Identifying Francisella tularensis Genes Required for Growth in Host Cells

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Francisella tularensis is a highly virulent Gram-negative intracellular pathogen capable of infecting a vast diversity of hosts, ranging from amoebae to humans. A hallmark of F. tularensis virulence is its ability to quickly grow to high densities within a diverse set of host cells, including, but not limited to, macrophages and epithelial cells. We developed a luminescence reporter system to facilitate a large-scale transposon mutagenesis screen to identify genes required for growth in macrophage and epithelial cell lines. We screened 7,454 individual mutants, 269 of which exhibited reduced intracellular growth. Transposon insertions in the 269 growth-defective strains mapped to 68 different genes. FTT_0924, a gene of unknown function but highly conserved among Francisella species, was identified in this screen to be defective for intracellular growth within both macrophage and epithelial cell lines. FTT_0924 was required for full Schu S4 virulence in a murine pulmonary infection model. The ΔFTT_0924 mutant bacterial membrane is permeable when replicating in hypotonic solution and within macrophages, resulting in strongly reduced viability. The permeability and reduced viability were rescued when the mutant was grown in a hypertonic solution, indicating that FTT_0924 is required for resisting osmotic stress. The ΔFTT_0924 mutant was also significantly more sensitive to β-lactam antibiotics than Schu S4. Taken together, the data strongly suggest that FTT_0924 is required for maintaining peptidoglycan integrity and virulence.

Francisella tularensis is a Gram-negative facultative intracellular pathogen capable of infecting over 250 hosts, ranging from amoebae to humans (1). F. tularensis subspecies tularensis is highly virulent in mammals, and as few as 25 organisms can cause a potentially fatal infection in humans (2). The bacterium infects a variety of cell types, including, but not limited to, macrophages, dendritic cells, neutrophils, epithelial cells, fibroblasts, and hepatocytes (3–7). F. tularensis replicates to high levels within these cell types, but the specific mechanisms F. tularensis uses to invade and replicate within host cells remain poorly understood.

It is essential for F. tularensis to replicate within host cells to successfully establish an infection and cause disease. Intracellular replication requires phagosomal escape, followed by adaptation to and replication in the host cytosol. Upon internalization by a host cell, the bacterium degrades the phagosome within 30 min and enters the cytosol (8). Escape from the phagosome is an essential step in the Francisella life cycle, and mutations in the Francisella pathogenicity island, which encodes an alternative secretion system, fail to escape the phagosome (9–11). Other factors have been identified that are required for efficient F. tularensis vacuolar escape; however, the specific mechanisms necessary for vacuolar escape have yet to be determined (12–14). Once in the cytosol, bacteria replicate to high levels before the host cell undergoes apoptotic cell death (15–18). Mutants that escape the phagosome but fail to replicate within the cytosol have been identified, including mutants with disruptions in purine biosynthesis genes, dipA or ripA (12, 19, 20). Francisella mutants defective for phagosomal escape or cytotoxic replication exhibit reduced virulence in murine infection models, indicating that both processes are essential for F. tularensis virulence.

A large portion of the Francisella genome encodes proteins of unknown function. Several genes of unknown function, including dipA, ripA, and FTT_1676, as well as others, are conserved across Francisella species and are required for intracellular growth and virulence (12, 20, 21). To better understand Francisella pathogenesis, it is important to identify and elucidate the mechanisms by which these genes affect bacterial intracellular growth and virulence. Here, we perform a large-scale transposon mutagenesis screen to identify novel virulence factors required for intracellular growth. Because F. tularensis infects a diverse set of host cell types and likely requires different mechanisms for entry and replication within distinct cell types, we screened our library of mutants within both macrophage-like (J774) and alveolar-epithelial (TC-1) cell lines. To validate the efficacy of the screen, we identified FTL_1286, a previously unidentified gene, and investigated whether it is required for virulence. FTL_1286 is a gene of unknown function that is conserved among Francisella species. To identify mechanisms required for F. tularensis pathogenesis, we wanted to further elucidate the function of FTL_1286 and its homolog in the highly virulent Schu S4 strain, FTT_0924, in intracellular growth and virulence.

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**MATERIALS AND METHODS**

**Bacterial strains.** *F. tularensis* subspecies *holarctica* LVS was obtained from the Centers for Disease Control and Prevention in Atlanta, GA, and *F. tularensis* subspecies *tularensis* Schu S4 was obtained from BEI Resources. Each *F. tularensis* strain was cultured using chocolate agar plates supplemented with 1% IsoVitalex (chocolate agar), brain heart infusion broth supplemented with 1% IsoVitalex (BHI), or Chamberlain's defined medium (CDM) (22). *Escherichia coli* DH10B was used for cloning and cultured using Luria-Bertani (LB) broth or LB agar. For selection, kanamycin was used at 50 μg/ml for *E. coli* strains and 10 μg/ml for *F. tularensis*, or hygromycin was used at 200 μg/ml for *E. coli* and *F. tularensis*. All cultures were grown at 37°C.

**Cell culture.** J774A.1 (ATCC TIB-67) cells are a murine macrophage-like cell line and were maintained in 75-cm² tissue culture flasks containing Dulbecco modified Eagle medium with glucose at 4.5 g/liter supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. TC-1 (ATCC CRL-2785) is a murine alveolar epithelial cell line, and TC-1 cells were maintained in 75-cm² tissue culture flasks containing RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 1.3 g of sodium bicarbonate/liter, and 0.1 mM nonessential amino acids. All tissue culture lines were maintained at 37°C and 5% CO₂.

**Plasmid vectors and allelic exchange.** The luminescent reporter plasmid (pBB1) was created by cloning the Photorhabdus luminescens luxCDABE operon from pXBR173 (23) into the low-copy shuttle plasmid pMP831 (24). In-frame, markerless deletions in *F. tularensis* LVS and *F. tularensis* Schu S4 were constructed using pEDLS0, a modified version of the suicide vector pMP812 containing an origin of transfer (25). Suicide vectors were mated into *F. tularensis* LVS or *F. tularensis* Schu S4 using *E. coli* S17-1Apir, and primary recombinants were selected with 200 μg of polymyxin B/ml and 10 μg of kanamycin/ml. Primary recombinants were grown overnight without selection in BHI and then plated on chocolate agar containing 10% sucrose. Complementing vectors were constructed by cloning into pJ3, which was derived from the plasmid pMP831 (24), respectively, each constitutively expressing luxCDABE. Green fluorescent protein (GFP) and PhoA fusion constructions were constructed by cloning the fusions into pEDL17 to allow controlled expression by anhydrotetracycline addition (26).

**Mapping transposon insertion sites.** Colonies of identified transposon insertion mutants were suspended in water and boiled for 10 min to release the genomic DNA. The lysates were centrifuged to remove the bacterial debris, and the remaining supernatant was used as the template DNA for amplification. Regions surrounding the transposon were amplified by using a primer specific to the transposon and random primers containing a tagged sequence [GGACACGCGTCGAA] as template DNA for amplification. Regions surrounding the transposon insertion sites were sequenced (Genewiz) for the following products were sequenced (Genewiz): lgl, codon-optimized kana-pMP831 (24). In-frame, markerless deletions in *F. tularensis* Schu S4, ΔFFT_0924, or ΔFFT_0924 p0924 strain intranassally. Mice were sacrificed at 2 h, 1 day, 3 days, 4 days, or 7 days postinfection to determine organ burdens in the lung, liver, and spleen. Organ samples were homogenized using a Biojector (Bioject), and homogenates were serially diluted and plated onto chocolate agar. Individual colonies were counted after incubation for 4 days at 37°C to quantify organ burdens.

**Subcellular fractionations.** Portions (50 ml) of cultures grown overnight in CDM were lysed via mechanical lysis with 0.1-mm-diameter silicon beads in a bead beater, and crude membrane fractions were pelleted from whole-cell lysates by centrifugation at 100,000 × g for 2 h. Each supernatant was removed for the soluble fraction. The membrane pellet was resuspended in 150 mM NaCl, 10 mM Tris, 0.5% Sarkosyl (pH 7.5) and adjusted overnight. The solubilized membrane fraction was then pelleted at 100,000 × g for 1.5 h. The soluble inner membrane fraction was removed, and the Sarkosyl insoluble fraction was washed and then resuspended in 150 mM NaCl–10 mM Tris (pH 6.5). All fractions were standardized to equal protein by using a bicinchoninic acid (BCA) assay (Pierce), run on a 4 to 20% polyacrylamide gradient gel (Bio-Rad), and transferred to a nitrocellulose membrane. The membranes were probed with antibodies recognizing hemagglutinin (HA) peptide (Sigma), RipA (27), IgLC (BEI Resources), and TulH (BEI Resources) for primary probes and antibodies conjugated to fluorophores as secondary probes (LI-COR Biosciences). Blots were visualized with an Odyssey infrared imaging system (LI-COR Biosciences).

**GFP and PhoA fusion localization assay.** Overnight cultures were grown in CDM broth containing 100 μg of anhydrotetracycline/ml. GFP fluorescence assays were performed by pelleting 1.5 ml of an overnight culture and resuspending samples in 50 ml Tris–HCl (pH 8.0), 200 mM NaCl, and 15 mM EDTA. A total of 200 μl of resuspended pellet was transferred to a black-walled 96-well dish (Nunc), and the plate was read using a Tecan plate reader with an excitation filter of 485 nm and an emission filter of 512 nm. PhoA activity assays were performed by pelleting and resuspending overnight cultures in 1 M Tris–HCl (pH 8.0), 1 M ZnCl₂, 0.01% sodium dodecyl sulfate, and 5% chloroform. The resuspended cultures were permeabilized for 5 min at 37°C. Then, 100 μl of permeabilized culture was transferred to a 96-well dish, and 20 μl of 0.4% *p*-nitrophenyl phosphate in 1 M Tris–HCl (pH 8.0) was added, followed by incubation at 37°C, and the OD₄₀₅ was measured every 5 min using a Tecan Infinite 200 series plate reader for 16 h. The time point to reach an arbitrary OD₄₀₅ was recorded. PhoA activity was calculated as (OD₄₀₅ × 1,000)/(min × OD₄₀₅ × ml of culture volume).

**Growth curves.** Overnight cultures of *F. tularensis* grown in CDM were diluted to an OD₀₆₀ of 0.05 and seeded into 200 μl of CDM or BHI well per well in a 96-well plate (Corning). Cultures were inoculated in an Infinite 200M Pro series Tecan plate reader (Tecan) at 37°C with orbital shaking. The OD₀₆₀ was measured every 15 min for 48 h. A final concen...
was resuspended in PBS (to an OD600 of 1), and a lawn of bacteria were incubated at 37°C for 2 days before the diameters of the inhibition biotics or compounds (Becton Dickinson) were placed on the agar. Plates were diluted to an OD600 of 0.05 in 10 ml of CDM with or without sucrose. Aliquots were removed at specific time intervals to measure the OD.

Intracellular growth was measured by infecting 10^3 J774 or TC-1 cells with 10^7 F. tularensis bacteria in a 96-well black-walled, clear-bottom plate for 2 or 4 h, respectively. Extracellular bacteria were then removed from the cells, fresh medium containing 25 μg of gentamicin/ml was added, and the plate was placed in the Tecan plate reader with the conditions of 37°C and 5% CO₂ maintained. The luminescence was measured every 15 min for 48 h.

Disc diffusions. Disc diffusions were performed on modified Mueller-Hinton agar supplemented with 1% tryptone, 0.5% NaCl, 0.05% t-cysteine freebase, 1% glucose, and 0.00025% iron pyrophosphate. F. tularensis was resuspended in PBS (to an OD600 of 1), and a lawn of bacteria was swabbed onto each plate. Paper discs impregnated with specific antibiotics or compounds (Becton Dickinson) were placed on the agar. Plates were incubated at 37°C for 2 days before the diameters of the inhibition zones were measured.

Fluorescence microscopy. J774 cells were plated onto 8-well chamber slides (Nunc) at 3 x 10^5 cells per well and allowed to replicate overnight. F. tularensis Schu S4-GFP or ΔFTT_0924-GFP was used to infect the J774 cells at an MOI of 100 for 2 h, the bacteria were removed, and fresh medium containing 25 μg of gentamicin/ml was added to the cells. To determine bacterial replication at 24 h postinfection, the medium was removed and washed once with PBS, and 4% paraformaldehyde was added for 30 min to fix the cells. The slide was then washed in 50 mM ammonium chloride to remove residual fixative. To stain the eukaryotic plasma membrane, the slide was incubated in 10 μg of wheat germ agglutinin/ml conjugated to AF647 for 10 min and then washed with PBS. DAPI (4’,6’-diamidino-2-phenylindole)-containing mounting medium (Vectorshield; Vector Labs) was then added to stain the nucleus.

To determine viability of intracellular bacteria, J774 macrophages were infected with Schu S4-GFP or ΔFTT_0924-GFP strains at an MOI of 100. At 2 h postinfection, the bacteria were removed, and fresh medium containing 25 μg of gentamicin/ml was added to the infected cells. At 16 h postinfection, the infected J774 macrophages were stained as described previously (28). Briefly, infected macrophages were permeabilized for 15 min in PBS containing 0.1% saponin and 3% bovine serum albumin (permeabilization buffer). Next, the infected cells were stained with 2.6 μM PI and an anti-F. tularensis lipopolysaccharide (LPS) antibody (as an internal permeabilization control) for 12 min. The cells were then washed three times with permeabilization buffer and fixed in 4% paraformaldehyde. The fixed cells were mounted with DAPI-containing mounting medium (Vectashield; Vector Labs) to stain the nucleus.

Transmission electron microscopy. Wild-type F. tularensis Schu S4 and ΔFTT_0924 cells were grown overnight in CDM containing 300 mM sucrose to maintain the structural integrity of the mutant strain. Bacteria were then resuspended into fresh CDM containing 300 mM sucrose at an OD600 of 0.1 and allowed to grow for an additional 6 h. Bacteria were then pelleted and resuspended in fixative buffer (2% glutaraldehyde, 2% paraformaldehyde, 300 mM sucrose, and 150 mM sodium phosphate, pH 7.4) for 4 h at 4°C overnight. Samples were then fixed in 0.15 M sodium phosphate and 1% osmium tetroxide. Fixed samples were dehydrated through a series of treatments with 30, 50, 75, 90, and 100% ethanol and propylene oxide and embedded in Spurr’s low-viscosity epoxy resin (Polysciences). Sections (70 to 80 nm) were cut with a diamond knife and mounted on 200 mesh Formvar/carbon-coated copper grids and stained with 4% aqueous uranyl acetate and Reynolds’ lead citrate. Samples were observed using a LEO EM910 transmission electron microscope operating at 80 kV (Carl Zeiss Microscopy, LLC), and digital images were acquired using a Gatan Orius SC1000 CCD Digital Camera with Digital Micrograph 3.11.0 (Gatan, Inc.).

RESULTS

F. tularensis luminescence reporter functions as a surrogate measurement for intracellular growth. F. tularensis LVS expressing pJB1, a luminescence reporter plasmid constitutively expressing Photobacterium luminescens operon luxCDABE, was used to determine whether luminescence could act as a reporter for intracellular growth within host cells. J774A.1 macrophages (J774) were infected with either LVS, LVS harboring pJB1 (LVS LUX), or ΔripA mutant, a mutant that does not grow inside macrophages, harboring pJB1 (ΔripA LUX) (20). Luminescence was measured at 4 and 24 h postinoculation; after each reading, the intracellular bacteria were enumerated by dilution plating. From 4 to 24 h postinoculation, both LVS and LVS LUX had increased intracellular numbers of roughly 100-fold, indicating that pJB1 did not significantly affect intracellular growth (Fig. 1). There was roughly a 100-fold increase in luminescence between 4 and 24 h postinoculation in LVS with pJB1, indicating that increased luminescence was directly proportional to the increase in intracellular bacterial numbers. There was no increase in luminescence in the LVS vector-only control between 4 and 24 h, showing that there were minimal background levels for this reporter system. Also, the absence of a significant change in luminescence or intracellular burden between 4 and 24 h for ΔripA LUX further indicated that luminescence can be used as an accurate measurement for intracellular growth.

Transposon mutagenesis screening identifies growth-deficient mutants in J774A.1 macrophages or TC-1 epithelial cells using a luminescence reporter system. LVS LUX was transformed with an EZ::TN (KAN-2) transposome complex (Epican-crete) containing a kanamycin resistance cassette that was codon optimized for F. tularensis (Blue Heron). A total of 7,614 individual mutants were picked and propagated in BHI broth overnight. Of these, 160 mutants failed to grow or luminesce and were discarded from further experiments, leaving 7,454 mutants to be tested for intracellular growth. J774 macrophages and TC-1 epithelial cells propagated in a 96-well format were infected with each individual mutant. The initial luminescence was measured at 4 h (J774) or 6 h (TC-1) and at 24 h to determine the intracellular growth. A total of 356 mutants from the primary screening exhibited an intracellular growth defect in either or both cell types.
These 356 mutants were reevaluated in triplicate for intracellular growth in both J774 and TC-1 cells using the same luminescence reporter system. Of these, 269 mutants from the primary screen repeated a growth defect. The transposon insertion sites for all 269 mutants were identified by amplifying the region surrounding the transposon insertion by semidegenerate PCR, followed by DNA sequence analysis (Genewiz), and comparison to the LVS whole-genome sequence (see Table S2 in the supplemental material). The 269 transposon insertions mapped to 74 genes and 3 pseudogenes. To further eliminate false positives, representative mutants with genes with only one transposon insertion were tested in standard gentamicin protection assays, identifying 68 genes and 2 pseudogenes as being required for intracellular growth (Table 1 and Fig. 2). To the best of our knowledge, 14 of the 68 genes were not previously identified as required for virulence or intracellular growth in any strain of Francisella.

The FTL_1286 and FTT_0924 loci are required for intracellular growth. One transposon mutation mapped to FTL_1286, a gene of unknown function that has not been identified as a virulence factor for any Francisella species. The mutant with the transposon mutation exhibited no growth within J774 macrophage cells but had observable growth within the TC-1 epithelial cells (see Table S2 in the supplemental material). To determine whether the FTL_1286 locus was required for intracellular growth, a markerless, in-frame deletion was constructed of the FTL_1286 locus and growth in macrophages was determined. In J774 cells, the mutant with the transposon mutation disrupting the FTL_1286 locus and ΔFTL_1286 expressing pBl3 (shuttle vector expressing P. luminescens luxCDABE) had no observable intracellular growth, as determined by the luminescence reporter system (Fig. 3A). However, growth of the ΔFTL_1286 pBl3 complemented strain grew to similar levels as wild-type LVS in macrophages, indicating that FTL_1286 is required for the intracellular replication of LVS (Fig. 3A). Since there is 100% amino acid sequence identity between FTL_1286 and the FTT_0924 homolog in the highly virulent Schu S4 strain, we hypothesized that FTT_0924 was also required for intracellular growth of Schu S4.

Deletion of the homologous FTT_0924 locus in the Schu S4 strain resulted in reduced intracellular growth in J774 cells that was complemented by expressing FTT_0924 in trans (Fig. 3B) in the mutant strain. Interestingly, the LVS mutant with the transposon mutation in FTL_1286 exhibited some growth within the TC-1 epithelial cells; however, the ΔFTL_1286 strain of LVS and the ΔFTT_0924 strain of Schu S4 exhibited no observable growth within epithelial cells, indicating that the transposon mutation did not fully abolish the function of FTL_1286 or that the transposon mutant acquired a mutation partially compensating for the genetic disruption (Fig. 3D and see Table S2 in the supplemental material).

To verify that FTT_0924 was required for intracellular growth and did not simply affect the bacterial luminescence reporter, we performed gentamicin protection assays in J774 and TC-1 cells, and the intracellular burdens were quantified by dilution plating (Fig. 3C and D). In both J774 and TC-1 cells, the ΔFTT_0924 strain did not exhibit intracellular growth, and intracellular growth could be complemented to near wild-type levels by expressing FTT_0924 in trans. Fluorescence microscopy analysis of GFP-expressing strains was also performed to visualize intracellular growth of the ΔFTT_0924 mutant (Fig. 3E and F).

FTT_0924 is required for virulence in mice. Since FTT_0924 is required for F. tularensis Schu S4 intracellular growth, we wanted to determine whether FTT_0924 was required for Schu S4 virulence in a mouse pulmonary infection model. C57BL/6 mice were inoculated intranasally with 100 CFU of the wild-type Schu S4, ΔFTT_0924, or ΔFTT_0924 pBl294 strain. At 2 h and at 1, 3, 4, and 7 days postinoculation, the bacterial burdens were enumerated from the lungs, livers, and spleens of the infected mice. At 2 h postinfection, the lungs of mice were harvested and dilution plated. Equal numbers of bacteria were recovered from the lungs of mice infected with each bacterial strain, indicating that the mutant and wild-type organisms accessed the lung at equivalent efficiencies (data not shown). At day 1, mice infected with the ΔFTT_0924 mutant had significantly reduced burdens in the lungs compared to the wild-type and complemented strains. No bacteria were detected in livers or spleens of animals infected with any strain at day 1. By day 3, mice infected with the ΔFTT_0924 mutant had significantly reduced burdens in the lungs and spleens.

### Table 1
Numbers of genes identified by mutant phenotype

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<tr>
<th>Growth phenotype</th>
<th>No. Genes</th>
<th>No. Pseudogenes</th>
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<td>1</td>
</tr>
<tr>
<td>Intermediate</td>
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<td>1</td>
</tr>
<tr>
<td>Growth in macrophages</td>
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<td>0</td>
</tr>
<tr>
<td>Growth in epithelial cells</td>
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<td>0</td>
</tr>
<tr>
<td>Total</td>
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<table>
<thead>
<tr>
<th>Bacterial processes</th>
<th>No. Genes</th>
<th>Pseudogenes</th>
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<td>Purine biosynthesis</td>
<td>transporters</td>
<td>1</td>
</tr>
<tr>
<td>Essential processes</td>
<td>carbon metabolism</td>
<td>1</td>
</tr>
<tr>
<td>Aromatic amino acid biosynthesis and transport</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Biotin biosynthesis</td>
<td>LPS biosynthesis</td>
<td>1</td>
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compared to the wild-type and complemented strains. The ΔFTT_0924 strain did not robustly infect the livers of the animals, whereas there were significant bacterial burdens observed in both the wild-type and complemented strains (Fig. 4). Interestingly, there was no change in bacterial burden between day 3, day 4, and day 7 in mice infected with the ΔFTT_0924 mutant in any organ tested, indicating that the ΔFTT_0924 mutant had reached a burden where bacterial growth and killing occurred at equal rates, whereas significant growth was observed in mice infected with the wild-type and complemented strains (Fig. 4). No data were taken for animals infected with wild-type Schu S4 or the complemented strain at day 7 because animals infected with these strains became moribund at day 4, which required the animals to be euthanized. Overall, these data indicate that ΔFTT_0924 was required for full virulence of F. tularensis Schu S4 in a pulmonary murine infection model.

ΔFTT_0924 is required for resisting osmotic stress during growth in liquid culture. To characterize the ΔFTT_0924 mutant, we monitored the general growth characteristics of the ΔFTT_0924 strain in liquid culture. The Schu S4 and ΔFTT_0924 strains were grown in CDM for 36 h, and the OD_{600} was measured every 6 h. After 36 h of growth, Schu S4 reached an OD_{600} of 2.75, while the ΔFTT_0924 mutant reached an OD_{600} of 1.05, indicating a defect for growth in liquid culture (Fig. 5A). To determine whether there was any difference in the number of viable bacteria at each point, wild-type Schu S4 and ΔFTT_0924 strains were enumerated by dilution plating. Strikingly, there were 100- to 1,000-fold fewer viable bacteria per OD_{600} in cultures with the ΔFTT_0924 mutant (Fig. 5B). We next sought to determine whether the viability defect of the ΔFTT_0924 mutant strain occurred in other conditions, including growth on solid agar or in PBS suspension. This viability defect was not observed after bacterial growth on chocolate agar plates, determined by resuspending plate-grown bacteria in PBS to an OD_{600} of 1 and dilution plating, indicating that the mutant strain specifically did not tolerate growth in liquid culture (Fig. 5C). Further, this viability defect in liquid culture was only observed with actively replicating bacteria since suspension and shaking in PBS for 24 h at 37°C did not...
not reduce the numbers of viable CFU recovered for either the Schu S4 or the \( \text{H9004} \) FT\_0924 strain (Fig. 5D).

One reason for the reduced viability in liquid culture might be that the \( \text{H9004} \) FT\_0924 mutant is sensitive to osmotic stress from a hypotonic solution. To test this hypothesis, we increased the osmolarity of the medium to prevent the influx of water into the bacterial cytoplasm, resulting in bacterial swelling and eventual lysis, by adding the nonionic osmolite sucrose to the medium.

![Graph showing bacterial viability](image)

**FIG 4** Effect of FT\_0924 on Schu S4 growth and dissemination in a mouse model of infection. C57BL/6 mice were infected intranasally with 100 CFU of the Schu S4 (WT), \( \text{H9004} \) FT\_0924, or \( \text{H9004} \) FT\_0924 p0924 strains. At 2 h and days 1, 3, 4, and 7 postinfection, organs were harvested, homogenized, serially diluted, and plated on chocolate agar to determine bacterial burdens. A horizontal dashed line indicates the limit of detection of the assay. **, \( P < 0.01 \); ***, \( P < 0.0001 \) (as determined by the Mann-Whitney U test).

![Graph showing bacterial growth](image)

**FIG 5** Bacterial viability of \( \text{H9004} \) FT\_0924 cells during *in vitro* growth. Schu S4 and \( \text{H9004} \) FT\_0924 cells were grown in CDM, and the OD\text{600} (A) and the viable bacteria per OD\text{600} (B) were measured. (C) The bacterial viability was measured after 24 h of growth on chocolate agar, resuspension in PBS, and dilution plating. (D) Schu S4 and \( \text{H9004} \) FT\_0924 cell viability was measured by inoculating PBS at an OD\text{600} of 1; samples were then allowed to shake for 24 h and dilution plated. The OD\text{600} (E) and the viable bacteria per OD\text{600} (F) were quantified at 24 h postinoculation from Schu S4 and \( \text{H9004} \) FT\_0924 cells grown in CDM with various amounts of sucrose. All of the results are representative of at least three independent experiments. *, \( P < 0.05 \); **, \( P < 0.01 \); n.s., not significant (as determined by using the Student t test).
Wild-type Schu S4 and ΔFTT_0924 strains were inoculated at an OD600 of 0.05 in CDM supplemented with either 0, 100, or 300 mM sucrose and then grown for 24 h at 37°C. Both OD600 and the numbers of viable bacteria were measured after 24 h of growth to determine whether growth in high-osmolarity medium rescued the viability defect of the ΔFTT_0924 mutant after growth in liquid culture. In medium containing sucrose, the ΔFTT_0924 mutant reached a terminal OD600 equal to that of the wild type, indicating a rescue of the ΔFTT_0924 mutant growth defect determined by OD600. Likewise, the number of viable mutant bacteria per OD600 was restored to near wild-type levels in the presence of sucrose (Fig. 5E and F), suggesting that ΔFTT_0924 peptidoglycan integrity was compromised, resulting in membrane permeability and an increased susceptibility to osmotic stress.

ΔFTT_0924 mutant membrane is permeable under hypotonic conditions. Since ΔFTT_0924 mutant viability was rescued with the addition of sucrose, we wanted to determine whether the membranes of the mutant bacteria were permeable specifically during growth under hypotonic conditions. Wild-type and the ΔFTT_0924 strains were grown in CDM containing propidium iodide (PI), which is membrane impermeable and will enter the bacterial cell and incorporate into the chromosomal DNA only if the integrity of the bacterial membrane is compromised. Once PI binds DNA, it becomes fluorescent, and thus an increase in PI fluorescence indicates that the bacterial membranes are permeable. Wild-type and ΔFTT_0924 strains were grown in CDM, CDM with sucrose, and CDM with gentamicin, a bacteriolytic antibiotic providing a positive control for PI incorporation. The OD (Fig. 6A) and PI fluorescence (Fig. 6B) were measured simultaneously every 30 min for 24 h. Wild-type bacteria grown in CDM or CDM with added sucrose did not have any appreciable increase in PI fluorescence, whereas bacteria grown in CDM plus gentamicin (inoculated at a higher OD600 of 0.3) exhibited significant PI fluorescence (Fig. 6B). In contrast, the ΔFTT_0924 strain showed a significant increase in PI fluorescence within the first 4 h of growth in CDM, which did not occur in CDM with added sucrose (Fig. 6B), indicating that the ΔFTT_0924 strain’s bacterial membrane was permeable only when grown in a hypotonic solution. Further, this increase in membrane permeability (Fig. 6B) in CDM correlated with the decrease in viability within 6 h of growth in CDM (Fig. 5B), indicating that the cellular defect was observable immediately upon initiation of bacterial replication.

Since the membrane of the ΔFTT_0924 mutant was permeable during replication under hypotonic conditions, we next tested whether the ΔFTT_0924 cell membrane was also permeable in macrophages. J774 cells were infected with either wild-type Schu S4-GFP or the ΔFTT_0924-GFP mutant for 16 h, and the macrophages were then permeabilized with saponin and stained with PI. We found that 5% of the intracellular Schu S4-GFP and 40% of the ΔFTT_0924-GFP mutant stained positive for PI, indicating that the ΔFTT_0924 mutant exhibited a significant increase in membrane permeability in macrophages (Fig. 7). These data suggest that the observed viability defect of the ΔFTT_0924 mutant is the reason for reduced intracellular growth in host cells and likely in vivo.

The observed membrane permeability of the ΔFTT_0924 mutant suggests that it may be involved in maintaining the overall structure of F. tularensis. Bacteria growing in CDM with sucrose were fixed, and transmission electron microscopy was performed to determine whether the bacteria had any observable gross
morphological differences compared to wild-type bacteria. No obvious observable physiological differences in cell size, shape, membrane structure, or other defects were visible between the wild-type and \( FTT_0924 \) strains (see Fig. S1 in the supplemental material). This suggests that membrane permeability in hypotonic solution is not due to an altered bacterial structure of the \( FTT_0924 \) mutant.

**FTT_0924 protein localizes to the inner membrane facing the periplasm.** Determining FTT_0924 localization could provide insight into how the protein contributes to osmotic stress resistance. Membrane fractions were prepared from the \( \Delta FTT_0924 \) mutant expressing FTT_0924 protein with a C-terminal HA tag (FTT_0924-HA). Soluble, inner-membrane and outer-membrane fractions were probed with antibodies recognizing HA, IglC, RipA, or Tul4 to determine protein localization. FTT_0924 localized to the inner membrane fraction and fractionated with the same pattern as RipA, a known inner membrane protein (Fig. 8A) (20).

FTT_0924 localized to the inner membrane. Since there is only one predicted transmembrane domain (TMHMM) at the extreme N terminus, the majority of the protein must be located within the cytoplasm or periplasm. To discern between these two possibilities, we adapted a GFP/PhoA fusion system to function in *Francisella* species (27). Constructs expressing translational fusions of GFP or PhoA to the C terminus of FTT_0924 were constructed and expressed in *F. tularensis* Schu S4. Since GFP fluoresces only when expressed in the cytoplasm and PhoA is only active in the periplasm, cytoplasmic or periplasmic localization can be determined based on fluorescence/activity ratios expressed by the fusion construct strains. The GFP and PhoA activities were measured for each fusion, and the ratios of the GFP and PhoA activities were used to determine localization. BioF, involved in biotin biosynthesis, and BlaB (\( \beta \)-lactamase) were used as cytoplasmic and periplasmic controls, respectively. Fusion of the FTT_0924-GFP-expressing strain exhibited near-background levels of fluorescence and the FTT_0924-PhoA-expressing strain lysate exhibited PhoA activity, indicating periplasmic location of the expressed fusion proteins (Fig. 8B). Together, these data indicate that FTT_0924 was localized in the inner membrane and facing the periplasmic space, where FTT_0924 may be directly involved in preventing bacterial lysis from osmotic stress during bacterial replication.

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**FIG 7** FTT_0924 is required to maintain membrane integrity in host cells. (A to C) Representative Schu S4-GFP cells (green) (A), PI staining (red) (B), and merged (C) images, including DAPI staining (blue) of J774 macrophages infected with Schu S4-GFP at 16 h postinfection. (D to F) Representative \( \Delta FTT_0924 \)-GFP cells (green) (D), PI staining (red) (E), and merged (F) images, including DAPI staining (blue) of J774 macrophages infected with the \( \Delta FTT_0924 \)-GFP mutant at 16 h postinfection. All samples were also stained with anti-*Francisella* LPS as an internal permeabilization control (data not shown). (G) Percent colocalization of GFP bacteria and PI staining from intracellular bacteria. The data were analyzed from \( n > 200 \) infected macrophages from two independent experiments. ***, \( P < 0.001 \) (as determined by using the Student \( t \) test).

**FIG 8** Localization of FTT_0924. (A) Inner membrane (IM), outer membrane (OM), soluble (Sol), and whole-cell lysate (WCL) samples were prepared via Sarkosyl extraction from Schu S4 and \( \Delta FTT_0924 \) p0924-HA cells (FTT_0924-HA). Proteins that reside in the cytosol (IglC), inner membrane (RipA), and outer membrane (Tul4) served as fraction purity controls. Lanes were loaded with equal amounts of protein, as determined by BCA assay. (B) Schu S4 cells expressing BioF, BlaB, or FTT_0924 as translational fusions to PhoA or GFP were tested for GFP or PhoA activity, and the results are presented as the ratio of the two activities. The data represent at least three independent experiments.
TABLE 2 Sensitivity of Schu S4, ΔFTT_0924 mutant, and ΔacrB mutant strains to specific compounds

<table>
<thead>
<tr>
<th>Antibiotic or reagent</th>
<th>Class</th>
<th>Inhibition zone mean diameter (mm) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Schu S4</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>Quinolone</td>
<td>32.7 ± 0.6</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Fluoroquinolone</td>
<td>36.7 ± 0.7</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>Macrolide</td>
<td>19 ± 0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Tetracycline/polyketide</td>
<td>21 ± 0</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>Intercalator</td>
<td>15.7 ± 0.6</td>
</tr>
<tr>
<td>SDS</td>
<td>Detergent</td>
<td>14 ± 0</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>β-Lactam</td>
<td>6 ± 0</td>
</tr>
<tr>
<td>Amoxicillin + clavulanic acid</td>
<td>β-Lactam</td>
<td>6 ± 0</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Aminoglycoside</td>
<td>21.3 ± 0.6</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Aminoglycoside</td>
<td>26 ± 0</td>
</tr>
<tr>
<td>Rifampin</td>
<td>Rifamycin</td>
<td>22.3 ± 0.6</td>
</tr>
</tbody>
</table>

The ΔFTT_0924 mutant is sensitive to β-lactam antibiotics. Because FTT_0924 is localized in the periplasm and is sensitive to osmotic stress, we hypothesize that FTT_0924 is involved in regulating peptidoglycan remodeling and therefore that the ΔFTT_0924 mutant would be sensitive to specific stresses affecting the cell wall and peptidoglycan. We therefore tested the sensitivity of the ΔFTT_0924 mutant to specific antibiotics and other compounds via disc diffusion assays. Suspensions of wild-type Schu S4, the ΔFTT_0924 mutant, or the ΔacrB mutant with a defect in the drug efflux system, were swabbed onto each compound were placed on each plate. The diameters of the mutant strains to specific compounds

**DISCUSSION**

Luminescence is a commonly used reporter in prokaryotic systems and was recently first used in F. tularensis (23). Here, we used bacterial luminescence as a reporter for growth within macrophages where bacterial burden increased proportionally with luminescence. Using this reporter system, we screened 7,454 transposon mutants for growth in macrophage and epithelial cell lines. Of these mutants, 269 with transposon insertions had a growth defect in one or both cell types, which mapped to 74 specific genes. The list of 74 genes was further narrowed to 68 genes by targeted phenotypic screening of the transposon mutants. The F. tularensis LVS genome contains about 1,800 genes, and testing 7,454 transposon insertion mutants should identify an average of four transposon insertion mutations per gene if there was an unbiased distribution of transposon insertions. The 269 transposon mutations that impacted intracellular growth mapped to 68 genes, ~4-fold fewer genes. All 8 genes in the purine biosynthesis pathway (that are predicted as nonredundant and not essential) and 5 of 6 genes required for biotin biosynthesis were identified in this screen, suggesting that the library of 7,454 mutants contained minimal bias of insertion locations with a high level of genomic coverage (30).

The majority of mutants identified in the screen had similar growth defects in both J774 and TC-1 cell lines (disruptions in 50 of 68 genes identified). The majority of genes involved in purine biosynthesis and transporters did not replicate to any detectable level in either cell type, reiterating the known dependence on purine biosynthesis and dependence on host nutrients for intracellular growth and virulence (7, 19, 31, 32). Mutants that grew to intermediate levels in both cell types included those involved in essential bacterial processes, carbon metabolism and DNA replication and repair. It is possible that the defect in intracellular replication of many of these mutants could be attributed to an overall fitness defect and are not specifically required within a host cell. Interestingly, the screen identified two predicted pseudogenes as required for intracellular growth, both predicted to be disrupted genes encoding transporters. This finding suggests that these and potentially other predicted pseudogenes may not be inactive and may encode functional proteins or untranslated elements required for virulence or other cellular processes.

Mutants with growth defects in macrophages but not TC-1 epithelial cells included those with insertions in genes encoding proteins involved in LPS and biotin biosynthesis. Mutants with mutations in the LPS biosynthesis pathway likely did not show observable luminescence in the J774 cells due to the hypercytotoxic phenotype previously observed (33). Interestingly, these mutants grew to wild-type levels in the TC-1 epithelial cells, indicating that functional LPS is not essential for intracellular growth in all cell types and that the host recognition receptors or signaling pathways that result in hypercytotoxicity may be myeloid specific. Mutants with defects in biotin biosynthesis grew in TC-1 but not in J774 cells because the tissue culture medium used for TC-1 cells was supplemented with biotin, whereas the medium used for J774 cells was not. Growth of the biotin biosynthetic mutants in J774 cells was restored by supplementing the medium with biotin (data not shown). The limited number of mutants that grew in macrophages but not in epithelial cells had defects in synthesis or the uptake of aromatic amino acids. It is possible that the reduced growth of these mutants in TC-1 cells was due to the lower concentrations of phenylalanine, tyrosine, and tryptophan in TC-1.
medium than in J774 medium. The growth defects observed in the purine, biotin, and aromatic amino acid biosynthetic pathways illustrate that several essential nutrients are limiting in the cytosolic compartment.

We here identified FTT_0924 as a factor required for osmotic stress resistance, intracellular growth within macrophages and epithelial cells, and virulence in a pulmonary murine infection model. FTT_0924 is highly conserved among all Francisella species and encodes a 132-amino-acid protein of unknown function but which contains no sequence similarity to proteins outside the Francisella genus. We found that FTT_0924 is required to maintain resistance to osmotic stress in liquid culture during replication and that deletion of the FTT_0924 locus results in membrane permeability in a hypotonic solution. In solution, the OD600 of the \( \Delta \)FTT_0924 mutant was maintained, but viability was severely reduced, suggesting that the mutant bacteria are leaky but that full bacterial lysis does not occur. This viability defect can be rescued when the osmolarity of the solution is increased, indicating that the viability defect and membrane permeability only occur in hypotonic solution. Interestingly, the total solute concentration of our defined medium is 456 mM, and the isotonic solute concentration of a J774 cell is roughly 290 mM, so the host cell cytosol is a hypotonic environment for \( F. \) tularis (34). This observation correlates with the data demonstrating that the \( \Delta \)FTT_0924 mutant membrane was permeable to PI in J774 macrophages, suggesting that the intracellular growth and virulence defect of a \( \Delta \)FTT_0924 mutant is due to loss of membrane integrity from osmotic stress experienced within the host cytosol.

In Schu S4 cells, FTT_0924 localizes with the inner membrane and faces the periplasm, placing it in an optimal location to directly modulate peptidoglycan dynamics. The \( \Delta \)FTT_0924 mutant is also hypersensitive to \( \beta \)-lactam antibiotics, further suggesting the role of FTT_0924 in peptidoglycan stability. Transmission electron microscopy analysis revealed no gross morphological differences in the bacterial shape, size, or membrane structure in the \( \Delta \)FTT_0924 strain compared to wild-type Schu S4; thus, the mutant bacteria maintained a structure similar to that of the wild type. Together, these data demonstrate that FTT_0924 plays a role in maintaining the integrity of the peptidoglycan but that it is not required for maintaining gross bacterial structure.

To survive and replicate, bacterial pathogens must adapt to and resist stresses within different host environments. Identifying the specific stresses pathogens must overcome within host cells will help us to define the mechanisms required for intracellular growth and virulence and identify potential targets for therapeutics. We performed a large-scale genetic screen to identify genes required for \( F. \) tularis intracellular growth. We identified several genes (genes not previously recognized as required for intracellular growth and virulence) involved in metabolite biosynthesis and uptake, indicating that the host cell cytoplasm is limiting in several essential nutrients. We also identified FTT_0924, a gene of unknown function, and determined that FTT_0924 is involved in modulating peptidoglycan dynamics. By further investigating the role of FTT_0924 in peptidoglycan dynamics, we can understand how \( F. \) tularis modulates its peptidoglycan and elucidate how optimal peptidoglycan dynamics is required for intracellular growth and virulence. Identifying new genes required for \( F. \) tularis intracellular growth, such as specific amino acid biosynthetic genes and FTT_0924, and defining the role of these genes in intracellular replication will help to define the specific mechanisms of how \( F. \) tularis infects and replicates within host cells and causes disease.

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