invitrogen) to facilitate IPTG-induced protein expression. Propagated bacteria bearing expressed protein were lysed in Cell Lytic 2x lysis reagent, and cleared lysates added to a glutathione-agarose suspension (Sigma). Mixtures were transferred to gravity flow columns and washed with PBS-T (PBS, 1% Triton X-100) (Sigma) to remove unbound proteins. GST-tagged proteins were eluted from columns in 10 mM reduced glutathione (pH 9.5) and fractions containing protein were dialyzed and concentrated as above. A Bradford Assay determined the final concentrations of purified proteins.

2.5 Production of polyclonal rabbit serum

One mg of each respective dialyzed His₆-tagged protein was shipped to Cocalico Biologicals (Reamstown, PA) for polyclonal antibody production in New Zealand White rabbits. Animals were immunized with 100 μ g protein mixed with Complete Freund's Adjuvant, and boosted with 50 μ g protein mixed with Incomplete Freund's Adjuvant on days 14, 21, and 49 post-initial injection. Sera from pre-bleeds and test-bleeds were screened for PilE5- and PilE6-specific antibodies by western blot against recombinant GST-tagged proteins, as well as *Francisella* whole cell lysates, to determine specificity.

2.6 Extraction of protein-specific antibodies from polyclonal rabbit serum

CNBr-activated Sepharose 4B beads (Sigma) were suspended and coupled to 1 mg GST-PilE5 or GST-PilE6 as per manufacturer's general protocol. Protein-specific antibodies were recovered from 1.5 ml polyclonal rabbit serum, respectively, dialyzed and concentrated in Centricon Plus-20 devices. The Bradford assay was used to determine final antibody concentrations.

2.7 Construction of *Francisella* nonpolar deletion and complementation mutants

Approximately 1.5 kb genomic DNA fragments upstream (5') and downstream (3') from each gene of interest were amplified by PCR and cloned into the kanamycin-resistant *sacB* suicide vector pGIR463 (a gift from Girija Ramakrishnan). Resulting plasmids were introduced into competent Schu S4 and LVS via electroporation [27]. Kanamycin-resistant

transformants were plated onto MH agar supplemented with 5% sucrose to resolve plasmid-specific regions and the targeted gene of interest from the chromosome. Primers corresponding to gene-specific flanking regions were used to confirm gene deletions from chromosomal DNA isolated from sucrose resistant colonies (Gentra Systems, Minneapolis, MN). RT-PCR was performed for deletion mutants to confirm nonpolarity of deletions.

Complementation plasmids were constructed by inserting PCR-amplified full length genes into the pFNLTP6-*groE-gfp* vector conferring kanamycin resistance [28]. Plasmids were transformed into their respective deletion mutants as above, and kanamycin-resistant colonies were screened for plasmid presence using a QIAprep Spin Miniprep Kit (Qiagen) followed by agarose gel electrophoresis and visualization with ethidium bromide and UV illumination. All inserted gene sequences were verified by DNA sequencing prior to bacterial transformation.

2.8 Transmission electron microscopy

Schu S4, LVS, and deletion mutant strains grown from frozen stocks were streaked to MH agar and grown for approximately 40 hrs at 37°C. Prior to use, carbon-coated formvar grids (Electron Microscopy Sciences, Hatfield, PA) were glow-discharged at 5 $\mu\text{Å}$ - using a sputter coater to render them hydrophilic. These glow-discharged grids were gently touched to single bacterial colonies and then fixed with 0.5% glutaraldehyde (Fisher Scientific, Pittsburgh, PA). Grids were washed with PBS and water before staining with freshly made 2% uranyl acetate. Negative staining was visualized with a JEOL 1230 transmission electron microscope at 6000x magnification.

2.9 Adherence assays

Murine macrophage-like J774A.1 (ATCC TIB-67) cells were propagated in high-glucose Dulbecco modified Eagle medium supplemented with sodium pyruvate, 10% fetal bovine serum, and penicillin-streptomycin (cDMEM) (Invitrogen) as per ATCC recommendations. For experimentation, J774A.1 cells were seeded in 24-well tissue culture-treated polystyrene plates (Sigma) approximately 24 hrs prior to infection. Immediately prior to infection cells were washed with cDMEM- (lacking antibiotics), then incubated in the same medium containing 1 $\mu\text{g/ml}$ cytochalasin D (Sigma) for 1 hr to inhibit their intrinsic phagocytic activity. Media was aspirated and bacteria (in cDMEM- containing 1 $\mu\text{g/ml}$ cytochalasin D) were added to 2.5×10^5 cells at an MOI of approximately 100:1. Plates were centrifuged at $500 \times g$ for 5 min to facilitate bacterial contact with mammalian cells, and incubated at 37°C. After 1 hr wells were washed with cDMEM- to facilitate the removal of non-adherent bacteria. Control wells were further incubated in media containing gentamicin (25 $\mu\text{g/ml}$) (Sigma) for 2 hrs to kill any extracellular bacteria; this facilitated detection of any intracellular bacteria and confirmed the efficiency of cytochalasin D in preventing uptake. Mammalian cells were lysed in 0.1% sodium deoxycholate (Sigma) in cDMEM- for 5 min, and initial and recovered adherent bacteria were quantitated by plating serial dilutions and enumerating CFUs after 48 hrs.

2.10 Statistical analysis of adherence

In vitro adherence of wild-type and mutant *Francisella* strains was analyzed in triplicate across a minimum of three independent experiments. All numerical values representing adherence as a percentage of respective starting inoculum were converted to log approximations and compared using the General Linear Model within the MiniTab®15 Software Package. This model uses multiple comparisons to calculate the mean adherence for each wild-type and mutant strain, and intrinsically adjusts for variability to fit the data. Due to the inherent biological variability across our experiments, this model allowed our statistical analysis to incorporate data from all experiments collectively. Here, we report the

calculated adherence capacity of each mutant strain as a ratio compared to its respective wild-type parent strain, for which the mean adherence has been designated “1”; 95% confidence intervals are graphically represented by error bars. While this pattern of adherence was consistent across multiple independent trials, there was a significant amount of biological variability among repeated experiments.

2.11 Confocal microscopy

J774A.1 cells were seeded to 24-well plates containing glass cover slips (Fisher), and adherence assays performed simultaneously to those described above. Following 1 hr incubation with bacteria, cells were washed with cDMEM- to remove non-adherent bacteria and then fixed by the addition 4% paraformaldehyde (Sigma). Following fixation, cells were blocked in 10% goat serum (in PBS), and then incubated with *F. tularensis* rabbit antiserum (Becton-Dickinson, Franklin Lakes, NJ) (diluted 1:5000 in 10% horse serum in PBS). Following washes, mammalian cells were permeabilized by the addition of 0.1% Triton X-100, blocked with 10% goat serum and then incubated with *F. tularensis* mouse antiserum (diluted 1:5000 in 10% horse serum in PBS) for 1 hr at room temperature. Cells were washed with PBS and then secondary antibodies, goat-anti-rabbit AlexaFluor 488 and goat-anti-mouse AlexaFluor 546 (Invitrogen) (diluted 1:1000 in 10% horse serum in PBS), were added to cells and incubated in the dark at room temperature for 1 hr. Following PBS washes, cover slips were mounted to microscope slides (Fisher) using Fluoromount G (Southern Biotech, Birmingham, AL). Images were captured using a Zeiss LSM 510 laser scanning confocal microscope and analyzed with the LSM Image Browser software. Surface-associated bacteria are stained yellow by this procedure.

2.12 In vivo bacterial infection

Six- to 8- week old C57BL/6 or Balb/C mice (Jackson Laboratory, Bar Harbor, Maine) were injected subcutaneously or intradermally with 2.2×10^6 – 5.0×10^8 CFU of LVS derived strains and 9 to 20 CFU of Schu S4 derived strains in a volume of 50–100 μ l. An aliquot of each strain, frozen in MH broth at -80°C , was thawed per experiment; calculated CFUs after thawing did not vary significantly over the course of experiments. Dilutions of stock strains were calculated to approximate the desired infectious dose per each injection; actual doses of inoculated bacteria were confirmed by simultaneous plate count CFU determination. All materials were diluted in 1x PBS prior to injection. Mice were monitored for at least 21 days post-infection, and were euthanized when signs of irreversibly mortality became apparent.

For dissemination experiments, C57BL/6 mice were injected with approximately 2×10^7 CFUs and sacrificed on days 3, 6 and 9 post-injection. A portion of the liver was homogenized in PBS, and then diluted to determine the number of CFUs/gram of liver. Statistical significance between the means was determined using the Student's *t*-test. All mouse studies were approved by the University of Virginia's Animal Care and Use Committee.

3. Results

3.1 Schu S4 and LVS produce Pile4, Pile5, and Pile6 proteins

Following *in silico* identification of the three type IV pilin subunit homologs within the Schu S4 and LVS genomes, we and others confirmed by RT-PCR that all open reading frames were transcribed in both subspecies ([11], and data not shown). However, whether protein products were translated from their respective mRNAs was uncertain. We raised polyclonal antibodies against recombinant LVS His₆-Pile5 and His₆-Pile6, and used them in Western blots against GST-tagged recombinant Schu S4 proteins, and recombinant GST-

tagged LVS Pile4 to confirm that antibody recognition was specific to each respective recombinant pilin homolog. Fig. 1A demonstrates that Pile1-Pile6 were all recombinantly expressed in *E. coli*, while 1B shows anti-Pile5 was specific for only GST-Pile5, and 1C shows anti-Pile6 was specific for only GST-Pile6. These specific antibodies were used in subsequent Western blots to verify Pile5 and Pile6 expression in whole cell lysates from both Schu S4 and LVS (Fig. 2). Expression of Pile5 (Fig. 2A), and Pile6 (Fig. 2B) was confirmed in wild-type strains, while protein absence was confirmed in respective deletion strains (designated “Δ”), and reconstituted protein expression was confirmed in *trans*-complemented strains (designated “c”). These *trans*-complemented strains expressed their respective proteins above wild-type levels, a direct result of using the strong *groE* promoter to drive protein expression.

An antibody recognizing *F. novicida* Pile4 was generously provided by David Thanassi (State University of New York at Stonybrook) to assess the expression of this protein in our various wild-type and mutant strains. Unfortunately, in our hands this antibody failed to recognize Pile4 from whole cell lysates of either Schu S4 or LVS. It did recognize the GST-tagged form of LVS and Schu S4 Pile4 recombinantly expressed in an *E. coli* background (data not shown). The reason for this is unclear; perhaps a structural alteration between recombinant and native proteins dictates antibody recognition. Though we could not use Western blot to confirm that Pile4 was expressed (or absent) within our various strains, negative stain transmission electron microscopy (discussed in Section 3.2) demonstrated a critical role for this protein in pilus formation in both Schu S4 and LVS.

3.2 Pile4 is essential for surface fiber expression by Schu S4 and LVS

Negative stain transmission electron microscopy (TEM) is a conventional method by which bacterial surface appendages, such as type IV pili, can be observed. We used TEM to visually confirm the presence of pilus-like fibers on the surface of LVS ([11]) and demonstrate that Schu S4 also expressed similar fibrous surface-associated appendages that resemble bundled type IV pili (Figs. 3 and 4). Fig. 3 shows that, in the Schu S4 background, there was no observable phenotypic effect on pilus expression in the absence of *pilE5* (Schu S4Δ*PilE5*) or *pilE6* (Schu S4Δ*PilE6*). However, in the absence of *pilE4* (Schu S4Δ*PilE4*) the surface-associated fibers were absent, implicating Pile4 as either the major pilin subunit or a protein critical to pilus assembly. Analysis of the corresponding LVS derivatives (Fig. 4) revealed the same results; fibers were absent in the *pilE4* mutant (LVSΔ*PilE4*), but were present and phenotypically similar to wild-type fibers in the *pilE5* (LVSΔ*PilE5*) or *pilE6* (LVSΔ*PilE6*) mutants. We also confirmed the absence of fibers in the LVS *pilT::HimarFT* mutant (previously described in [22, 29]). In other bacteria PilT functions as retractile ATPase, and mutation of pilT typically results in hyperpiliation and longer than normal pili [22].

Complementation of the *pilE4* mutation restored pilus expression in both Schu S4 (Schu S4Δ*PilE4*c) and LVS (LVSΔ*PilE4*c); complementation of these deletion mutants with the empty pFNLTP6-*groE-gfp* control vector did not restore pilus expression (data not shown). Attempts to isolate and purify the protein component(s) of the surface-associated fibers to directly ascertain the identity of the pilin subunit, or any of its relevant associated factors, were unsuccessful.

Notably, Pile4 differs significantly in amino acid sequence and length between Schu S4 and LVS (202 amino acids versus 305 amino acids, respectively) [21]. The first 198 N-terminal amino acids of LVS Pile4 are nearly identical (only three amino acid substitutions) to Schu S4 Pile4. The C-terminal 103 amino acids of LVS Pile4 are identical to those of *F. novicida*, in which Pile4 has previously been implicated as the putative major pilin subunit protein.

3.3 Adherence of LVS and Schu S4 deletion mutants to J774A.1 cells

Type IV pili are often implicated in adherence to host cells during the first stages of infection. To determine whether pilin-related homologs, including the putative major pilin subunit PilE4, and pilus-like fiber expression in *Francisella* were important to host cell adherence, we used the J774A.1 macrophage-like cell line to study the adherence capacities of both wild-type and deletion mutant strains. The results from four independent experimental trials indicated that LVSΔPilE4, LVSΔPilE5, LVSΔPilE6, and *pilT::HimarFT* were all significantly more adherent to J774A.1 cells compared to LVS ($p < 0.0005$; 1.78, 3.76, 4.54, and 4.58 times more adherent, respectively, Fig. 5A). We also performed confocal microscopy on samples used in the adherence assays described above. This was done to confirm that any quantitative alterations in adherence were not artifacts of cell clumping (resulting from altered pilus expression) or increased affinity for the polystyrene tissue culture plate. The microscopic images in Fig. 5B qualitatively confirmed the enhanced adherence results presented by CFU enumeration; J774A.1 cells are visible via DIC imaging, and their associated attached bacteria are stained in yellow. Notably, the adherence of wild-type and mutant strains appeared independent of pilus expression because both nonpilated (LVSΔPilE4, *pilT::HimarFT*) and pilated (LVSΔPilE5, LVSΔPilE6) mutant strains all exhibited enhanced adherence compared to wild-type LVS.

While the LVS mutants demonstrated increased adherence to J774A.1 cells compared to wild-type LVS, the adherence demonstrated by Schu S4 mutant strains was similar (only 1.18–1.40 times greater) to that of wild-type bacteria (Fig. 6A). Only Schu S4ΔPilE6 possessed significantly increased adherence to J774A.1 cells compared to wild-type ($p < 0.005$), while Schu S4ΔPilE4 and Schu S4ΔPilE5 did not differ significantly compared to wild-type ($p > 0.06$ and $p > 0.8$, respectively). Results from CFU enumeration were confirmed qualitatively by confocal microscopy (Fig. 6B). The number of mutant bacteria associated with host cells was not statistically different for any mutant when compared to wild-type bacteria (data not shown). As we observed for LVS, the pilus-like fibers did not appear to contribute significantly to host cell adherence by Schu S4; both nonpilated (Schu S4ΔPilE4) and pilated (Schu S4 wild-type, Schu S4ΔPilE5) strains exhibited statistically similar levels of adherence to host cells.

The *trans*-complemented LVS strains did not restore wild-type levels of adherence (data not shown). We hypothesize that the overproduction of pilin protein products following *trans*-complementation (shown in Fig. 2) interferes with the normal function of these proteins in some capacity. It is possible that our genetic manipulations resulted in second site mutations; however, we verified the nonpolarity of our deletion mutations (with one exception) using RT-PCR. The Schu S4 *pilE4* deletion may be a polar deletion mutant, as we detected decreased transcription of the downstream gene (Fig. S1). This downstream gene encodes a protein 83 amino acids in length; it possesses no putative conserved domains and is not annotated in the genome. It appears unlikely that the loss of this putative transcript had a significant effect on the *pilE4* mutant phenotype in Schu S4, because adherence was unaffected in this mutant and complementation with the *pilE4* gene restored the pilus expression (Fig. 3).

3.4 LVS virulence *in vivo* is attenuated in the absence of PilE5, PilE6, and PilT

To test the impact of the deletion of pilin homologs on virulence *in vivo*, mice were injected intradermally or subcutaneously with LVS, LVSΔPilE5, LVSΔPilE6, or *pilT::HimarFT* (Fig. 7). All mice injected with wild-type LVS bacteria ($n=6$) died or were euthanized by Day 5 following intradermal infection. 64% of mice injected with LVSΔPilE4 ($n=11$) died or were euthanized by Day 10, marking a subtle increase (36%) in survival compared to wild-type, while mice injected with LVSΔPilE5 ($n=10$), LVSΔPilE6 ($n=10$), and

pilT::HimarFT (n=5) had survival rates of 70%, 90%, and 80%, respectively. Both pilated (LVSΔPile5, LVSΔPile6) and nonpilated (*pilT::HimarFT*) mutant strains exhibited similar degrees of disease attenuation compared to wild-type LVS. However, the two phenotypically nonpilated strains, *pilT::HimarFT* and LVSΔPile4, exhibited markedly different survival rates by the intradermal route (80% versus 36%, respectively). Growth curves were performed for all deletion and wild-type strains, and confirmed that the deletion derivatives grew similarly to the wild-type strain in culture (data not shown).

Following subcutaneous infection, all mice injected with wild-type bacteria (n=5) died or were euthanized by Day 9 post-infection. Mice injected with LVSΔPile4 (n=6) were all deceased by Day 6 post-infection, marking no significant difference in disease progression compared to the wild-type LVS strain. However, parallel to the intradermal route, those mice infected subcutaneously with LVSΔPile5 (n=11), LVSΔPile6 (n=11) and *pilT::HimarFT* (n=11) all demonstrated significantly increased survival (82%) compared to those mice infected with either LVSΔPile4 or wild-type LVS.

We performed dissemination experiments to further explore and verify the apparent virulence defects. Mice were intradermally inoculated with 2.2×10^6 – 3.0×10^7 CFU, and then sacrificed on days 3, 6 and 9 post-infection (Fig. 8). The number of bacteria present in the liver on these days was determined by colony counts. On day 3 post-infection, there was no statistical difference among bacterial burdens in the livers of mice infected with either wild-type LVS or deletion strains, though there was a trend towards lower numbers in LVSΔPile5- and LVSΔPile6-infected mice. These findings suggest that the deletion of these three pilin genes does not markedly affect the ability of LVS to disseminate to the liver. LVSΔPile4 infected mice had on average, higher numbers of bacteria in the liver on day 6 post-infection ($p < 0.05$). Although these higher numbers persisted on day 9, they were not significant; notably, only two mice remained in the day 9 group of LVSΔPile4-infected mice because the other mice died or were euthanized on days 5 and 7 post-infection. On day 6 post-infection, both LVSΔPile5 and LVSΔPile6 had statistically lower numbers of bacteria in the liver compared to LVS ($p < 0.03$). On day 9, three out of four LVSΔPile5-infected mice had undetectable levels of bacteria in the liver, while only one of the four LVSΔPile6-infected mice had apparently cleared the infection. These results, coupled to those from intradermal and subcutaneous infection experiments, support the conclusion that Pile4, and the pilus structures it comprises, lacks a significant role in LVS virulence in mice. These results also suggest the remaining pilin homologs Pile5, Pile6 and PilT (whose absence yielded significantly lower virulence compared to wild-type LVS) function in some capacity unrelated to pilus formation and structure.

3.5 Schu S4 virulence in vivo is not affected by pilin homolog deletions

To test the role of the pilin genes in Schu S4 we introduced approximately 20 CFUs of each strain through both intradermal and subcutaneous routes of infection (confirmed by CFU enumeration via plate counts). By day 7 post-infection all mice receiving wild-type Schu S4 (n=3), Schu S4ΔPile5 or Schu S4ΔPile6 (n=9, respectively) by either route had died or were euthanized (Fig. 9). Surprisingly, there was some limited survival among mice infected with the Schu S4ΔPile4 mutant; 16.7% of mice survived intradermal challenge (n=12) while 28.6% of mice survived subcutaneous challenge (n=7). However, in trials with surviving mice, the infectious dose of Schu S4ΔPile4 was approximately 9 CFUs, roughly half the desired infectious dose of 20 CFUs. In trials where mice received >9 CFUs of Schu S4ΔPile4, all mice died or were euthanized by day 7 post-infection. Survival against a lower challenge dose (16.9% and 28.6%, respectively) suggests Schu S4ΔPile4 may demonstrate slight virulence attenuation; surviving mice infected with this strain never exhibited signs of illness, though their spleens were enlarged upon sacrifice at Day 21 post-infection.

4. Discussion

Pili-like structures have been observed on the surface of *F. novicida* [21] and LVS [11]. We confirmed the presence of pili on LVS and demonstrated that the *F. tularensis* Schu S4 strain also possesses pili. There are six potential pilin-encoding genes in *Francisella* [11]. Through deletion mutagenesis and complementation we have shown that only *pilE4* is required for pilus formation in both LVS and Schu S4. The *PilE4* homolog in *F. novicida* has also previously been implicated as the major pilin subunit [21]. *pilE1*, *pilE2*, and *pilE3* are presumably dispensable to pilus formation, because LVS remains piliated despite the natural absence of all three of these genes (our study and [11]). TEM of our Schu S4 and LVS deletion mutants also revealed that *PilE5* and *PilE6* were not required for pilus expression in these strains.

Five of the pilin-like proteins (*PilE1*-*PilE3*, *PilE5*, *PilE6*) resemble the pilin subunits of *P. aeruginosa* and *Neisseria gonorrhoeae* (classified type A), while *PilE4* bears more similarity to the type B pilin subunits of *Vibrio cholerae* and enteropathogenic *E. coli* [11, 13, 30]. Type IV pilin proteins are classified as type A or type B according to amino acid sequence similarity and length [13, 30]. Type A pilins typically have a shorter leader sequence targeted for cleavage, and shorter mature polypeptide sequences with an N-methylated phenylalanine at the N-terminus (variable in type B pilins) (see Fig. S2). The scattered distribution of type IV pilin-related genes in *Francisella* mirrors that found in *P. aeruginosa* and other type A type IV pilus-producing organisms, implying that *Francisella* spp. assemble one or more subunits with type A homology into the pilus structure. Specifically, the presence of the *pilNOPQ* operon found in *Francisella* spp. is indicative of type A type IV pilus assembly; to date this organized operon has been identified only in those organisms expressing pili assembled from type A pilin subunits [31]. In addition, the presence of *pilT* is generally not associated with type B pilus-producing organisms (with the exception of EPEC [32]), further implicating a type A type IV pilus for *Francisella* subspecies. However, the *Francisella* pili have a bundled appearance, which is characteristic of type B type IV pili. To our knowledge, a type IV pilus system with type A organization, but expressing a fiber comprised of type B subunits, has not been previously documented. Heterologous expression of *pilE1*-*pilE5* in *N. gonorrhoeae* lacking its endogenous pilin subunit indicated that *pilE1*, but not the remaining pilin subunit genes from both Schu S4 and *F. novicida*, was capable of producing pilus-like surface appendages [33]. The inability of *PilE4* to form a pilus in this system may have been because *N. gonorrhoeae* assemble type A type IV pilin subunits, and *PilE4* resembles a type B pilin subunit.

The specific function of the *Francisella* pili still remains an enigma. Type IV pili are found in a wide variety of bacteria, both Gram-negative and Gram-positive, and exhibit diverse functions including DNA competence, twitching motility, aggregation, and attachment to host cells and surfaces [31]. Thus far, *Francisella* type IV pili have demonstrated little to no involvement in these activities, particularly in the Schu S4 background. While *PilE4* is essential for pilus formation, the presence of this protein, and the pilus fiber it comprises, does not appear to be an important component of host cell attachment *in vitro*. We found that the LVS *pil* mutants had significantly enhanced adherence to J774A.1 cells compared to wild-type LVS. In contrast, Chakraborty *et al.* found that LVS *pilT* and *pilF* mutants, two pilus-deficient mutants, were defective in attachment to several cell types compared to wild-type LVS across several cell types [22]; however, the extent of this deficiency varied considerably across the cell lines under study. The observed hyperadherence may vary with cell types. We have also examined adherence to A549 cells (data not shown). Adherence of the *pil* gene mutants to these cells was either unchanged or increased when compared to wild-type bacteria. Hyperadherence and the virulence capacities of LVS mutant strains *in vivo* were independent of the piliated phenotype; the virulence of LVSΔ*PilE4* (nonpiliated)

was similar to wild-type, while LVSΔPile5 and LVSΔPile6 (piliated), and *pilT::HimarFT* (nonpiliated), demonstrated significant attenuation in mice (>70%) compared to wild-type LVS. Although perhaps counter-intuitive, there was also a correlation between hyperadherence and attenuation. The LVSΔPile4 strain was only modestly hyperadherent (1.78 fold increase) while LVSΔPile5, LVSΔPile6 and *pilT::HimarFT* mutants were significantly more adherent (increases of 3.76, 4.54, and 4.58, respectively). All together our results suggest that a defect in any one of these genes alters the surface of the bacteria with respect to its ability to interact with host cells, perhaps by altering the protein composition of the outer membrane. The fact that multiple genes produce the same phenotype is consistent with their participation in a common system. When screening a transposon library of *F. novicida* for mutants that were hypercytotoxic to J774A.1 cells, Lai *et al.* isolated 11 mutants that had mutations in *pil* genes [34]. Despite exhibiting a hypercytotoxic phenotype *in vitro*, seven of the *pil* mutants were attenuated *in vivo*, including *pilE1* (*pilA*), *Pile5* (*pilE*), and *pilE6* (*fimT*). Although Lai *et al.* did not characterize any *pil* mutants further, hypercytotoxic mutants that were further characterized appeared to be hyperadherent by confocal microscopy techniques. In addition, they demonstrated that fixed mutant bacteria had increased uptake into J774A.1 cells compared to fixed wild-type cells. The authors suggest that a surface structure is exposed on mutant bacteria that promotes uptake, and that this structure is not exposed on wild-type bacteria. We speculate that the lack of Pile5, Pile6, or PilT changes the nature of the bacterial surface, perhaps by exposing or unmasking some other protein on the surface.

The Schu S4 mutant strains tested in this study were not significantly attenuated *in vivo*, indicating that *pilE4*, *pilE5*, and *pilE6* did not contribute significantly to the pathogenicity of Schu S4 in mice. Notably, a Schu S4 *pilT* mutant is also virulent *in vivo* [35]. It is not unprecedented for LVS mutation(s) to yield attenuating effects that contrast those observed in an *F. tularensis* Schu S4 background. The deletion of an RND-like efflux pump has no effect on the virulence of Schu S4 [36], while a similar deletion in LVS is attenuating [37]. In addition, despite similar susceptibilities to H₂O₂ and comparable *in vitro* replication patterns, a *katG* (catalase-peroxidase) mutation in LVS is highly attenuated for virulence in mice, yet the same mutation of the orthologous gene in Schu S4 behaves like wild type [38]. Discrepancies in the impact of specific genes on the virulence of LVS and Schu S4 are likely due, at least in part, to individual or cumulative effects of single nucleotide polymorphisms (SNPs), deletions, genomic rearrangements, and pseudogenes that differ between the two strains [6, 16]. However, with respect to the genes putatively involved in type IV biogenesis and structure, specific differences are also likely to impact the function and role of this system across the two strains. The most notable difference between Schu S4 and LVS, along with some sequence variation between pilin orthologs, is the absence of the three pilin-related genes *pilE1–3* in LVS and other *F. holarctica* isolates.

Restoration of *pilE1*, also known as *pilA*, to *F. holarctica* has been shown to increase its virulence [35], and the deletion of *pilE1* in Schu S4 decreases virulence [35]. In *F. novicida*, *pilE1* is required for protein secretion but not type IV pilus assembly [17, 21, 39]. It is clear that *pilE1* is not essential for the expression of pili in LVS; LVS is a natural *pilE1* mutant that retains pilus expression [11]. The differential effects of *pilE5* and *pilE6* mutations on LVS and Schu S4 virulence may be explained by the presence or absence of *pilE1*; in Schu S4, Pile1 may be able to functionally compensate for the loss of Pile5 or Pile6.

It is apparent that products of the *pil* genes are involved in complex process or activities that are affected by the strain background, the presence or absence, or composition of other *pil* genes. Thus far, active type II-like protein secretion has only been demonstrated in *F. novicida* [17]. The apparent inability of Type A strains like Schu S4 to secrete proteins through this system is difficult to explain because, unlike LVS, Schu S4 possesses a full

complement of the required genes. Perhaps the secretion of proteins by this subspecies is regulated or induced by specific environmental conditions. Forslund *et al.* showed that Pile1 contributes to virulence of *F. tularensis* in some capacity because the deletion of *pile1* attenuates its virulence [35]; participation in type II secretion in Schu S4 is the mostly likely function for this protein. We speculate that Pile5 and Pile6 also act as part of this secretion system, perhaps in a regulatory role.

While type IV pilus-like structures are present on the surface of *F. tularensis* subspecies, they do not appear to play a significant role in virulence. Biofilm formation has been observed in *F. novicida*, but a relationship to pilus formation has not been documented [40]. It is certainly possible the novel the *Francisella* type IV pili are useful for persistence in environmental reservoirs (e.g. ticks, amoebas) or specific host organisms. Some or all of the pilin homologs not directly involved in type IV pilus expression may participate in a novel type II secretion system that shares at least some portion of its biosynthetic machinery with the type IV pilus system. Further characterization of these pilin proteins, and their functional roles, should uncover their role in the virulence of *F. tularensis* subspecies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

PilE5 and PilE6 antisera are specific for their respective proteins. The six Schu S4 putative pilin genes (in addition to LVS *pilE4*) were cloned into expression vector pGST-Parallel1 and transformed into *E. coli* DH5 α to express recombinant pilins bearing N-terminal GST tags. Whole *E. coli* lysates were transferred onto PVDF following 15% SDS-PAGE. Western blotting with anti-GST antibody (A) revealed that all recombinant pilins were expressed by *E. coli*. Anti-PilE5 antisera (B) and anti-PilE6 antisera (C) revealed that each antibody was specific for recognition of the respective pilin protein against which it was raised. The protein products expressed in *E. coli* migrated close to the predicted sizes of their respective GST fusions, which were: PilE1 39.5 kD, PilE2 41.4 kD, PilE3 47.7 kD, PilE4 47.9 kD, PilE4LVS 59.7 kD, PilE5 40.7 kD, PilE6 46.9 kD.

Fig. 2. Schu S4 and LVS nonpolar deletion mutants do not express their respective deleted proteins. Whole cell lysates representing approximately 3.5×10^7 (LVS) or 7×10^7 (Schu S4) bacteria were transferred onto PVDF following 15% SDS-PAGE. Membranes were incubated with anti-PileE5 (A) or anti-PileE6 (B) antisera to confirm protein expression in each wild-type strain, as well as their absence or reconstituted presence in deletion and complementation strains, respectively. Anti-GroEL antiserum (C) was used to approximate equal loading of samples. Deletion strains are designated “Δ”, while trans-complemented strains are designated “c”.

Fig. 3.

Pile4 is essential for pilus-like fiber expression on Schu S4. Negative stain TEM demonstrated that wild-type Schu S4, Schu S4ΔPile5, and Schu S4ΔPile6 exhibited similar levels of piliation, while Schu S4ΔPile4 demonstrated a complete lack of pili. The absence of pili in Schu S4ΔPile4, coupled to their reconstitution in the *trans*-complemented Schu S4ΔPile4c, suggested that Pile4 was either the major pilin subunit or an accessory protein necessary for the expression of type IV pili in *F. tularensis*. Images shown here at 6000x magnification are representative of a minimum of 20 fields of observation.

Fig. 4.

PilE4 is essential for pilus-like fiber expression on LVS. Negative stain TEM demonstrated that wild-type LVS, LVS Δ PilE5, and LVS Δ PilE6 exhibited similar levels of piliation, while LVS Δ PilE4 and the *pilT::HimarFT* mutant demonstrated a complete lack of pili. The reconstitution of pilus expression upon PilE4 *trans*-complementation (LVS Δ PilE4c) suggested that, as in Schu S4, PilE4 was either the major pilin subunit or a necessary accessory protein for type IV pilus assembly in LVS. Images shown here at 6000x magnification are representative of a minimum of 20 fields of observation.

Fig. 5.

LVS pilE4, *pilE5*, *pilE6*, and *pilT* mutants demonstrate increased adherence to J774A.1 cells. *LVS*Δ*PilE4*, *LVS*Δ*PilE5*, *LVS*Δ*PilE6*, and the *pilT::HimarFT* mutant demonstrated increased adherence compared to wild-type *LVS* in adherence assays with cytochalasin D-treated J774A.1 cells. Adherent bacteria were enumerated via recovered CFU counts, and the ratio of adherence capacity for each mutant strain compared to wild-type was calculated using the General Linear Model. A value of "1" represents wild-type *LVS* adherence to cells (approximately 0.07% of starting inoculum) (A). CFU enumeration results were confirmed qualitatively by confocal microscopy (B), in which bacteria adherent to cells were stained yellow, and quantitatively in (C). For these measurements, the number of bacteria associated with 50 mammalian cells was counted and then averaged for each strain. Notably, both nonpiliated (*LVS*Δ*PilE4*, *pilT::HimarFT*) and pilated (*LVS*Δ*PilE5*, *LVS*Δ*PilE6*) mutant strains demonstrated increased adherence compared to wild-type *LVS*, indicating adherence was independent of piliation. The data shown represent the cumulative results of four independent experiments in which MOIs were approximately 100:1.

Fig. 6.

Adherence of Schu S4 *pile4*, *pile5*, and *pile6* mutants to J774A.1 cells. Schu S4 *pile4*, *pile5*, and *pile6* mutants demonstrated adherence similar to wild-type Schu S4 in adherence assays with cytochalasin D-treated J774A.1 cells. Adherent bacteria were enumerated via recovered CFU counts, and the ratio of adherence capacity for each mutant strain compared to wild-type was calculated using the General Linear Model. A value of “1” represents wild-type Schu S4 adherence to cells (approximately 0.60% of starting inoculum) (A). CFU enumeration results were qualitatively confirmed by confocal microscopy (B) in which bacteria adherent to J774A.1 cells were stained yellow. Notably, both nonpiliated (Schu S4ΔPile4) and piliated (Schu S4ΔPile5) mutant strains demonstrated statistically similar levels of adherence compared to wild-type Schu S4 ($p>0.06$ and $p>0.08$, respectively), indicating adherence was independent of piliation. The data shown represent the cumulative results of seven independent experiments in which MOIs were approximately 100:1.

Fig. 7. PilE5, PilE6, and PilT contribute to LVS virulence *in vivo*. Balb/C mice were inoculated subcutaneously (1×10^8 CFU) or intradermally (5×10^8 CFU) with wild-type LVS, LVSΔPilE4, LVSΔPilE5, LVSΔPilE6, or LVS *pilT::HimarFT* bacteria. Mice infected subcutaneously with LVSΔPilE4 did not survive bacterial challenge, though mice infected intradermally with LVSΔPilE4 demonstrated a slight increase (36%) in survival compared to wild-type LVS. Mice infected with LVSΔPilE5, LVSΔPilE6, or *pilT::HimarFT* via either route demonstrated significantly attenuated disease progression (at least 70% survival) compared to mice infected with wild-type LVS. There were no deaths after day 9. Surviving mice were followed for more than 21 days.

Fig. 8. Dissemination to and replication of LVS *pilE4*, *pilE5* and *pilE6* mutants in the liver. Mice were intradermally infected with wild-type LVS (3.9×10^6 CFUs), LVS Δ *PilE4* (2.2×10^6 CFUs), LVS Δ *PilE5* (7.1×10^6 CFUs), or LVS Δ *PilE6* (3.0×10^7 CFUs). Livers were harvested on days 3, 6, and 9 post-infection, and bacterial loads determined by serial dilution and plate count. Means statistically different from that of LVS are indicated by “*” ($p < 0.05$) and “***” ($p < 0.02$).

Fig. 9.

Pile4, Pile5, and Pile6 do not significantly contribute to Schu S4 virulence *in vivo*. C57BL/6 mice were inoculated subcutaneously or intradermally with approximately 20 CFU of wild-type Schu S4, Schu S4 Δ Pile4, Schu S4 Δ Pile5, or Schu S4 Δ Pile6 bacteria. Mice receiving Schu S4 Δ Pile5 and Schu S4 Δ Pile6 demonstrated no significant difference in time to death from those receiving the parent Schu S4 strain via either route of infection. Four mice receiving Schu S4 Δ Pile4 survived infection challenge; these mice were challenged with only half the infectious dose (9 CFU) of those mice that had died or were euthanized (20 CFU) indicating Pile4, in addition to Pile5 and Pile6, is not significantly important to Schu S4 virulence *in vivo*. There were no deaths after day 9. Surviving mice were followed for more than 21 days.

Table 1

Bacteria strains or plasmids used in this study

Bacterial Strain or Plasmid	Description	Source
<i>E. coli</i> DH5- α	<i>fhuA2</i> Δ (<i>argF-lacZ</i>) <i>U169 phoA glnV44</i> Φ 80 Δ (<i>lacZ</i>) <i>M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	Invitrogen
Schu S4	<i>F. tularensis</i> sbsp. <i>tularensis</i>	CDC, Ft. Collins, CO
NMA04(S)	Schu S4 Δ <i>pilE4</i>	This Study
NMA04(S)c	NMA04(S) pFNLTP6- <i>groE-S4pilE4</i> ⁺	This Study
NMA05(S)	Schu S4 Δ <i>pilE5</i>	This Study
NMA06(S)	Schu S4 Δ <i>pilE6</i>	This Study
Live Vaccine Strain(LVS)	Derived from <i>F. tularensis</i> subsp. <i>holartctica</i>	Karen Elkins, FDA
NMA04(L)	LVS Δ <i>pilE4</i>	This Study
NMA04(L)c	NMA04(L) pFNLTP6- <i>groE-LpilE4</i> ⁺	This Study
NMA05(L)	LVS Δ <i>pilE5</i>	This Study
NMA05(L)c	NMA05(L) pFNLTP6- <i>groE-LpilE5</i> ⁺	This Study
NMA06(L)	LVS Δ <i>pilE6</i>	This Study
NMA06(L)c	NMA06(L) pFNLTP6- <i>groE-LpilE6</i> ⁺	This Study
<i>pilT::HimarFT</i>	LVS <i>pilT::HimarFT</i>	Maier [29]
Plasmids		
pGIR463	<i>sacB</i> suicide vector, Ap ^R , Kan ^R	Ramakrishnan [40]
pFNLTP6- <i>groEgfp</i>		Maier [28]
pFNLTP6- <i>groE-S4pilE4</i> ⁺	pFNLTP6- <i>groE-S4pilE4</i>	This Study
pFNLTP6- <i>groE-LpilE4</i> ⁺	pFNLTP6- <i>groE-LVSpilE4</i>	This Study
pFNLTP6- <i>groE-LpilE5</i> ⁺	pFNLTP6- <i>groE-LVSpilE5</i>	This Study
pFNLTP6- <i>groE-LpilE5</i> ⁺	pFNLTP6- <i>groE-LVSpilE6</i>	This Study
pCRT7/NT-TOPO		Invitrogen
pHIS-PilE5	pCRT7/NT-TOPO-LVSpilE5	This Study
pHIS-PilE6	pCRT7/NT-TOPO-LVSpilE6	This Study
pGST-Parallel1		Sheffield [26]
pGST-S4PilE1	pGST-Parallel1- <i>S4pilE1</i>	This Study
pGST-S4PilE2	pGST-Parallel1- <i>S4pilE2</i>	This Study
pGST-S4PilE3	pGST-Parallel1- <i>S4pilE3</i>	This Study
pGST-S4PilE4	pGST-Parallel1- <i>S4pilE4</i>	This Study
pGST-S4PilE5	pGST-Parallel1- <i>S4pilE5</i>	This Study
pGST-S4PilE6	pGST-Parallel1- <i>S4pilE6</i>	This Study
pGST-LPilE4	pGST-Parallel1-LVSpilE4	This Study