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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 Δ*FTT_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 Δ*FTT_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 cytosolic 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-cm^2 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-cm^2 tissue culture flasks containing RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 1.5 g of sodium bicarbonate/liter, and 0.1 mM nonessential amino acids. All tissue culture lines were maintained at 37°C and 5% CO_2.

Plasmid vectors and allelic exchange. The luminescent reporter plasmid (pJB1) was created by cloning the *Photorhabdus luminescens luxCDABE* operon from pXB173 (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 pEDL50, 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* S17-1λpir, 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 pJB3, which was derived from the plasmid pMP831 (24), respectively, each constitutively expressing *luxCDABE*. Green fluorescent protein (GFP) and PhoA fusion constructs 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 [GGACACGCGTCGA CTAGTGG(N_{10})AA]. The amplified products from the PCR were used as a template in a subsequent reaction by using a primer complementary to the tag (GGACACGCGTCGACTAGTGG) and a primer specific to the transposon. The following products were sequenced (Genewiz) by using a primer to amplify outward from the transposon.

Transposon mutagenesis screen. An EZ::TN ⟨KAN-2⟩ transposome complex (Epicentre) containing an *F. tularensis* codon-optimized kanamycin cassette (Blue Heron) was electroporated into *F. tularensis* LVS harboring pJB1. Transposon mutants were selected on chocolate agar plates containing 10 μg of kanamycin/ml and 200 μg of hygromycin/ml. Individual transposon mutants were grown in BHI broth, with 10 μg of kanamycin/ml and 200 μg of hygromycin/ml, overnight in 96-well, black-walled, clear-bottom plates (Corning). The following day, overnight cultures were screened for growth at an optical density at 600 nm (OD_{600}) and luminescence using an Infinite M200 Series plate reader (Tecan). Mutants that failed to grow or luminesce were removed from future experiments. Overnight cultures were diluted 1:50 into 200 μl of either J774 medium or TC-1 medium. A 50-μl portion of the dilution was added to either 10^5 J774 cells or 10^5 TC-1 cells in 96-well, black-walled, clear-bottom, tissue culture-treated plates, which yielded an average multiplicity of infection (MOI) of 100. At 2 h postinfection for J774 cells and 3 h postinfection for TC-1 cells, the bacteria were removed, and 200 μl of their respective tissue culture media containing 25 μg of gentamicin/ml was added to each infected well. Luminescence was read at 4, 6, and 24 h using the Tecan plate reader in order to determine the intracellular growth.

Gentamicin protection assays. *F. tularensis* LVS strains cultured for 3 days on chocolate agar containing 1% IsoVitaleX were used to make overnight cultures in CDM. The following day, the cultures were diluted to 100 Klett units (10^9 CFU/ml) in phosphate-buffered saline (PBS). This suspension was diluted 1:10 into either J774 medium or TC-1 medium, and 1 ml was added to 10^6 J774 or TC-1 cells, respectively, resulting in an MOI of 100. At 2 h postinfection for J774 cells and 3 h postinfection for TC-1 cells, the bacteria were removed, and 1 ml of medium containing 25 μg of gentamicin/ml was added to the infected wells. At 2 h after the gentamicin treatment, the medium was removed and washed once with PBS, and the cells were scraped up, vortexed hard for 1 min, and dilution plated to enumerate the intracellular bacteria.

Mouse infections. Groups of four 6- to 8-week-old C57BL/6 mice (Jackson Laboratory) were infected with approximately 100 CFU of the *F. tularensis* Schu S4, Δ*FTT_0924*, or Δ*FTT_0924* p0924 strain intranasally. 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. Organs 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 agitated 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 Tul4 (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 ng of anhydrotetracycline/ml. GFP fluorescence assays were performed by pelleting 1.5 ml of an overnight culture and resuspending samples in 50 mM 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 mM $ZnCl_2$, 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 OD_{420} was measured every 5 min using a Tecan Infinite 200 series plate reader for 16 h. The time taken to reach an arbitrary OD_{420} was recorded. PhoA activity was calculated as (OD_{420} × 1,000)/(min × OD_{600} × ml of culture volume).

Growth curves. Overnight cultures of *F. tularensis* grown in CDM were diluted to an OD_{600} of 0.05 and seeded into 200 μl of CDM or BHI per well in a 96-well plate (Corning). Cultures were incubated in an Infinite 200M Pro series Tecan plate reader (Tecan) at 37°C with orbital shaking. The OD_{600} was measured every 15 min for 48 h. A final concen-

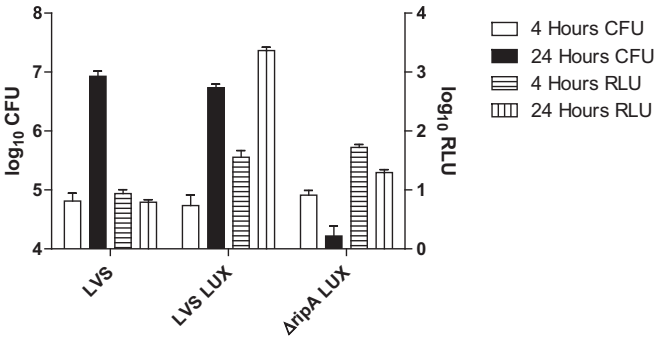

FIG 1 Luminescence as an indication for intracellular growth. The intracellular replication of LVS, LVS LUX, and LVS Δ*ripA* LUX in J774 macrophage cells was measured via luminescence (right axis) and dilution plating (left axis) for CFU at 4 and 24 h postinfection. RLU, relative light units. Each sample was read for luminescence; the infected macrophages were then lysed, and the intracellular bacteria were enumerated by dilution plating. The bars represent three independent experiments performed in triplicate.

tration of 2.5 μM propidium iodide (PI) was used to measure membrane permeability, and the fluorescence was measured using a 535-nm excitation and a 617-nm emission. For sucrose viability rescues, overnight cultures were diluted to an OD_{600} 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 10^5 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% CO_2 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% L-cysteine freebase, 1% glucose, and 0.00025% iron pyrophosphate. *F. tularensis* was resuspended in PBS (to an OD_{600} 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 3×10^4 cells per well and allowed to replicate overnight. *F. tularensis* Schu S4-GFP or Δ*FTT_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 (Vectashield; Vector Labs) was then added to stain the nucleus.

To determine viability of intracellular bacteria, J774 macrophages were infected with Schu S4-GFP or Δ*FTT_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 Δ*FTT_0924* cells were grown overnight in CDM containing 300 mM sucrose to maintain the structural integrity of the mutant strain. Bacteria were then reseeded into fresh CDM containing 300 mM sucrose at an OD_{600} 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 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 *Photorhabdus 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 Δ*ripA* mutant, a mutant that does not grow inside macrophages, harboring pJB1 (Δ*ripA* 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 Δ*ripA* 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 (Epicentre) 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 discounted 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.

TABLE 1 Numbers of genes identified by mutant phenotype

Growth phenotype	No.		Bacterial processes
	Genes	Pseudogenes	
No growth	20	1	Purine biosynthesis; transporters
Intermediate growth	30	1	Essential processes; carbon metabolism
Growth in macrophages only	5	0	Aromatic amino acid biosynthesis and transport
Growth in epithelial cells only	13	0	Biotin biosynthesis; LPS biosynthesis
Total	68	2	

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 Δ*FTL_1286* expressing pJB3 (shuttle vector expressing *P. luminescens luxCDABE*) had no observable intracellular growth, as determined by the luminescence reporter system (Fig. 3A). However, growth of the Δ*FTL_1286* p1286 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 Δ*FTL_1286* strain of LVS and the Δ*FTT_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).

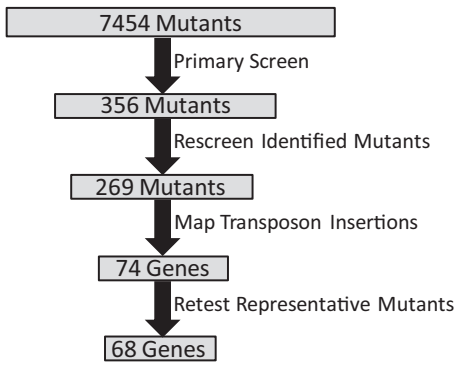

FIG 2 Screening procedure used to identify genes required for intracellular growth. Each mutant identified in the primary screen with reduced growth in J774 or TC-1 cells was rescreened in triplicate in J774 and TC-1 cells. Transposon insertions were mapped in rescreened mutants with growth defects in either or both cell lines. If only one transposon insertion mapped to a specific gene, the mutant was rescreened in a standard gentamicin protection assay.

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 Δ*FTT_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 Δ*FTT_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, Δ*FTT_0924*, or Δ*FTT_0924* p0924 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 Δ*FTT_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 Δ*FTT_0924* mutant had significantly reduced burdens in the lungs and spleens

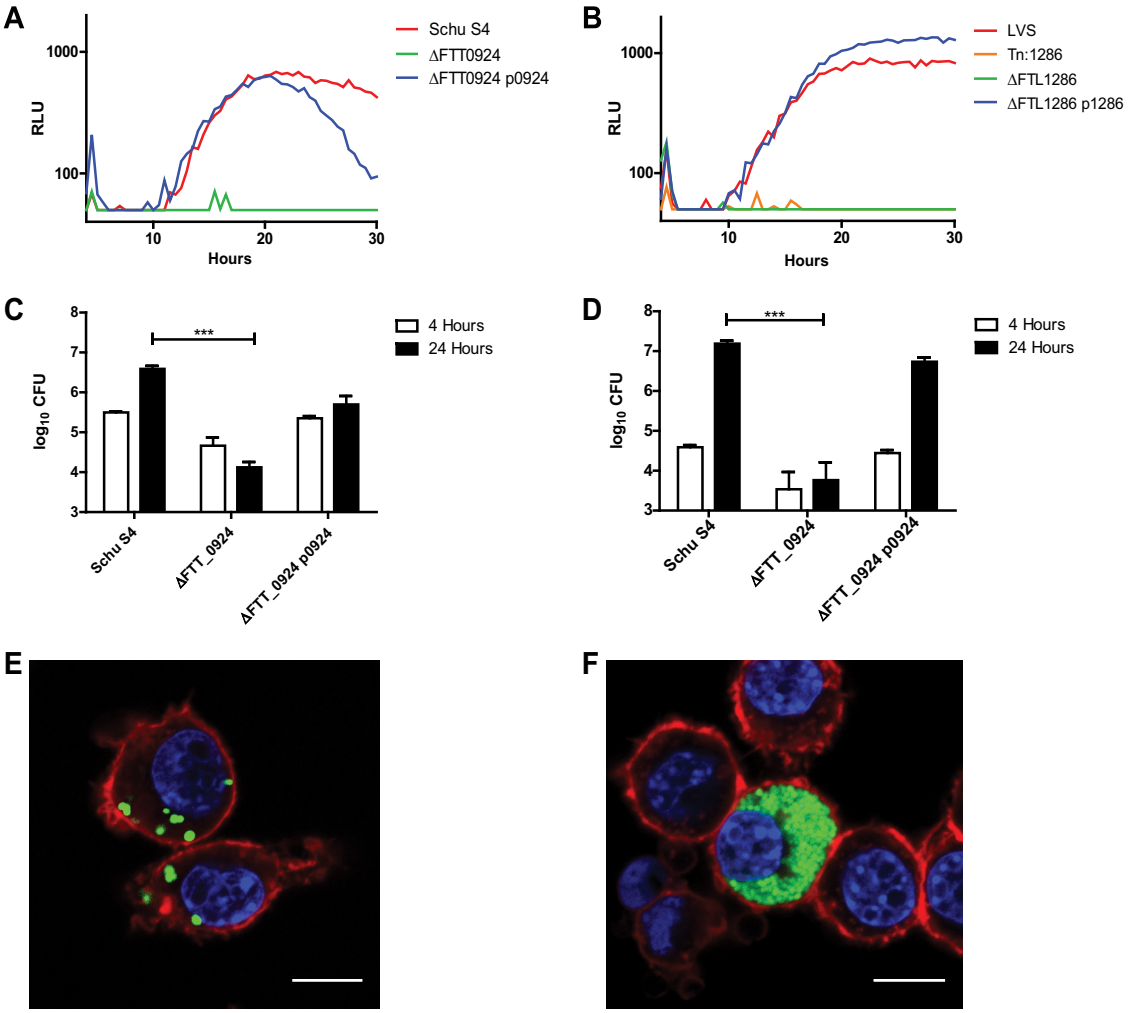

FIG 3 Impact of *FTL_1286* and *FTT_0924* on intracellular growth. (A and B) Intracellular growth of LVS LUX, Tn:*FTL_1286*, Δ*FTL_1286* LUX, and Δ*FTL_1286* p1286 strains (A) and Schu S4, Δ*FTT_0924* LUX, and Δ*FTT_0924* p0924 strains (B), as indicated via luminescence every 30 min. (C and D) J774 (C) or TC-1 (D) cells infected with Schu S4, Δ*FTT_0924*, or Δ*FTT_0924* p0924 strains measured at 4 and 24 h by dilution plating. (E and F) Representative images ($n = 30$) of J774 cells at 24 h postinfection with the Δ*FTT_0924*-GFP (E) or Schu S4-GFP (F) strain. Scale bars, 10 μm. Red indicates wheat germ agglutinin staining, blue indicates DAPI, and green indicates GFP bacteria. Three independent experiments were performed for each panel.

compared to the wild-type and complemented strains. The Δ*FTT_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 Δ*FTT_0924* mutant in any organ tested, indicating that the Δ*FTT_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 *FTT_0924* was required for full virulence of *F. tularensis* Schu S4 in a pulmonary murine infection model.

***FTT_0924* is required for resisting osmotic stress during growth in liquid culture.** To characterize the Δ*FTT_0924* mutant, we monitored the general growth characteristics of the Δ*FTT_0924* strain in liquid culture. The Schu S4 and Δ*FTT_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 Δ*FTT_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 Δ*FTT_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 Δ*FTT_0924* mutant (Fig. 5B). We next sought to determine whether the viability defect of the Δ*FTT_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

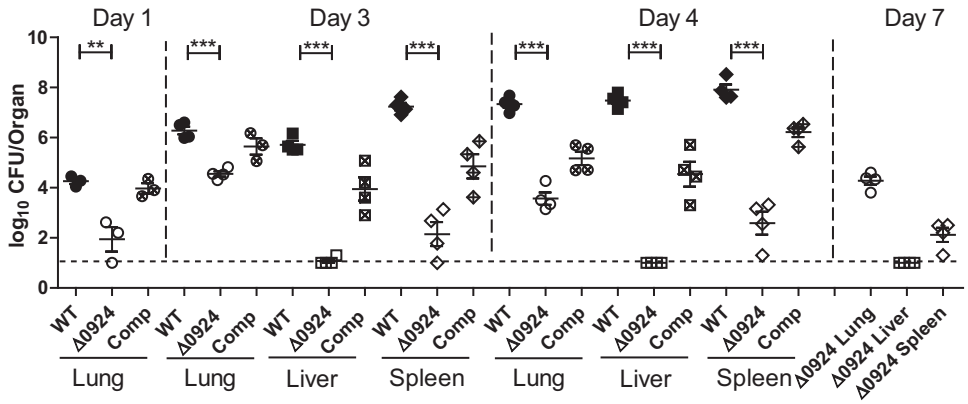

FIG 4 Effect of *FTT_0924* on Schu S4 growth and dissemination in a mouse model of infection. C57BL/6 mice were infected intranasally with 100 CFU of the Schu S4 (WT), Δ*FTT_0924*, or Δ*FTT_0924* p0924 strains. At 2 h and days 1, 3, 4, and 7 postinfection, organs were harvested, homogenized, serially 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).

not reduce the numbers of viable CFU recovered for either the Schu S4 or the Δ*FTT_0924* strain (Fig. 5D).

One reason for the reduced viability in liquid culture might be that the Δ*FTT_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.

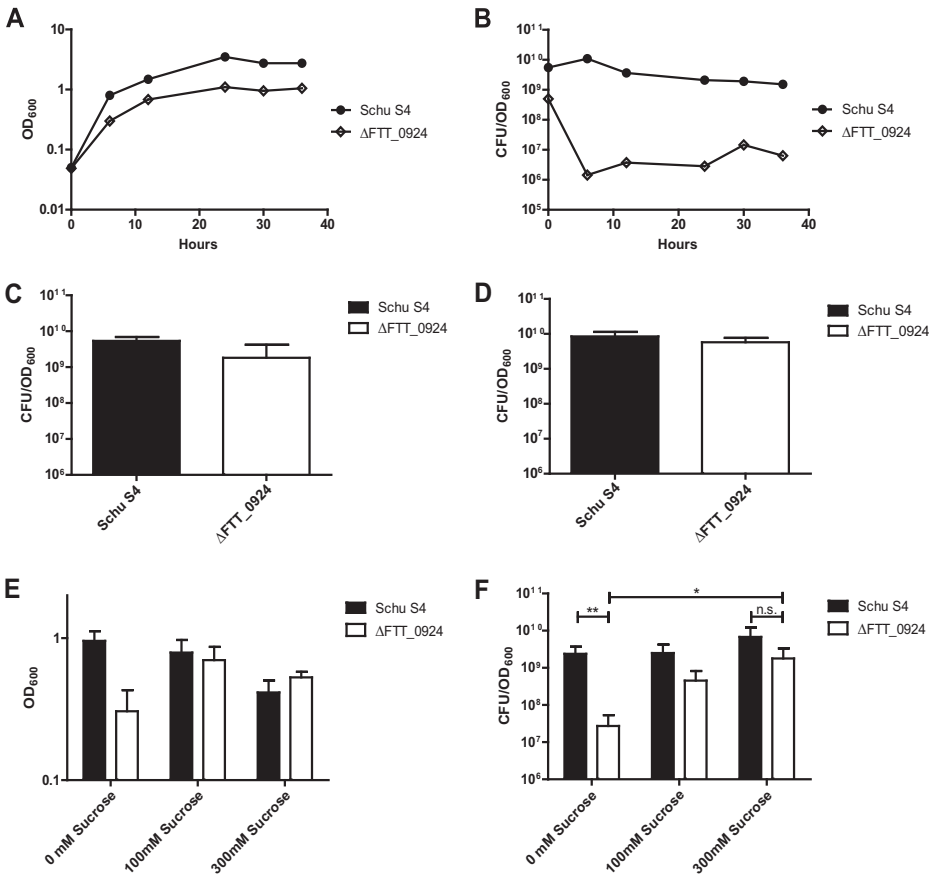

FIG 5 Bacterial viability of Δ*FTT_0924* cells during *in vitro* growth. Schu S4 and Δ*FTT_0924* cells were grown in CDM, and the OD_{600} (A) and the viable bacteria per OD_{600} (B) were measured. (C) The bacterial viability was measured after 24 h of growth on chocolate agar, resuspension in PBS, and dilution plating. (D) Schu S4 and Δ*FTT_0924* cell viability was measured by inoculating PBS at an OD_{600} of 1; samples were then allowed to shake for 24 h and dilution plated. The OD_{600} (E) and the viable bacteria per OD_{600} (F) were quantified at 24 h postinoculation from Schu S4 and Δ*FTT_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).

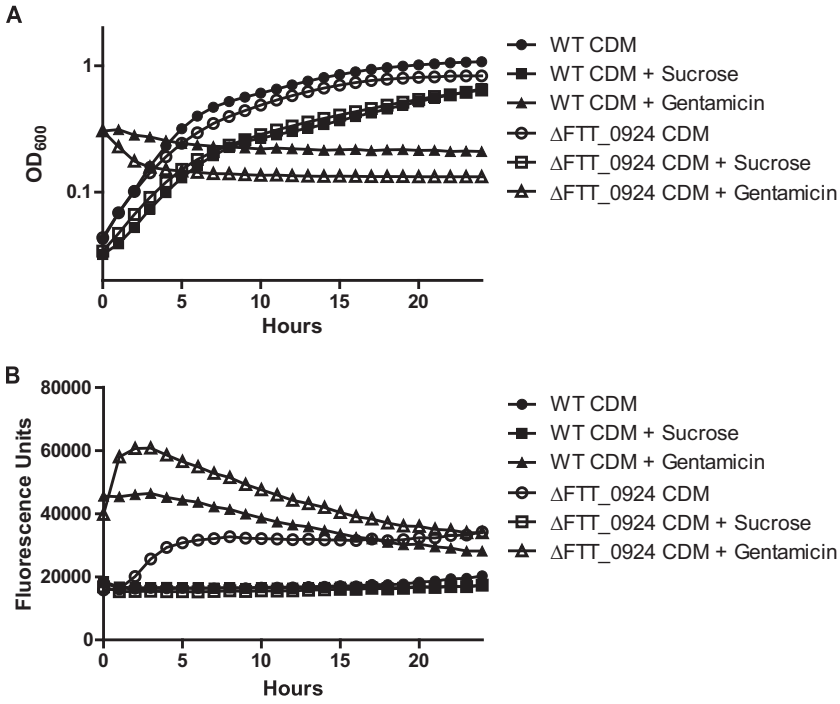

FIG 6 Membrane permeability of Schu S4 and Δ*FTT_0924* cells in liquid culture. Schu S4 and the Δ*FTT_0924* mutant were grown in CDM, CDM plus 300 mM sucrose, or CDM plus 50 μg of gentamicin/ml; each medium also contained 2.5 μM PI. (A) The OD_{600} of Schu S4 and Δ*FTT_0924* cells was measured every 30 min over 24 h. (B) The fluorescence from PI incorporation into bacterial DNA of Schu S4 and Δ*FTT_0924* cells was measured every 30 min over 24 h. All of the results are representative of at least three independent experiments.

Wild-type Schu S4 and Δ*FTT_0924* strains were inoculated at an OD_{600} of 0.05 in CDM supplemented with either 0, 100, or 300 mM sucrose and then grown for 24 h at 37°C. Both OD_{600} 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 Δ*FTT_0924* mutant after growth in liquid culture. In medium containing sucrose, the Δ*FTT_0924* mutant reached a terminal OD_{600} equal to that of the wild type, indicating a rescue of the Δ*FTT_0924* mutant growth defect determined by OD_{600}. Likewise, the number of viable mutant bacteria per OD_{600} was restored to near wild-type levels in the presence of sucrose (Fig. 5E and F), suggesting that Δ*FTT_0924* peptidoglycan integrity was compromised, resulting in membrane permeability and an increased susceptibility to osmotic stress.

Δ*FTT_0924* mutant membrane is permeable under hypotonic conditions. Since Δ*FTT_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 Δ*FTT_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 Δ*FTT_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 OD_{600} of 0.3) exhibited significant PI fluorescence (Fig. 6B). In contrast, the Δ*FTT_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 Δ*FTT_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 Δ*FTT_0924* mutant was permeable during replication under hypotonic conditions, we next tested whether the Δ*FTT_0924* cell membrane was also permeable in macrophages. J774 cells were infected with either wild-type Schu S4-GFP or the Δ*FTT_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 Δ*FTT_0924*-GFP mutant stained positive for PI, indicating that the Δ*FTT_0924* mutant exhibited a significant increase in membrane permeability in macrophages (Fig. 7). These data suggest that the observed viability defect of the Δ*FTT_0924* mutant is the reason for reduced intracellular growth in host cells and likely *in vivo*.

The observed membrane permeability of the Δ*FTT_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

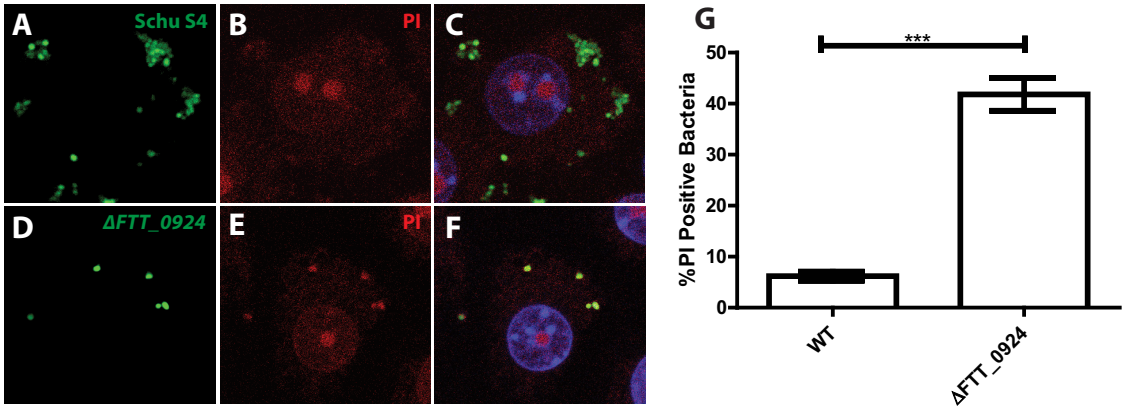

FIG 7 *FTT_0924* is required to maintain membrane integrity in host cells. (A to C) Representative Schu S4-GFP cells (green) (A), PI staining (red) (B), and merged (C) images, including DAPI staining (blue) of J774 macrophages infected with Schu S4-GFP at 16 h postinfection. (D to F) Representative Δ*FTT_0924*-GFP cells (green) (D), PI staining (red) (E), and merged (F) images, including DAPI staining (blue) of J774 macrophages infected with the Δ*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).

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 Δ*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 Δ*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 Δ*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 (β-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 bacteria replication.

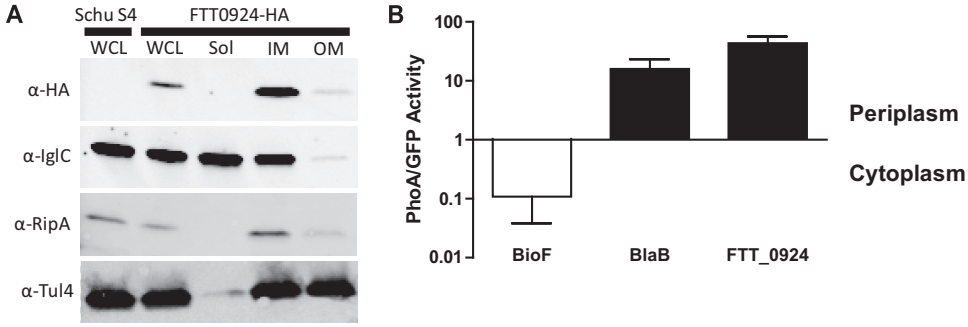

FIG 8 Localization of FTT_0924. (A) Inner membrane (IM), outer membrane (OM), soluble (Sol), and whole-cell lysate (WCL) samples were prepared via Sarkosyl extraction from Schu S4 and Δ*FTT_0924* p0924-HA cells (FTT_0924-HA). Proteins that reside in the cytosol (IglC), inner membrane (RipA), and outer 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, Δ*FTT_0924* mutant, and Δ*acrB* mutant strains to specific compounds

Antibiotic or reagent	Class	Inhibition zone mean diameter (mm) ± SD		
		Schu S4	Δ*FTT_0924* mutant	Δ*acrB* mutant
Nalidixic acid	Quinolone	32.7 ± 0.6	31 ± 1	33.7 ± 0.7
Ciprofloxacin	Fluoroquinolone	36.7 ± 0.7	37.7 ± 0.7	38.7 ± 0.7
Erythromycin	Macrolide	19 ± 0	20.3 ± 1.5	34.3 ± 1.2
Tetracycline	Tetracycline/polyketide	21 ± 0	21.3 ± 0.6	26 ± 0
Ethidium bromide	Intercalator	15.7 ± 0.6	12.3 ± 0.6	21 ± 0
SDS	Detergent	14 ± 0	17 ± 0	29.3 ± 0.6
Ceftriaxone	β-Lactam	6 ± 0	24.3 ± 2.3	6 ± 0
Amoxicillin + clavulanic acid	β-Lactam	6 ± 0	17.3 ± 2.5	6 ± 0
Kanamycin	Aminoglycoside	21.3 ± 0.6	26 ± 0	23 ± 0
Gentamicin	Aminoglycoside	26 ± 0	31.3 ± 0.6	26.3 ± 0.6
Rifampin	Rifamycin	22.3 ± 0.6	35.3 ± 0.6	26 ± 0

The Δ*FTT_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 Δ*FTT_0924* mutant would be sensitive to specific stresses affecting the cell wall and peptidoglycan. We therefore tested the sensitivity of the Δ*FTT_0924* mutant to specific antibiotics and other compounds via disc diffusion assays. Suspensions of wild-type Schu S4, the Δ*FTT_0924* mutant, or the Δ*acrB* strain, a *Francisella* mutant with a defect in the drug efflux system, were swabbed onto modified Mueller-Hinton agar plates, and paper discs containing each compound were placed on each plate. The diameters of the zones of inhibition were measured after 2 days of incubation at 37°C. The Δ*acrB* mutant displayed increased sensitivity to several hydrophobic drugs as previously described, whereas the Δ*FTT_0924* mutant displayed a different pattern of antibiotic sensitivities, indicating that the sensitivities of the Δ*FTT_0924* mutant do not affect the function of the known drug efflux mechanisms (Table 2) (29). Interestingly, the Δ*FTT_0924* strain displayed significantly increased sensitivity to β-lactam antibiotics compared to the wild type. It should be noted that the Δ*FTT_0924* mutant was also sensitive to macrolide antibiotics and rifampin, which may be a result of either damaged peptidoglycan or an indication that *FTT_0924* may play a role in other cellular processes. Overall, these data further demonstrate that *FTT_0924* is required to maintain peptidoglycan integrity and dynamics.

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 results 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 OD_{600} of the Δ*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. tularensis* (34). This observation correlates with the data demonstrating that the Δ*FTT_0924* mutant membrane was permeable to PI in J774 macrophages, suggesting that the intracellular growth and virulence defect of a Δ*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 Δ*FTT_0924* mutant is also hypersensitive to β-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 Δ*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. tularensis* 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. tularensis* modulates its peptidoglycan and elucidate how optimal peptidoglycan dynamics is required for intracellular growth and virulence. Identifying new genes required for *F. tularensis* 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. tularensis* infects and replicates within host cells and causes disease.

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