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Type IV Pili in *Francisella tularensis*: Roles of *pilF* and *pilT* in Fiber Assembly, Host Cell Adherence, and Virulence[▽]

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***Francisella tularensis*, a highly virulent facultative intracellular bacterium, is the causative agent of tularemia. Genome sequencing of all *F. tularensis* subspecies revealed the presence of genes that could encode type IV pili (Tfp). The live vaccine strain (LVS) expresses surface fibers resembling Tfp, but it was not established whether these fibers were indeed Tfp encoded by the *pil* genes. We show here that deletion of the *pilF* putative Tfp assembly ATPase in the LVS resulted in a complete loss of surface fibers. Disruption of the *pilT* putative disassembly ATPase also caused a complete loss of pili, indicating that *pilT* functions differently in *F. tularensis* than in model Tfp systems such as those found in *Pseudomonas aeruginosa* and *Neisseria* spp. The LVS *pilF* and *pilT* mutants were attenuated for virulence in a mouse model of tularemia by the intradermal route. Furthermore, although absence of pili had no effect on the ability of the LVS to replicate intracellularly, the *pilF* and *pilT* mutants were defective for adherence to macrophages, pneumocytes, and hepatocytes. This work confirms that the surface fibers expressed by the LVS are encoded by the *pil* genes and provides evidence that the *Francisella* pili contribute to host cell adhesion and virulence.**

Francisella tularensis is a highly virulent, gram-negative, facultative intracellular bacterium that causes the zoonotic disease tularemia (14). Human illness can range from an ulceroglandular form to more serious conditions, including pneumonic, typhoidal, and meningitic tularemia (48, 63). Due to its high infectivity and potential for airborne transmission, *F. tularensis* has been designated a category A agent of bioterrorism (12). There are four subspecies of *F. tularensis*: *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, *F. tularensis* subsp. *mediasiatica*, and "*F. tularensis* subsp. *novicida*," the first two being clinically important (48). *F. tularensis* subsp. *tularensis* (the type A biovar) causes the most severe form of tularemia, whereas *F. tularensis* subsp. *holarctica* (the type B biovar) is less virulent and produces milder disease symptoms in humans. *F. tularensis* subsp. *novicida* is infectious only for immunocompromised individuals; however, it causes tularemia-like symptoms in mice. The live vaccine strain (LVS), which was developed in the former Soviet Union, belongs to *F. tularensis* subsp. *holarctica* (12) and is now widely used to study tularemia because it remains pathogenic for certain animals and causes a lethal infection in mice that closely mimics the human disease (2, 13).

F. tularensis is able to escape the endosomal-lysosomal trafficking pathway within phagocytes (32, 59) and evades host innate immune responses, a characteristic attributable in part to an unusual lipopolysaccharide structure (15, 58) and a capsule that protects the bacteria from serum-mediated lysis (57). A duplicated pathogenicity island and a pair of regulatory genes (*mglA* and *mglB*) play important roles in the organism's escape from the phagosome and replication within the cytosol of host cells (4, 32, 60). Examination of the available *Francisella* genome sequences reveals a lack of secreted proteins or secretion systems that are typically present in intracellular pathogens (31, 56). However, some potential pathways for the secretion of virulence factors have been identified. Orthologs of the TolC outer membrane protein required for multidrug efflux and protein secretion by the type I secretion pathway are present in *Francisella* strains, and *tolC* was shown to be a virulence determinant of the LVS (19). Recently, it was proposed that the *Francisella* pathogenicity island encodes a type VI secretion system (43). The type VI system is a newly identified secretion pathway that appears to be widespread among bacterial pathogens, including *Pseudomonas aeruginosa* and *Vibrio cholerae* (42, 52). *F. tularensis* strains also contain orthologs of *pil* genes required for the biogenesis of type IV pili (Tfp) (18, 31). Tfp are multifunctional, filamentous surface fibers expressed by a wide variety of bacteria, including *P. aeruginosa*, *Neisseria* spp., enteropathogenic *Escherichia coli*, and *V. cholerae* (6, 38, 45). Tfp functions include surface motility, microcolony and biofilm formation, host cell adhesion, and natural transformation (1, 26). For many gram-negative pathogens, disruption of Tfp assembly results in reduced virulence (25, 33). The LVS expresses surface fibers resembling Tfp, although the identity of these fibers was not confirmed (18). As discussed below, a subset of the *pil* genes of *F. tularensis* subsp. *novicida* were shown to participate in protein secretion into culture supernatant fractions (22), suggesting that these genes might function as a secretion system in addition to or instead of Tfp biogenesis.

The biogenesis and regulation of Tfp is complex, involving a

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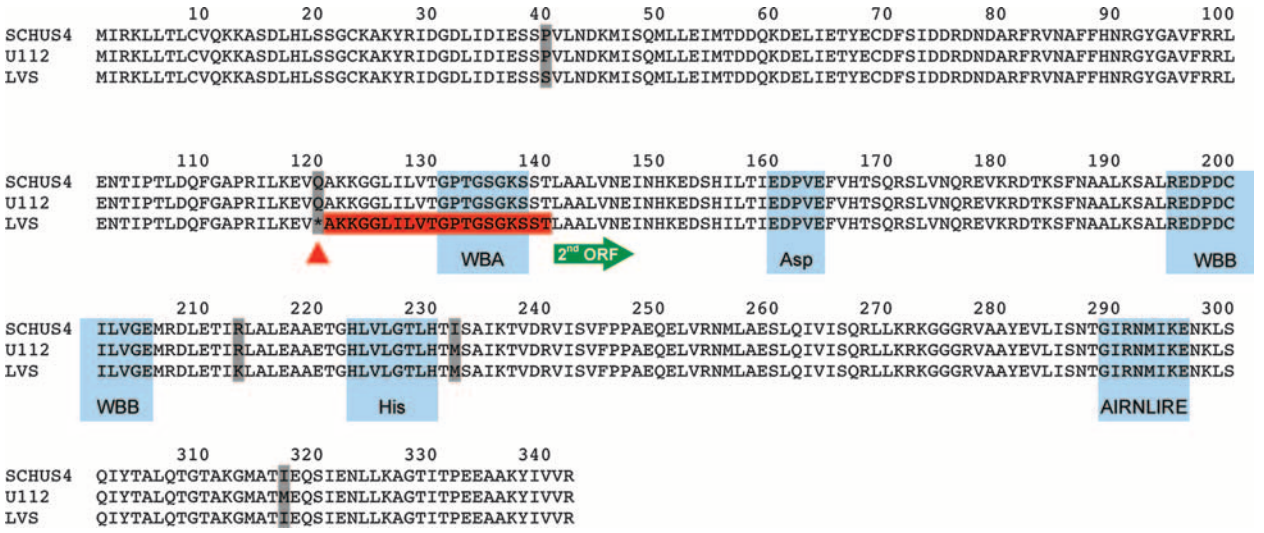

FIG. 1. Analysis of *pilT* ORFs from *F. tularensis* SchuS4 (*F. tularensis* subsp. *tularensis*; FTT0088), U112 (*F. tularensis* subsp. *novicida*; FTN_1622), and LVS (*F. tularensis* subsp. *holarctica*; FTL_1770 and FTL_1771). The amino acid sequences of the ORFs are aligned, with nonconserved residues boxed in gray. The red triangle marks the location of the stop codon mutation in the LVS. The stop codon is followed by a stretch of 20 residues predicted not to be translated (boxed in red), which is followed by the predicted start site for the second ORF, indicated by the green arrow. The locations of the Walker box A (WBA), aspartate box (Asp), Walker box B (WBB), histidine box (His), and AIRNLIRE motifs are indicated. The motifs were identified by visual inspection of the PilT sequences and manual alignment with the motifs as previously defined in related family members (3, 54).

large number of proteins that are related to the type II secretion system of gram-negative bacteria and filamentous phage assembly (11, 38, 45). We use here the nomenclature originally proposed for the *F. tularensis* Tfp components (18), which corresponds to that used for *Neisseria*. Alternate designations will be indicated in parenthesis where appropriate. The *F. tularensis pil* genes are distributed in clusters or individually at different locations in the genome (18, 31). One *F. tularensis* cluster contains the genes *pilNOPQ*. PilQ is a member of a large family of proteins termed secretins, which forms channels in the bacterial outer membrane for secretion of the pilus fiber. A second cluster contains the genes *pilFG*, in which PilF (PilB) is a nucleotide-binding protein required for energizing pilus assembly and secretion, and PilG (PilC) is an integral cytoplasmic membrane protein of the pilus biosynthetic machinery (39, 65). The genes *pilT* and *pilD* occur at separate locations in the genome. PilD is the cytoplasmic membrane peptidase responsible for processing the prepilin subunits (39, 65). PilT proteins form a subgroup of bacterial type II secretion ATPases and belong to the RecA superfamily of hexameric ATPases (69). PilT functions as an antagonist of pilus assembly and is critically involved in twitching motility and pilus retraction, mediating bacterial surface translocation, and interactions between the bacteria and host tissues (41). ATP hydrolysis by PilT is proposed to mediate pilus disassembly from the fiber base at the cytoplasmic membrane, causing retraction (69). A *Pseudomonas pilT* mutant strain is hyperpiliated due to loss of pilus retraction but significantly less infectious than the wild-type strain in mouse models of corneal infection and acute pneumonia (10, 73). Interestingly, the *F. tularensis* LVS *pilT* gene contains a point mutation that introduces a premature stop codon, dividing the gene into two open reading frames (ORFs) (Fig. 1) (18). This nonsense mutation appears to be common to other *F. tularensis* subsp. *holarctica* strains, including FSC352, FSC354, and FSC200 (16, 50, 55). In contrast, the *pilT* sequences in *F. tularensis* subsp. *novicida* (U112) and *F. tularensis* subsp. *tularensis* (SchuS4) are intact and lack similar mutations (Fig. 1).

Gil et al. identified five genes in SchuS4 that could code for the major pilus subunit protein (*pilE1* to *pilE5*) (18). In the LVS, *pilE1* (*pilA*), *pilE2* (*pilE*), and *pilE3* (*pilV*) are not likely to be functional due to mutations and deletions within these genes (16, 18). However, the *pilE4* and *pilE5* genes of the LVS appear to be intact. Forslund et al. demonstrated that PilE1 (PilA) is an important virulence determinant of *F. tularensis* subsp. *holarctica* strains by the subcutaneous route of infection in mice (16). In a recent study by Hager et al. (22), seven proteins, including a protease (PepO), two chitinases (ChiA and ChiB), a chitin-binding protein (CbpA), a predicted β-glucosidase (BglX), and two proteins with unknown functions, were detected in culture supernatant fractions of *F. tularensis* subsp. *novicida*. Mutations in the Tfp genes *pilF* (*pilB*), *pilG* (*pilC*), *pilQ*, and *pilE1* (*pilA*) abolished secretion of these proteins by *F. tularensis* subsp. *novicida*, whereas mutation of *pilT* did not affect secretion (22). The Hager et al. study revealed that the *F. tularensis pil* genes function as a type II-like system for the secretion of soluble proteins.

We show here that *pilF* and *pilT* are required for expression of the surface fibers by *F. tularensis* LVS, confirming that these fibers are encoded by the *pil* genes. The finding that *pilT* is required for pilus assembly in the LVS implies that *pilT* is functional despite being disrupted by mutation and indicates a role distinct from or in addition to pilus retraction. We demonstrate that the *F. tularensis* pili contribute to host cell adherence and are virulence determinants that are important for pathogenesis in mice by the intradermal route.

TABLE 1. Strains and plasmids used in this study

Bacteria or plasmid	Characteristics[a]	Reference or source
Strains		
E. coli		
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 (*lacZYA–argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1* λ$^-$	71
S17-1	*thi thr leu tonA lacY supE recA* RP4-2Tc::Mu, Kn::Tn7	61
F. tularensis		
LVS	Pm^r; vaccine strain; *F. tularensis* subspecies *holarctica*	ATCC 29684
DTB3	LVS Δ*ftlC*	19
DTLB	DTB3 Δ*pilF*	This study
LVS *pilT*::*HimarFT*		37
LVS *pilF*::*HimarFT*		37
Plasmids		
pGEM-T Easy	Ap^r; PCR cloning vector	Promega, Madison, WI
pPV	Ap^r Cm^r, *sacB*, *mob*; vector for allelic replacement in *F. tularensis*	21
pFNLTP6 gro-gfp	Ap^r Kn^r; contains *gfp* under the control of the *F. tularensis groE* promoter	36
pPVLB	pPV with upstream and downstream regions of *pilF*	This study
pGPB	pFNLTP6 gro-gfp with *gfp* replaced by *pilF*	This study
pMP633	Hyg^r; *E. coli-F. tularensis* shuttle vector with P_{groESL}-*hyg* cassette and ORF4-ORF5 of pFNL10	35
p633TL	pMP633 with *pilT* gene from LVS	This study
p633TN	pMP633 with *pilT* gene from *F. novicida* U112	This study

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Kn^r, kanamycin resistance, Pm^r, polymyxin B resistant; Hyg^r, hygromycin resistance.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in the present study are described in Table 1. Unless otherwise noted, the LVS was grown on Mueller-Hinton II chocolate agar plates (MHC; BD Biosciences) containing 1% hemoglobin and 1% IsoVitaleX (BD Biosciences) or in Mueller-Hinton broth (MHB; BD Biosciences) supplemented with 0.1% glucose, 2% IsoVitaleX, 0.025% ferric pyrophosphate, 0.625 mM $CaCl_2$, and 0.530 mM $MgCl_2$ and were incubated at 37°C with 5% CO_2 as described previously (18, 19). *E. coli* strains were grown on Luria-Bertani (LB) agar plates or in LB broth supplemented with 10 μg of chloramphenicol/ml, 100 μg of ampicillin/ml, or 50 μg of kanamycin/ml as appropriate. *F. tularensis* deletion mutants and complementation plasmids were constructed as described previously (19, 21). Strain DTB3 was derived from the LVS and contains a deletion of the *ftlC* gene (19). Due to its greater antibiotic sensitivity, strain DTB3 was used in the present study to facilitate creation of the *pilF* deletion mutant. Briefly, we used an allelic exchange protocol (19, 21) to generate the *pilF* deletion mutant. Regions upstream and downstream of *pilF* were PCR amplified and ligated together into the pPV suicide vector (21), which contains markers conferring chloramphenicol resistance and sucrose sensitivity. *E. coli* S17-1 was used to conjugate the suicide plasmid into strain DTB3, and chloramphenicol-resistant, sucrose-sensitive colonies were screened by PCR using primer pairs both internal and external to *pilF* (Table 2) to verify integration of the suicide plasmid into the target gene (Fig. 2A). Colonies were then grown on medium containing sucrose to select for the second recombination event and elimination of the vector sequences. Sucrose-resistant, chloramphenicol-sensitive colonies were again screened by PCR to verify deletion of the target gene and elimination of the vector sequences (Fig. 2A). We also verified that all intermediate and final strains were *F. tularensis* and not contaminants by using PCR to detect the *F. tularensis pilT* gene. The final Δ*pilF* strain was named DTLB. The *pilT*::*HimarFT* and *pilF*::*HimarFT* transposon mutants were isolated as described

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)[a]	Features
UF3254F	ACA A**GT CGA C**AT CAA TAT AAC TAA AGT CTC TAG GC	Primer for cloning upstream *pilF* with the SalI site
UF4240R	ATG A**CT GCA G**CT TTT GCG TGT AAA TAG TGC	Primer for cloning upstream *pilF* with the PstI site
DF5633F	ATT G**CT GCA G**AA GCT CAA AAA GAA GGA ATC G	Primer for cloning downstream *pilF* with the PstI site
DF6650R	ACT T**GT CGA C**TC CCG TTG CTA AAG ACA CC	Primer for cloning downstream *pilF* with the SalI site
FG4116F	GCA AAT GTT GGC ATA TTA CC	Downstream *pilF* primer (inside *pilC*)
FG6231F	AAA TCG CTG TTG GCA CCT C	Upstream *pilF* primer
FG4501R	ACT TTA TCT AGA GTT GTT CC	*pilF* internal primer
FG4609F	CTA ACT CGA AAG CTA TGT CC	*pilF* internal primer
SP6	CGA TTT AGG TGA CAC TAT AG	Sequencing insert in pGEM-Easy vector
T7	TAA TAC GAC TCA CTA TAG GG	Sequencing insert in pGEM-Easy vector
SAC1243R	TTC CTT TCG CTT GAG GTA CAG C	Detecting *sacB* (S1)
SAC787F	GCA AAC ACT GGA ACT GAA GAT GG	Detecting *sacB* (S2)
PILT186F	GCT GAT AGA AAC CTA CGA ATG TG	Detecting *pilT* (T1)
PILT682R	TCC CTA AAA CCA AAT GCC C	Detecting *pilT* (T2)
PilB F3845	CAA A**GC TAG C**AG CTA TAG CCT CCT CCT AAG CTA	Primer for cloning *pilF* with NheI site
PilB R5718	CCC T**GG ATC C**TT AAC TTA CAC GGT ATA CTT C	Primer for cloning *pilF* with BamHI site
PilTFCOM	GAA **AGA TAT C**CT ATT TAA GTA ATC AAT CTG A	Primer for cloning *pilT* with EcoRV site
PilTRCOM	AGG A**AC GCG T**TT ATC TTA CTA CAA TGT ATT TAG CGG C	Primer for cloning *pilT* with MluI site

[a] Boldface letters indicate restriction sites.

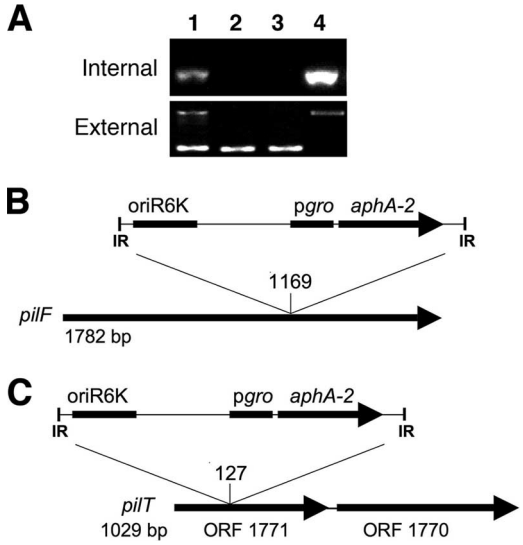

FIG. 2. LVS *pilF* and *pilT* mutants. (A) Construction and validation of the DTLB (Δ*pilF*) mutant. PCR was performed with primers internal or external to the *pilF* gene. Lanes: 1, intermediate strain; 2, final deletion strain DTLB; 3, allelic replacement plasmid pPVLB; 4, LVS. (B and C) Schematic representations of the *pilF* and *pilT* transposon insertion mutations. (B) The *HimarFT* transposon inserted at bp 1169 of *pilF*. (C) The *HimarFT* transposon inserted at bp 127 of the first *pilT* ORF (FTL_1771). The *aphA-2* gene for kanamycin resistance in the transposon is under control of the *groE* promoter, which could drive expression of downstream genes.

in Maier et al. (37). *HimarFT*-based mutagenesis of *F. tularensis* LVS resulted in random, single, stable insertions at high efficiency.

Plasmid pGPB, expressing *pilF*, was constructed by PCR, amplifying the gene from the LVS and cloning it into vector pFNLTP6-*gro-gfp* (36). This placed *pilF* under control of the *F. tularensis groE* promoter. The plasmid was transformed into DTLB by electroporation. PCR primers are described in Table 2. The PCR amplicons were purified by using a Qiagen (Valencia, CA) gel purification kit and sequenced by using a BigDye terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequences were analyzed by using the MacVector software program (Oxford Molecular, Madison, WI). The complementation plasmid p633TL, expressing *pilT* from the LVS, was constructed by PCR amplification of the *pilT* gene from the LVS (including both ORFs 1771 and 1770) with primer pairs PilTFCOM and PilTRCOM (Table 2). The PCR product was ligated into the pGEM-T Easy vector (Promega) and subsequently subcloned into vector pMP633 (35) using EcoRV and MluI. Plasmid p633TL was transformed into LVS *pilT*::*HimarFT* by electroporation. The complementation plasmid p633TN, expressing *pilT* from *F. tularensis* subsp. *novicida*, was constructed following the same strategy, except the PilTFCOM and PilTRCOM primers were used to amplify the *pilT* gene from *F. tularensis* subsp. *novicida* strain U112.

Cell lines and media. Murine bone marrow-derived macrophages (muBMDM) were obtained as described previously (7) and resuspended in bone marrow medium (Dulbecco modified Eagle medium [Invitrogen, Carlsbad, CA] containing 2 mM L-glutamine, 1 mM sodium pyruvate, 20% heat-inactivated fetal bovine serum [FBS; HyClone, Logan, UT], and 30% medium previously conditioned by L929 cells). The conditioned medium was obtained by plating 2×10^5 L929 cells in 75-cm^2 culture flasks in minimum essential medium (Invitrogen) containing 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids (Invitrogen), and 10% FBS and collecting the medium after 10 days. The A549 human lung carcinoma cell line was purchased from the American Type Culture Collection (ATCC) and grown in DMEM plus 10% FBS. The murine hepatocyte cell line FL83B, derived from the normal liver of a 15- to 17-day-old fetal mouse, was purchased from the ATCC. The hepatocytes were grown in F12K medium (ATCC) plus 10% FBS.

TEM. Single bacterial colonies were inoculated into Chamberlain's defined medium, prepared as described previously (18). These cultures were incubated at 37°C in 5% CO_2 with shaking at 100 rpm for 16 h. For transmission electron microscopy (TEM), bacteria were washed once with phosphate-buffered saline (PBS), adsorbed onto polyvinyl formal-carbon-coated grids (Ernest F. Fullam, Latham, NY) for 2 min, and fixed with 1% glutaraldehyde (Sigma-Aldrich, St. Louis, MO) for 1 min. The grids were washed twice with PBS and twice with water and then negatively stained with 0.5% phosphotungstic acid (Ted Pella, Inc., Redding, CA) for 35 s. The grids were viewed in a transmission electron microscope (FEI TECNAI 12 BioTwin G^{02}) at 80-kV accelerating voltage, and images were obtained by using a AMT XR-60 charge-coupled device digital camera system.

DNA microarray. The wild-type LVS, strain DTB3 (Δ*pilF*), and the *pilT*::*HimarFT* mutant were grown to early logarithmic phase (optical density at 600 nm [OD_{600}], ~0.2) in MHB at 37°C with 5% CO_2. The cultures were harvested by centrifugation at 6,000 × *g* for 15 min at 4°C. Total RNA was isolated with the RNeasy Midi kit (Qiagen, Valencia, CA) by following the manufacturer's protocol. During RNA extraction, treatment with the RNase-free DNase set (Qiagen) was used to remove any DNA contamination. RNA concentration was determined by spectrophotometry (OD_{260}), and RNA integrity was verified by agarose gel electrophoresis.

70-mer oligonucleotide microarrays representing all ORFs from SchuS4 (*F. tularensis* subsp. *tularensis*), LVS (*F. tularensis* subsp. *holarctica*), and F6168 (*F. tularensis* subsp. *novicida*) were obtained from The Institute for Genomic Research (TIGR). The microarray slides were prehybridized, washed, and dried immediately before hybridization using the protocol recommended by TIGR (http://www.tigr.org/tdb/microarray/protocolsTIGR.shtml). For hybridization, cDNA with 150 pmol Cy3 and cDNA with 150 pmol Cy5 were included in a 55 μl of hybridization solution containing 25% (vol/vol) formamide, 5× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), and 100 μg of sonicated salmon sperm DNA/ml. Hybridization was performed under lifter coverslips (25 by 60 mm; Erie Scientific) at 50°C in a humidified chamber for 16 to 20 h. Hybridized arrays were washed with gentle shaking as follows: twice briefly with 2× SSC–0.1% SDS at 50°C, twice for 10 min with 2× SSC–0.1% SDS at 50°C, twice briefly with 0.1× SSC–0.1% SDS at 50°C, twice for 10 min with 0.1× SSC–0.1% SDS, and four times briefly with 0.1× SSC at room temperature. Arrays were dried by placing them into a 50-ml Falcon tube containing tissue paper in the bottom and were centrifuged at 1,300 × *g* for 2 min at room temperature. Arrays were scanned by using an Axon 4000B scanner that was controlled using GenePix Pro 6 software with a pixel size of 10 μm and two-pass sequential line averaging. The laser power was set to 100%, and PMT gains were subjectively adjusted during prescanning to maximize the effective dynamic range and to limit image saturation. Lossless image files were stored for later analysis.

Microarray data was analyzed using the Limma module of the Bioconductor package for the R statistical environment (17, 62). The "normexp" method was used for background correction, followed by print tip loss normalization and between-array normalization of intensities. The microarray data for each gene was fitted to a linear model, and statistics were generated by using the lmFit and eBayes functions (17). The *P* values were adjusted for multiple testing using the Benjamini and Hochberg method within Limma. Genes with *P* values of <0.05 were considered differentially regulated. Annotations for microarray data were derived from TIGR gene array list files.

Intracellular infection experiments. muBMDM, A549, and FL83B cells were seeded in 24-well plates at concentrations of 1.5×10^5, 1×10^5, and 1×10^5 cells per well, respectively, and were used for experiments the next day. For each experiment, *F. tularensis* strains were streaked from frozen stocks to MHC, and a single colony was grown in Chamberlain's defined medium for 16 to 18 h at 37°C with shaking at 100 rpm in a 5% CO_2 atmosphere. Aliquots of the bacterial cultures were centrifuged, resuspended in the appropriate cell culture medium, and added at a multiplicity of infection of 50, 400, or 1,000 to the muBMDM, A549, or FL83B cells, respectively. Bacterial concentrations were initially estimated by determining the OD_{600} of the suspension culture, and actual numbers of viable bacteria were determined by CFU counts on MHC plates. Cell culture plates were centrifuged for 5 min at 800 rpm to facilitate contact between the cells and bacteria. After 2 h of coculture at 37°C, the cells were washed extensively and incubated with 5 μg of gentamicin/ml for 1 h to kill any remaining extracellular bacteria. To measure viable intracellular bacteria, a set of wells was lysed with water at 4°C for 10 min, and serial dilutions were plated to determine the CFU. The remaining wells were incubated for a total of 24 h in the appropriate cell culture medium before determination of CFU.

Cell adherence assay. muBMDM, A549, and FL83B cells were seeded in 24-well plates as for the intracellular infection experiments. The next day, the cells were treated with 1 μg of cytochalasin D (Sigma)/ml for 1 h to inhibit the internalization of bacteria inside the cells and then thoroughly washed with PBS. Aliquots of bacterial cultures were centrifuged, resuspended in the appropriate cell culture medium, and added at a multiplicity of infection of 100, 800, or 2,000

to the muBMDM, A549, or FL83B cells, respectively. Bacterial concentrations were initially estimated by determining the OD_{600} of the suspension culture, and actual numbers of viable bacteria were determined by CFU counts on MHC plates. Cell culture plates were centrifuged for 5 min at 800 rpm to facilitate contact between the cells and bacteria. After 2 h of coculture at 37°C, the cells were washed extensively and lysed with water at 4°C for 10 min, and serial dilutions were plated to determine CFU.

Mouse infection experiments. For virulence studies, groups of five 6- to 8-week-old C3H/HeN mice (Charles River) were used, and four independent experiments were performed. Mice were housed under conventional conditions and allowed to acclimatize for at least 7 days before infection. LVS, DTB3, DTLB, *pilT*::*HimarFT*, and DTLB/pGPB were grown in MHB overnight to an OD_{600} of 0.2 and diluted in PBS, and 100 μl was injected intradermally to give infectious doses of 10^7 or 10^6 CFU per mouse. The actual infectious doses were determined by viable count. The animals were monitored for 14 days.

All animal research protocols were approved by the Institutional Animal Care and Use Committee of Stony Brook University.

RESULTS

LVS *pilF* and *pilT* mutants. To investigate whether the Tfp genes in *F. tularensis* are involved in expression of the surface fibers observed by Gil et al. (18), we used an allelic replacement technique (21) to construct a deletion of the *pilF* (*pilB*) gene in the LVS and examined previously isolated *pilT*::*HimarFT* and *pilF*::*HimarFT* transposon mutants in the LVS (37). In characterized Tfp systems, *pilF* is required for pilus assembly, whereas *pilT* drives pilus retraction (3). To construct the *pilF* deletion mutant, we used strain DTB3, which is a derivative of the LVS containing a deletion of the *ftlC* gene (19). DTB3 is more sensitive to chloramphenicol due to reduced drug efflux, facilitating construction of the mutant. Importantly, DTB3 is not impaired for virulence in the mouse model of tularemia (see Fig. 5) and is not defective for replication in macrophages or other cell types (19) (G. J. Platz and D. G. Thanassi, unpublished data). Strain DTB3 was tested alongside the wild-type LVS in all assays used in the present study and exhibited no defects relative to the wild-type LVS. For clarity of the text, we only show results for the wild-type LVS, except for the mouse infection studies (see Fig. 5). Using strain DTB3, we were able to successfully delete *pilF*; this strain was designated DTLB. Proper construction of strain DTLB was validated both phenotypically and genotypically as described in Materials and Methods. Figure 2A shows the PCR verification of the deletion of *pilF* in DTLB. The LVS *pilF*::*HimarFT* and *pilT*::*HimarFT* transposon mutants were isolated and confirmed as described by Maier et al. (37). None of the mutant strains exhibited growth defects on solid or in liquid media (data not shown).

The transposon insertion locations in *pilF* and *pilT* are shown in Fig. 2. In *pilF*::*HimarFT*, the transposon was inserted toward the end of the gene, at bp 1169 of 1782 (Fig. 2B). In *pilT*::*HimarFT*, the transposon was inserted near the beginning of the gene, at bp 127 of 1029 (Fig. 2C). PilT is thought to be composed of two major structural domains. The N-terminal domain, containing ca. 100 to 115 amino acids, is required for membrane association and polar localization of PilT (8). The C-terminal domain, ~240 amino acids, contains sequences commonly associated with NTPase activity, including the Walker A phosphate-binding (P) loop and a loosely defined Walker B box (Fig. 1) (28). The C-terminal domain also contains Asp and His boxes defined for type II and type IV secretion ATPases (28, 51, 54). PilT has low NTPase activity in vitro (24, 46), but this activity is likely important for PilT function, since mutation of the P-loop lysine prevents twitching motility (3). Beyond the ~170 amino acids that make up the ATPase core, the C-terminal ~70 amino acids also contain a well-conserved PilT-specific AIRNLIRE motif which is required for pilus retraction but not ATPase activity (Fig. 1) (3). The nonsense mutation in the LVS *pilT* introduces a stop codon that divides *pilT* into two ORFs: FTL_1771 and FTL_1770 (Fig. 1). The stop codon is followed by 60 nucleotides, which is followed by the start codon of the second predicted *pilT* ORF (FTL_1770). Whether this second ORF is expressed is not known, since it contains a weaker TTG start codon and no obvious preceding Shine-Dalgarno sequence. Two virulent *F. tularensis* subsp. *holarctica* strains, OSU18 and FSC200, also share this same nonsense mutation in *pilT* (16, 50, 55). The intergenic region between the two ORFs of *pilT* includes the Walker A box (Fig. 1). However, the Walker Box B, the conserved Asp and His regions, and the AIRNLIRE motif are present in the second *pilT* ORF. Nevertheless, loss of the Walker A box would be expected to disable the NTPase activity of the protein (5, 8).

Expression of *Francisella* Tfp requires *pilF* and *pilT*. Strains DTLB (Δ*pilF*), *pilF*::*HimarFT*, and *pilT*::*HimarFT* were examined for the expression of surface fibers using whole bacteria, negative-stain TEM. The LVS expressed pilus fibers as previously demonstrated (18) (Fig. 3A and B). In contrast, the Δ*pilF* strain DTLB did not express any piluslike structures (Fig. 3C and D). Complementation of DTLB with a plasmid-borne copy of *pilF* restored expression of the pili (Fig. 3E and F). This demonstrates that the LVS surface fibers require *pilF* for expression, as found in other Tfp systems (29), and suggests that the fibers are indeed Tfp. Similar to strain DTLB, pilus expression by the LVS *pilF*::*HimarFT* mutant was dramatically reduced, although a few surface fibers were occasionally observed (data not shown). This low expression of fibers suggests that the transposon insertion, which occurred toward the end of the *pilF* gene (Fig. 2B), did not completely disable PilF function.

We also found a complete lack of pilus fibers on the surface of the LVS *pilT*::*HimarFT* mutant (Fig. 3G and H). This was surprising for two reasons. First, in other Tfp systems, PilT is only required for pilus retraction, and mutation of *pilT* results in hyperpiliation rather than the loss of pili (70). Second, as discussed above, the *pilT* gene in the LVS already contains a premature stop codon and was expected to be nonfunctional. Thus, a transposon insertion into an already nonfunctional gene should not cause an additional loss of function. To determine whether the phenotypes of the *pilT*::*HimarFT* and the DTLB (Δ*pilF*) mutants were caused by downregulation of other *pil* genes or some other transcriptional effect, we performed DNA microarray analysis of the LVS, DTLB, and *pilT*::*HimarFT* strains using an array specific for the LVS and other strains of *F. tularensis*. None of the known Tfp structural, assembly, or regulatory genes was positively or negatively affected by the *pilT* or *pilF* mutations compared to the LVS (data not shown). As expected, message corresponding to the *pilT* gene in *pilT*::*HimarFT* and the *pilF* gene in DTLB was decreased (2.8- and 6.4-fold, respectively). Thus, the Δ*pilF* and *pilT*::*HimarFT* mutations are nonpolar, suggesting that the mutant phenotypes are directly caused by disruption of these genes. To provide additional support for the specificity of the

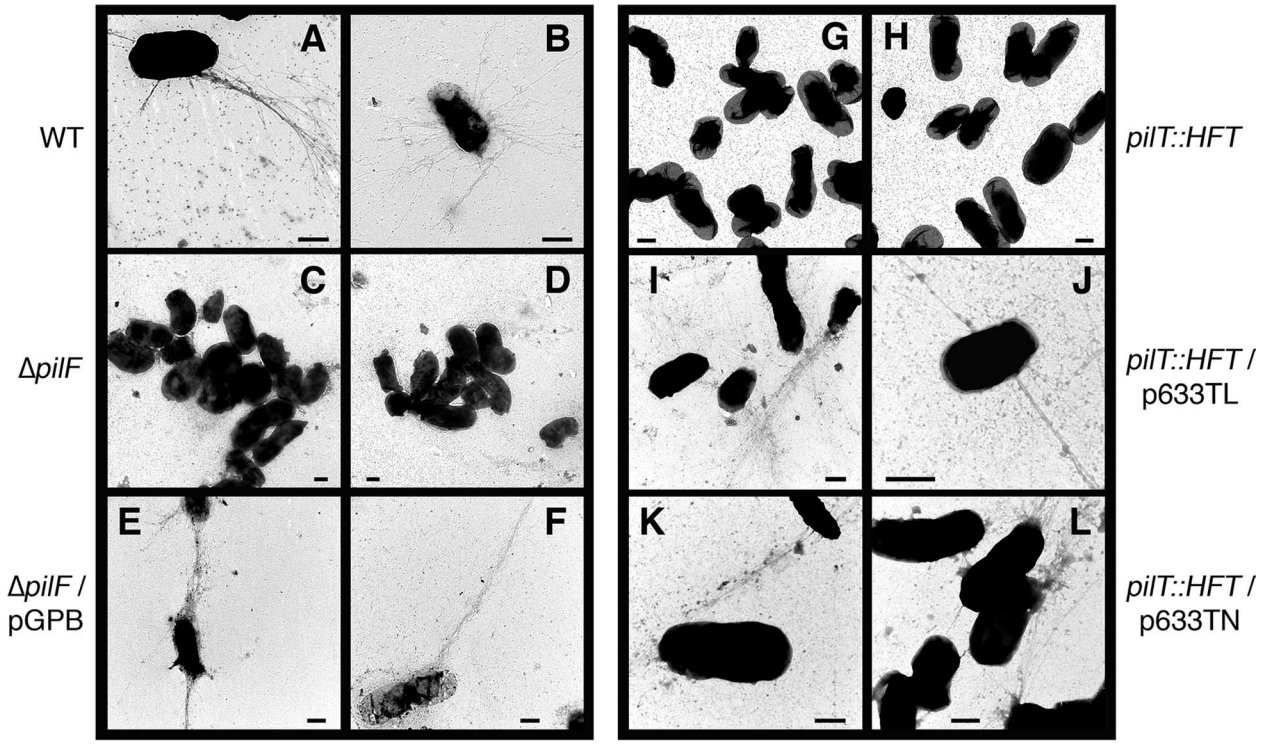

FIG. 3. Whole bacteria, negative-stain TEM of *F. tularensis* LVS. (A and B) The wild-type LVS expresses pilus fibers. (C and D) No fibers were detected on strain DTLB (Δ*pilF*). (E and F) Complementation of strain DTLB with plasmid pGPB (*pilF*$^+$) restored the expression of pili. (G and H) The *pilT*::*HimarFT* mutant expresses no pilus fibers. (I through L) Complementation of the LVS *pilT*::*HimarFT* mutant with *pilT* from either the LVS (p633TL; panels I and J) or *F. tularensis* subsp. *novicida* (p633TN; panels K and L) restored the expression of pili.

LVS *pilT*::*HimarFT* mutation, we complemented this strain with either plasmid p633TL, containing the complete *pilT* gene from the LVS (both ORFs 1771 and 1770), or plasmid p633TN, containing the intact *pilT* gene from *F. tularensis* subsp. *novicida* strain U112. Complementation with either plasmid restored expression of the pilus fibers, as shown by negative-stain TEM (Fig. 3I through L). This confirms that *pilT* is required for expression of the LVS surface fibers and shows that the LVS *pilT* gene is functional.

***Francisella* Tfp contribute to host cell adherence but are not required for intracellular replication.** Adherence to host epithelial tissues is an early step of bacterial pathogenesis that is required for successful colonization and subsequent infection (27, 47). Tfp serve as host cell adhesins for a number of bacterial pathogens (20, 66, 72). To investigate the role of Tfp in the interaction of *F. tularensis* with different cell types, we compared adhesion of the LVS, DTLB (Δ*pilF*), and the *pilT*::*HimarFT* strain to primary murine bone marrow-derived macrophages (muBMDM), A549 human lung epithelial cells, and FL83B murine hepatocytes. For these experiments, the cells were treated with cytochalasin D to inhibit internalization of the bacteria (see Materials and Methods). A recent study found that treatment of HEp-2 cells with cytochalasin D almost completely abrogated the uptake of *F. tularensis* (34). For each of the cell types tested, the Δ*pilF* and *pilT*::*HimarFT* mutants were significantly defective in adherence compared to the LVS (Fig. 4). Interestingly, the Δ*pilF* mutant consistently exhibited a greater defect in adherence compared to the *pilT* mutant, suggesting subtle differences in the phenotypes of these two mutants. Expression of *pilF in trans* restored the adherence of the strain DTLB back to levels similar to the wild-type LVS (Fig. 4), confirming that the adherence defect of this strain was specifically due to the the loss of *pilF*. These findings demonstrate that the *Francisella* pili contribute to host cell adherence and suggest they may mediate colonization of different tissues within the host.

The ability to survive and replicate intracellularly constitutes a major virulence determinant of *F. tularensis* (32, 44), and we next addressed the question of whether *pilF* and *pilT* are required for growth within muBMDM, A549, or FL83B cells. Mutant strains DTLB (Δ*pilF*) and *pilT*::*HimarFT* exhibited no defects in intracellular survival and replication compared to the wild-type LVS for each of the cell types tested (data not shown). Therefore, in contrast to their role in host cell adhesion described above, the *Francisella* pili are not required for intracellular replication.

Tfp are virulence factors of *F. tularensis*. The contribution of Tfp to the virulence of *Francisella* was investigated using a mouse model of tularemia. Mice were inoculated intradermally with the LVS, DTB3 (Δ*ftlC*), DTLB (Δ*pilF*), or the *pilT*::*HimarFT* mutant, and the mice were monitored for survival for 14 days. In the experiment shown in Fig. 5A, mice inoculated with 10^7 CFU of the LVS or DTB3 (the parent strain of DTLB) began succumbing to the infection 2 to 3 days after inoculation, with no surviving mice by days 7 to 8. In comparison, DTLB (Δ*pilF*) was highly attenuated, with 70% survival on day 5 and 60% survival through day 14 (Fig. 5A). The *pilT*::*HimarFT* mutant was similarly attenuated, with 60% survival

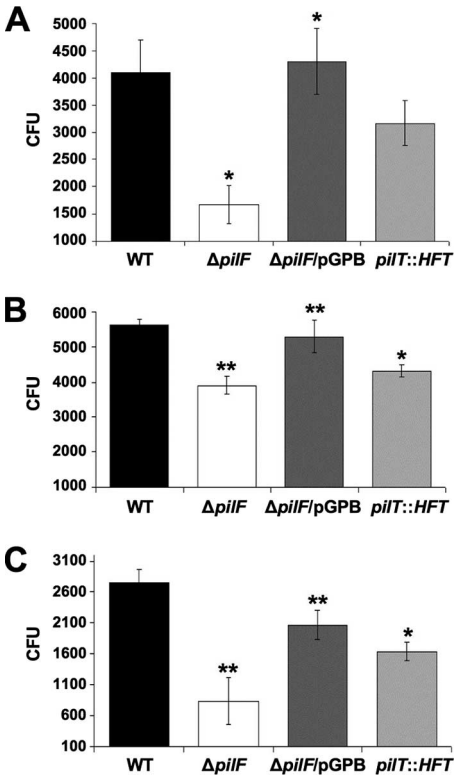

FIG. 4. *pilF* and *pilT* contribute to host cell adherence. Cells were treated with cytochalasin D to inhibit the internalization of bacteria and then infected with wild-type LVS (WT), DTLB (Δ*pilF*), DTLB complemented with *pilF* (Δ*pilF*/*pGPB*), or the *pilT*::*HimarFT* mutant. The graphs show the CFU of bacteria that adhered to murine bone marrow-derived macrophages (A), A549 human lung epithelial cells (B), and FL83B murine hepatocytes (C). Error bars indicate the standard deviation of triplicate samples. *P* values were calculated by using a paired *t* test, comparing DTLB or *pilT:HimarFT* with the wild-type LVS or comparing DTLB/pGPB with DTLB (*, $P < 0.05$; **, $P < 0.02$).

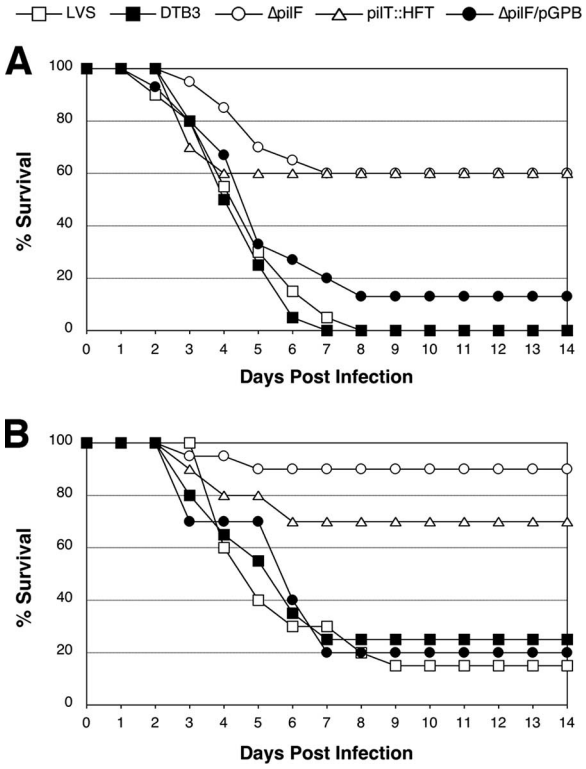

FIG. 5. *pilF* and *pilT* are virulence determinants of *F. tularensis*. C3H/HeN mice were infected intradermally with 10^7 CFU (A) or 10^6 CFU (B) of the LVS, DTB3 (Δ*ftlC*), DTLB (Δ*pilF*), *pilT*::*HimarFT*, or DTLB complemented with *pilF* (Δ*pilF*/*pGPB*). The mice were monitored for survival for 14 days. A total of 20 mice were used for each strain and each dose.

through day 14. Comparable results were obtained with an infectious dose of 10^6 CFU (Fig. 5B). At this dose, 90% of the mice inoculated with strain DTLB (Δ*pilF*) survived through day 14, whereas only 15 to 25% of mice inoculated with the wild-type LVS or strain DTB3 (Δ*ftlC*) survived for the duration of the study. The *pilT*::*HimarFT* mutant also exhibited attenuation at 10^6 CFU, with 70% survival through day 14. As noted above for the adhesion assay, subtle differences were observed between the Δ*pilF* and *pilT* mutants, with the Δ*pilF* mutant exhibiting a slightly higher level of attenuation, particularly at the lower infectious dose (Fig. 5B). Complementation of strain DTLB with plasmid pGPB (*pilF*) restored the virulence of this strain in the mouse infection model at both the 10^7 and the 10^6 CFU infectious doses (Fig. 5), confirming that the virulence defect was specifically due to loss of *pilF*. Overall, our results demonstrate that both *pilF* and *pilT* are important virulence determinants of the LVS and define a function for the *pil* genes in the pathogenesis of *F. tularensis* by the intradermal route.

DISCUSSION

We show here, through analysis of *pilF* and *pilT* mutations in the LVS, that the surface fibers expressed by *F. tularensis* are encoded by the *pil* genes, suggesting that these fibers are Tfp. We provide evidence that the pili are important for adherence to different host cell types, and we demonstrate that the *pil* genes function as virulence determinants of *F. tularensis*.

Whole-bacteria, negative-stain TEM of the Δ*pilF* strain DTLB revealed a complete absence of surface fibers, which matches results found for Tfp systems in other bacteria upon loss of *pilF* (29). Thus, PilF likely serves as the Tfp assembly ATPase in *F. tularensis*. Similarly, the LVS *pilF*::*HimarFT* mutant had a dramatic, although not complete, loss of surface fibers. The Walker box A motif in PilF, located at residues 336 to 348, precedes the transposon insertion site, and the presence of this intact motif may explain the low activity retained in the *pilF*::*HimarFT* mutant. Taking the above findings together with the demonstration by Hager et al. that the *pil* genes function as a type II-like system for the secretion of soluble proteins in *F. tularensis* subsp. *novicida* (22), we propose that the *F. tularensis pil* genes perform dual roles, both assembling Tfp and serving as a protein secretion system. This dual function makes sense given that *F. tularensis* lacks many secretion systems found in other intracellular pathogens and would need to make maximum use of its limited secretion capability. The type II secretion and Tfp biogenesis machinery are closely related and share many features (49, 68). Indeed, a dual function for Tfp machinery has been established in other gram-negative pathogens. The toxin coregulated pilus of *V. cholerae*

was shown to secrete a soluble virulence factor and the Tfp system in the ovine foot rot pathogen *Dichelobacter nodosus* was shown to secrete extracellular proteases (23, 30).

A striking observation made in the present study is the lack of any surface fibers in the LVS *pilT*::*HimarFT* mutant. This was surprising not only because mutation of *pilT* in other Tfp systems results in hyperpiliation rather than loss of pili (69) but also because *pilT* was already expected to be nonfunctional in the LVS due to disruption of its ORF by a premature stop codon. Nevertheless, because the *pilT*::*HimarFT* mutant failed to express Tfp and complementation with either the LVS or *F. tularensis* subsp. *novicida pilT* gene *in trans* restored pilus expression, it is likely that PilT is directly or indirectly required for pilus biogenesis. This paradox may be explained by several possibilities. The *HimarFT* transposon contains a *groE* promoter driving the *aphA-2* gene for kanamycin resistance (Fig. 2), and this promoter could drive expression of downstream genes. However, *pilT* is not part of an operon, is not followed by an ORF in the same direction, and our microarray analysis found that none of the other *pil* genes were down- or upregulated in the *pilT*::*HimarFT* mutant. A second possibility is that the *HimarFT* transposon resulted in generation of a *pilT* protein fragment that acted in a dominant-negative manner, interacting with and titrating away other factors required for pilus biogenesis. The *HimarFT* insertion occurred in the first predicted *pilT* ORF in the LVS, at bp 127 (Fig. 2C). Thus, a protein fragment containing the region of *pilT* preceding the insertion could be synthesized. In addition, the *aphA-2* gene in the *HimarFT* transposon could possibly drive expression of a fusion protein with the region of *pilT* downstream of the insertion. A third possibility, and one that we favor, is that *pilT* is functional in the LVS despite containing a premature stop codon. This possibility is supported by the fact that we could complement LVS *pilT*::*HimarFT* with the "mutated" LVS *pilT* gene. Thus, the LVS *pilT* gene is functional. The first *pilT* ORF (FTL_1771) in the LVS contains the N-terminal domain of PilT, which functions in membrane association and polar localization (8); this function may be important for pilus biogenesis in *F. tularensis*. Alternatively, there may be readthrough of the premature stop codon to produce a full-length PilT protein. We note that *F. tularensis* subsp. *novicida* contains an intact *pilT* gene (Fig. 1) and that the *F. tularensis* subsp. *novicida pilT* gene complemented the LVS *pilT*::*HimarFT* mutant, arguing that *pilT* function is preserved in the LVS despite the premature stop codon. Overall, our results show that the LVS *pilT* is required for pilus assembly and has evolved to perform a function distinct from or in addition to pilus retraction. Such a function for PilT implies that the paradigm of Tfp dynamics established in the *P. aeruginosa* and *Neisseria* spp. model systems may not be applicable to *F. tularensis*. Interestingly, a recent study in the gram-positive bacterium *Clostridium perfringens* also found that PilT is required for the assembly of Tfp (67).

Our studies with the LVS *pilF* and *pilT* mutants suggest that *F. tularensis* uses pili for adhesion to host cells. Both the Δ*pilF* and the *pilT*::*HimarFT* mutants were defective for adherence to macrophages, hepatocytes, and lung epithelial cells. In addition to adhesion, *F. tularensis* is capable of surviving and replicating inside these different cell types (9, 40, 53). However, the LVS *pilF* and *pilT* mutants did not exhibit any growth defects in the cell lines tested. Similar results were found for the intracellular pathogen *Legionella pneumophila*. The Tfp of *L. pneumophila* have a role in promoting adherence to mammalian and protozoan cells but are not involved in intracellular survival and replication (64). Significantly, we found that the LVS *pilF* and *pilT* mutants were attenuated for virulence in mice when inoculated by the intradermal route. These findings identify *pilF* and *pilT* as virulence factors of *F. tularensis* and support a role for the pili in pathogenesis. Interestingly, the LVS *pilT*::*HimarFT* mutant consistently exhibited milder adhesion and virulence phenotypes compared to the Δ*pilF* mutant, suggesting that these genes have overlapping but distinct functions. Hager et al. found that *pilF* (*pilB*), but not *pilT*, was required for protein secretion in *F. tularensis* subsp. *novicida* (22). Therefore, the LVS Δ*pilF* mutant may affect both Tfp expression and protein secretion, whereas the *pilT*::*HimarFT* mutant may only affect Tfp biogenesis. However, the PilE1 (PilA) potential pilin subunit was also required for protein secretion in *F. tularensis* subsp. *novicida* (22), but *pilE1* is not intact in the LVS (18, 31). Thus, the protein secretion function of the *pil* genes may not be operational in the LVS. Future studies are needed to address the specific roles of *pilF* and *pilT* in pilus biogenesis and whether the *pil* genes also mediate protein secretion in the LVS.

In summary, we have established that the surface fibers expressed by the LVS are encoded by the *pil* genes and provided evidence that the *Francisella* pili play important roles in attachment to host cells and virulence in the mouse infection model. We found that, in addition to *pilF*, *pilT* was required for pilus assembly. This suggests that the pilus machinery of *F. tularensis* functions differently than in established model systems. Therefore, identification and characterization of the complete pilus assembly pathway in *F. tularensis* will impact our understanding of pilus biogenesis, as well as elucidate the molecular mechanisms that make *Francisella* such a successful pathogen.

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