Supporting Information

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SI Methods and Materials

Mutagenesis and Complementation. To generate the kanamycin (kan)-marked *FTN_0818* deletion mutant, the regions of the chromosome 5′ and 3′ to *FTN_0818* were amplified by PCR, using primers found in Table S2. A kan-resistance cassette flanked by flippase recognition target (Flp-FRT) recombinase sites was introduced between these flanking regions using overlapping PCR. The sewn PCR construct was gel purified (Qiagen) and chemically transformed into competent U112 as described previously (1).

To create the *FTN_0818* clean deletion mutant, plasmid pLG72 encoding the flippase gene was transformed into the kan-marked *FTN_0818* mutant and clones in which the kan cassette had been deleted were isolated as previously described (1). To complement the deletion, constructs were made using overlapping PCR by amplification of the 5′ and 3′ regions, the WT gene, and a kan-cassette for selection and this construct was transformed into the *FTN_0818* clean deletion mutant. All strains were verified by PCR and sequencing (Eurofins EWG Operon).

The serine to alanine *FTN0818* point mutant (*FTN0818*-S151A) was constructed by overlapping PCR using a primer encoding a single amino acid change and using a chloramphenicol cassette for selection. This construct was transformed into the *FTN0818* kan-resistant deletion mutant and selected for on chloramphenicol plates chloramphenicol (3 μg/mL).

To generate a mutant lacking the *FTN_0818* ortholog, *FTL_1266*, in the *F. holarctica* LVS, we used a group II intron as previously described (1). We constructed primers targeting *FTL_1266* using the TargeTron Gene Knockout System (Sigma-Aldrich), and the PCR product was cloned into the *Francisella* targeting vector, pKEK1140, a generous gift from Karl Klose (University of Texas, San Antonio, TX). LVS was then transformed with the targeting vector and *FTN_1266* deletion clones (Δ*FTL_1266*) were isolated. All cloning primers are listed in Table S2.

WT *F. tularensis* (SchuS4) was obtained from the Centers for Disease Control and Prevention. SchuS4 was cultured in modified Mueller Hinton broth [MMH broth supplemented with 10 g/L tryptone, 0.1% glucose, 0.025% ferrous pyrophosphate, 0.1% L-cysteine, and 2.5% (vol/vol) calf serum] or in brain heart infusion (BHI) broth (BHI supplemented with 50 μg/mL hemin, 1.4% agar (wt/vol), and 1% (vol/vol) IsoVitalex (BBL). Counter selection for SchuS4-p*ΔFTT_0941* cointegrants was performed on cysteine heart agar (CHA) containing 5% sucrose, plus kanamycin at 10 μg/mL when necessary.

The Δ*FTT_0941* construct was created by PCR overlap extension using primers listed in Table S2. The PCR product was then ligated into plasmid p*ΔFTT_0941* was introduced by electroporation into electrocompetent SchuS4. Electrocompetent cells were prepared using an overnight MMH broth culture of SchuS4 to inoculate 50 mL of fresh MMH broth. The culture was then incubated, shaking at 37 °C until reaching an OD_{600} of 0.3, transferred to a 50-mL conical tube, pelleted at 4 °C, resuspended in 4 mL of prechilled 0.5 M sucrose, divided into 2 × 2 mL screw-capped vials, and centrifuged at 2,000 × *g* at 4 °C. The cells were then washed (×3) with 2 mL of prechilled 0.5 M sucrose. Next, the washed pellet was resuspended into 70 μL of 0.5 M sucrose and placed on ice, and 70 μL of the purified pFTT_0941 was added. The mixture was transferred to a prechilled 0.1-cm electroporation cuvette (BTX) and electroporated (1.5 kV, 25 μF, and 200 ohms). Immediately after, 1 mL of MMH broth was added, transferred to a 50-mL conical tube, and the cells were incubated, shaking at 37 °C for 2 h. Cells were collected by centrifugation, resuspended in 500 μL of MMH broth, and spread onto BHI agar plates containing 10 μg/mL kanamycin. After 3 d of incubation at 37 °C, colonies were patched onto fresh BHI agar plates, grown overnight, and used to inoculate 1 mL of MMH broth without antibiotics. The resulting broth culture was incubated with shaking at 37 °C until early log phase and spread onto CHA–5% sucrose plates. Sucrose-resistant colonies (4–5 d later) were then patched onto fresh CHA–sucrose plates and the plates incubated at 37 °C. The resulting colonies were then replica-plated onto BHI agar plates in the presence and absence of kanamycin. The resulting clones were restreaked onto BHI agar, and a single colony was selected, cultured, and screened by PCR for the *FTT_0941* deletion.

Infection stocks of *F. tularensis* SchuS4 were prepared by pelleting bacterial cells from midlog phase cultures via centrifugation, resuspending cells in fresh MMH with 20% glycerol, and cryogenically storing 100–200 μL aliquots at −80 °C. Infection stocks were enumerated by dilution plating several times over a span of 3 mo to ensure stability of the cryogenically preserved bacteria. To initiate infections, the frozen stocks were thawed and diluted to the desired concentration in sterile PBS.

Macrophages. Bone marrow–derived macrophages (mBMDMs) were prepared as described previously (1). Briefly, bone marrow was collected from the femurs of mice. Bone marrow cells were plated into sterile Petri dishes and incubated in DMEM supplemented with 10% heat-inactivated FBS and 10% macrophage colony-stimulating factor (M-CSF)–conditioned medium (collected from M-CSF–producing NIH 3T3 cells). Bone marrow cells were incubated at 37 °C with 5% CO_2 and harvested after 6 d. All mBMDMs were incubated before and during infection in 24-well plates at 37 °C with 5% CO_2.

Growth Curves. Bacteria were subcultured to an OD_{600} of 0.03 in TSB/0.2% cysteine or CHB (Teknova) (2) for *F. novicida*, whereas LVS cultures were grown in modified Mueller–Hinton (MH) broth described previously (1). Subcultures were read hourly using a SynergyMX BioTek plate reader (Applied Biosystems). Biotin (0.25 μM) (Merck) or pimelate (3 μg/mL) (Sigma-Aldrich) was added when appropriate.

Transmission Electron Microscopy. Infected mBMDMs were fixed with 1 mL of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) overnight at 4 °C. Cells were then postfixed in 1% buffered osmium tetroxide, dehydrated, and embedded in Eponate 12 resin (Ted Pella). Ultrathin sections were cut on a UC6rt ultramicrotome (Leica Microsystems) at 70–80 nm and counterstained with 4% aqueous uranyl acetate and 2% lead citrate. Sections were examined using a Hitachi H-7500 transmission electron microscope (Hitachi High Technologies of America) equipped with a Gatan BioScan CCD camera. Multiple fields were examined for a total of 100 bacteria per time point. The criterion for being considered within the phagosome was visualization of a phagosomal membrane that was at least 90% intact surrounding a bacterium.

Nutrient Supplementation Phenotypic Microarray. Metabolic profiling was performed according to modified Biolog guidelines (http://www.biolog.com). An 85% transmittance cell culture was made by using a damp cotton swab to transfer colonies from tryptic soy agar (TSA)/0.1% cysteine into 15 mL of modified CHB (Table S1) and measuring in a turbidimeter.

Inoculating media were prepared by mixing 50 mL of 1.2× mCHB (pH 5.5) with 360 μL of dye (Biolog; Dye Mix A, catalog no. 74221) and 360 μL of the 85% transmittance cells. Sterile water was added for a final volume of 60 mL. Phenotypic Microarray plate 5 (PM5) (Biolog; catalog no. 12141) was inoculated with 100 μL of the inoculating media per well. The plate was incubated in a Biolog Omnilog Phenotypic Microarray incubator for 48 h at 37 °C.

Immunoprecipitation. Bacteria were subcultured to an OD600 of 0.03 in TSB/0.2% cysteine or CHB and grown for 4 hr shaking at 37 °C, pelleted and resuspended in PBS. Bacteria were then freeze-thawed 3 times, centrifuged to discard unlysed bacteria, and the whole cell lysate (WCL) was applied to Protein G sepharose beads (Invitrogen, Frederick, MD) conjugated to anti-biotin antibody (Invitrogen). Eluted proteins were then quantified using the BCA protein assay (Thermo Fisher Scientific, Rockford, IL).

Immunofluorescence Microscopy. For immunofluorescence, mBMDMs were infected at an MOI of 100:1 and fixed at specified time points (3). Staining, imaging, and quantification of phagosomal escape were performed as previously described (3).

1. Llewellyn AC, Jones CL, Napier BA, Bina JE, Weiss DS (2011) Macrophage replication screen identifies a novel Francisella hydroperoxide resistance protein involved in virulence. *PLoS ONE* 6(9):e24201.
2. Chamberlain RE (1965) Evaluation of live tularemia vaccine prepared in a chemically defined medium. *Appl Microbiol* 13:232–235.
3. Jones CL, Weiss DS (2011) TLR2 signaling contributes to rapid inflammasome activation during F. novicida infection. *PLoS ONE* 6(6):e20609.

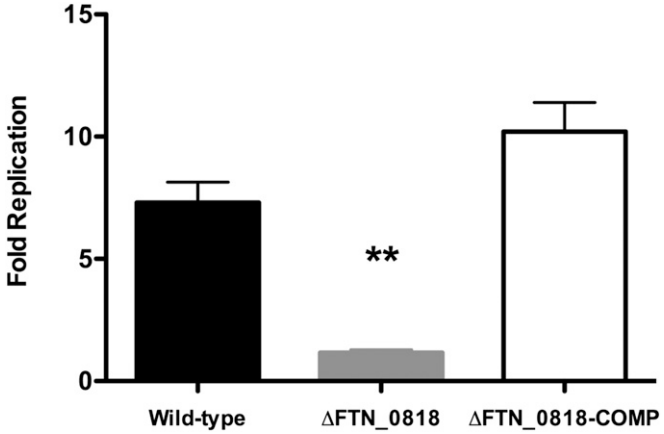


Fig. S1. FTN_0818 is required for replication in macrophages. Macrophages were infected with WT, *ΔFTN_0818*, and the *FTN_0818* complemented (ΔFTN_0818-COMP) *F. novicida* strains. Colony-forming units were quantified at 30 min and 7.5 h pi, and fold replication was calculated. $^{**}P < 0.001$.

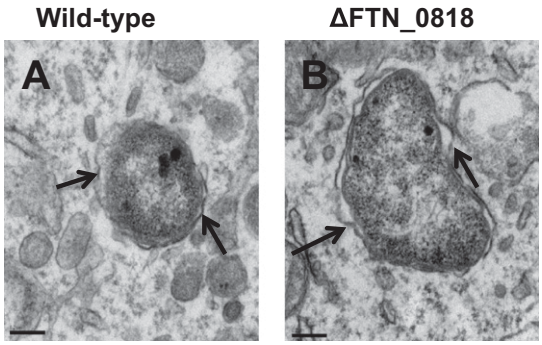


Fig. S2. *ΔFTN_0818* and WT *F. novicida* localize to the phagosome at 30 min pi. Macrophages were infected with WT (*A*) or *ΔFTN_0818* (*B*) *F. novicida*, and samples were fixed at 30 min pi for transmission electron microscopy (arrows, intact FCP).

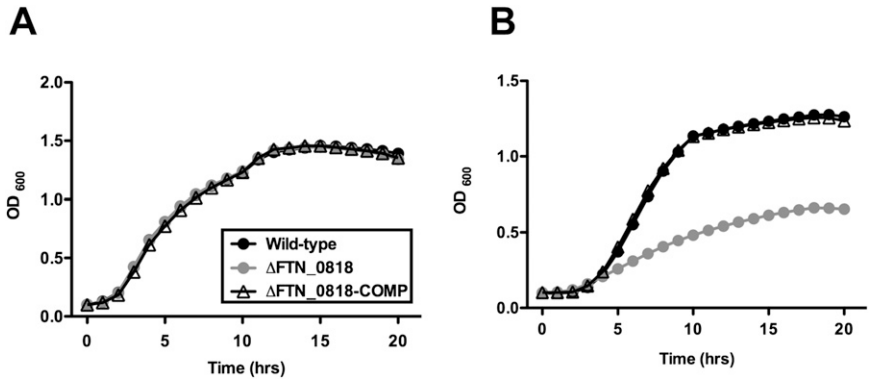
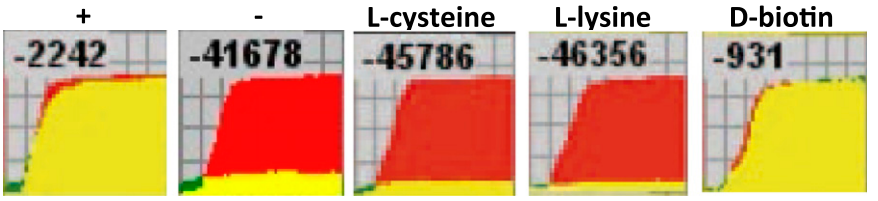


Fig. S3. FTN_0818 is required for replication in minimal media. *F. novicida* strains were grown overnight in TSB and subcultured into TSB (*A*) or CHB (*B*), and the OD_{600} was measured every hour (black circles, WT; gray circles, *ΔFTN_0818*; open triangles, ΔFTN_0818-COMP).



Test Compound	Area Wild-type	Area ΔFTN_0818	Area Difference (Wild-type - ΔFTN_0818)	Ratio (ΔFTN_0818/U112)
Positive Control	55672	53430	2242	0.9597
Negative Control	53454	11776	41678	0.2203
L-cysteine	53793	6344	45786	0.1179
L-lysine	49937	3581	46356	0.0717
D-biotin	50179	49248	931	0.9814

Fig. S4. Biotin can complement *ΔFTN_0818* growth in minimal media. WT or *ΔFTN_0818 F. novicida* strains were subcultured into modified CHB, added to Biolog Phenotypic Microarray plate 5 (PM5) and incubated for 48 h at 37 °C (WT growth, red; *ΔFTN_0818* growth, green; overlapping growth, yellow). Areas under the curve and ratio of WT/*ΔFTN_0818* growth are shown for the Biolog-positive and -negative controls, L-cysteine, L-lysine, and D-biotin. Growth to at least 80% of WT levels was used as a cutoff for full complementation.

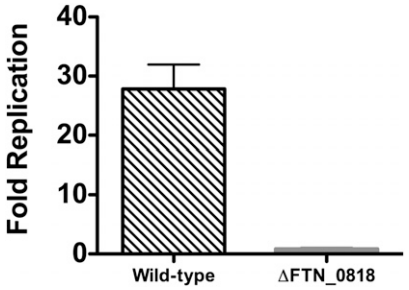


Fig. S5. *ΔFTN_0818* growth defect in macrophages is not rescued by pretreatment with biotin. WT and *ΔFTN_0818 F. novicida* strains were grown overnight in CHB supplemented with biotin and used to infect macrophages in the absence of biotin at a 10:1 (bacteria:macrophage) ratio. Intracellular colony-forming units were enumerated at 30 min and 5 h pi, and fold replication was calculated.

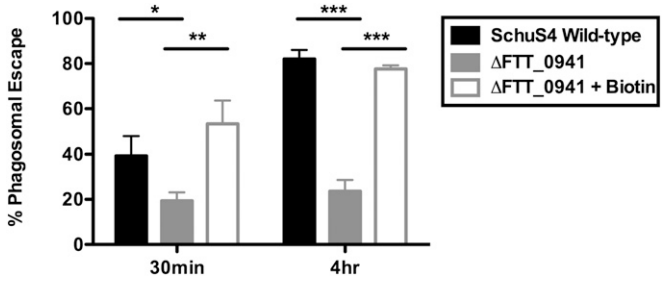


Fig. S6. FTT_0941, the FTN_0818 ortholog in *F. tularensis* SchuS4, is required for rapid FCP escape. Quantification of FCP escape at 30 min and 4 h pi in macrophages infected with WT *F. tularensis* SchuS4, *FTT_0941*, or *FTT_0941* supplemented with biotin (t = 0), by immunofluorescence microscopy. Two hundred bacteria were counted per sample, and colocalization of DAPI with LAMP-1 was used as a marker for phagosomal localization. *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$.

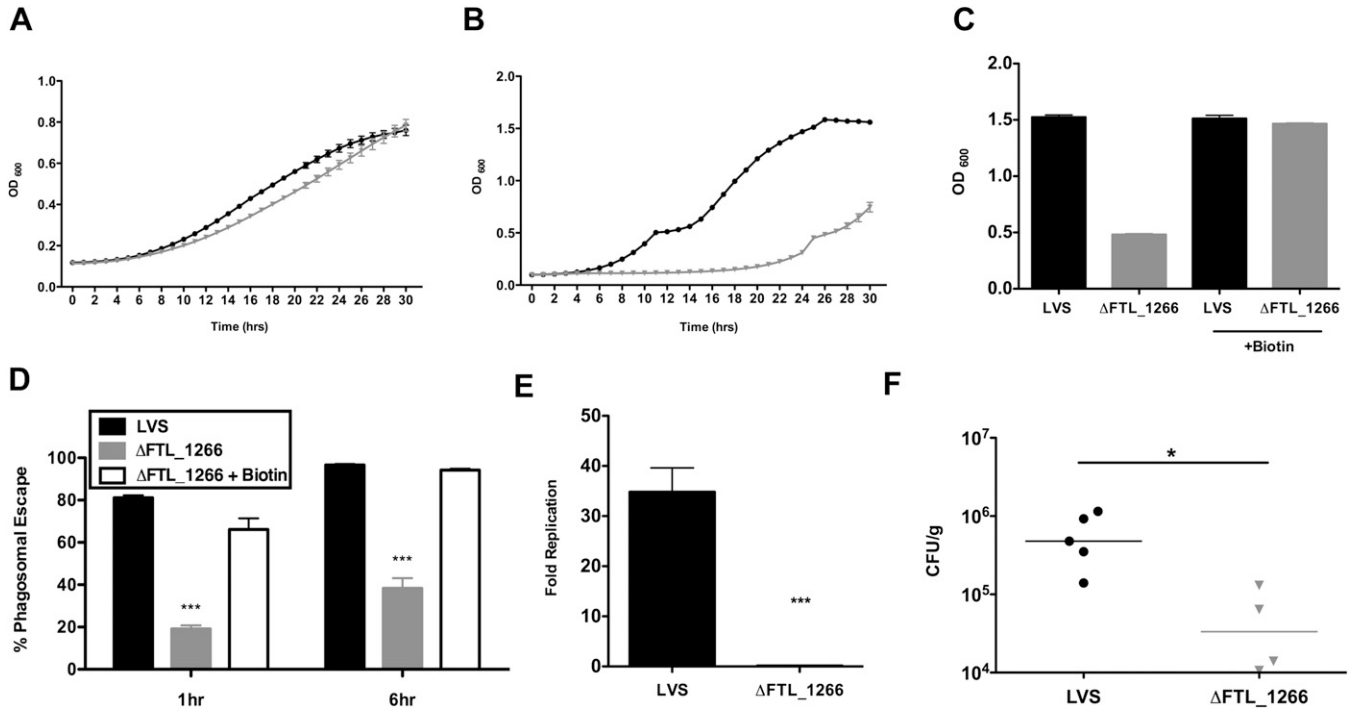


Fig. S7. FTL_1266, the LVS FTN_0818 ortholog, is required for escape from the FCP and in vitro and in vivo replication. WT (black circles) and *ΔFTL_1266* (gray triangles) LVS strains were grown in TSB (*A*) or CHB (*B*) for 30 h, and the OD_{600} was measured every hour. (*C*) WT and *ΔFTL_1266* LVS strains were grown for 30 h in CHB supplemented with and without biotin. (*D*) FCP escape was quantified at 1 and 6 h pi in macrophages infected with WT LVS, *ΔFTL_1266*, or *ΔFTL_1266* supplemented with biotin (t = 0), using immunofluorescence microscopy. Two hundred bacteria were counted per sample, and colocalization of DAPI with LAMP-1 was used as a marker for phagosomal localization. ***$P < 0.0001$. (*E*) Macrophages were infected with WT and *ΔFTL_1266* LVS strains, colony-forming units were enumerated at 30 min and 24 h pi, and fold replication calculated. (*F*) Mice were infected s.c. with 10^6 cfu of WT or the *ΔFTL_1266* LVS strains. At 48 h pi, spleens were harvested, and colony-forming units per gram was quantified. *$P < 0.05$.

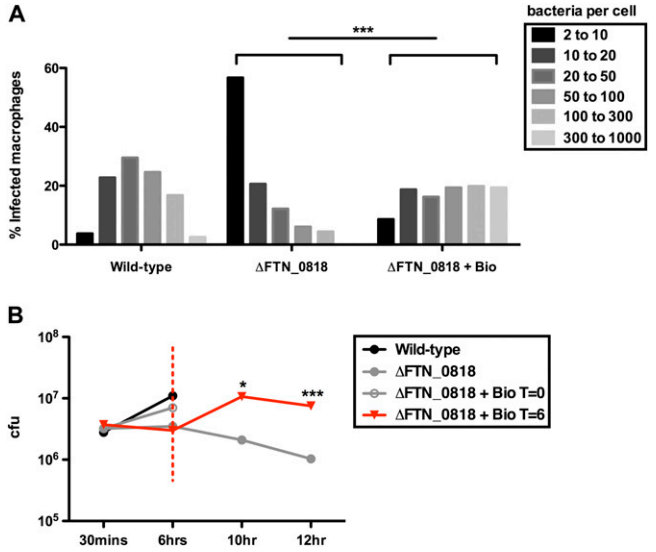


Fig. S8. FTN_0818 is required for *F. novicida* replication in the cytosol. (*A*) J774 cells were injected with GFP-expressing WT or the Δ*FTN_0818* mutant and infection followed for 24 h. Pictures were taken at 24 h with a live-cell imaging microscope. The number of bacteria/cell was determined by microscopic counting and categorized as indicated. The total number of infected cells for each different strain was the basis for the statistical comparisons. The non-parametric Wilcoxon rank-sum test was used to determine Δ*FTN_0818* without biotin compared to Δ*FTN_0818* with biotin was significantly different ($P < 2.2^{-16}$). (*B*) Macrophages were infected with WT, Δ*FTN_0818*, or Δ*FTN_0818* supplemented with biotin at time 0 (Bio T = 0) or at 6 h (Bio T = 6; indicated by the red dashed line). Intracellular colony-forming units were quantified for all strains/conditions at 30 min and 6 h pi. Additional time points were taken at 10 and 12 h pi for Δ*FTN_0818* and Δ*FTN_0818* + Bio T = 6. **P* < 0.05; ****P* < 0.0001.

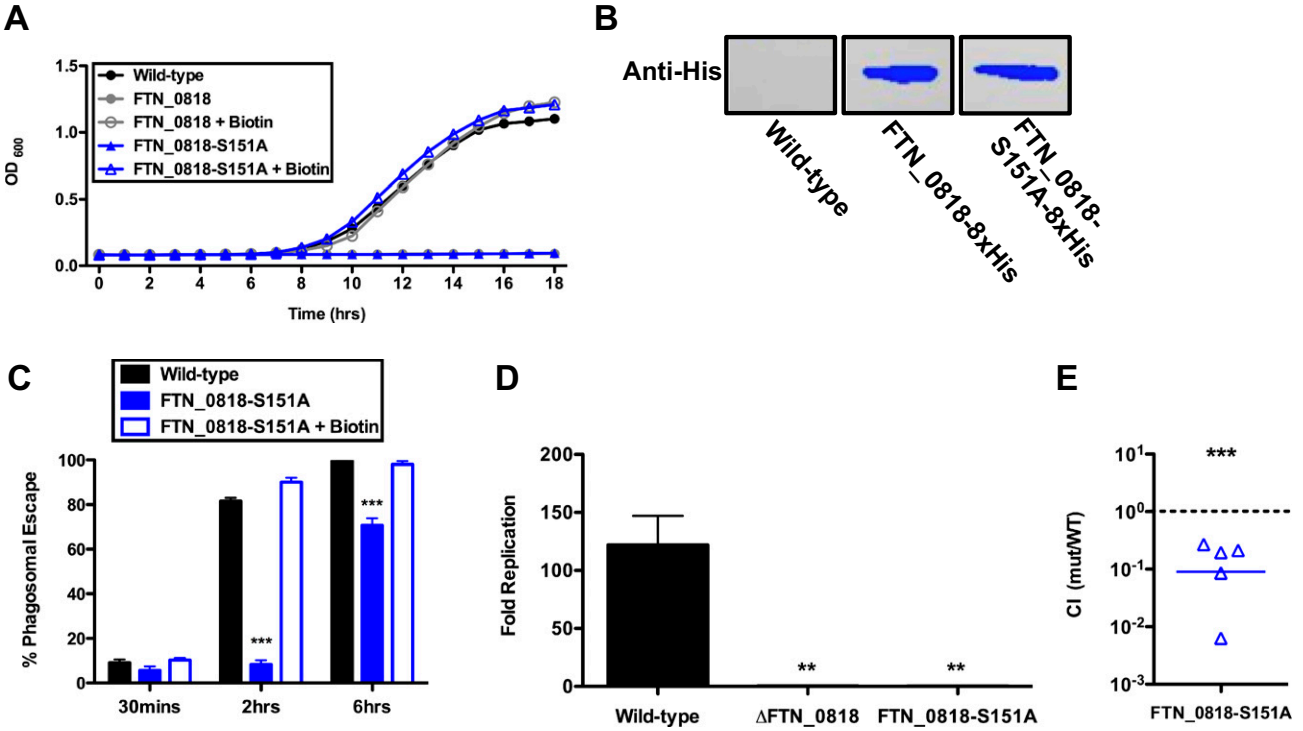


Fig. S9. Putative catalytic serine residue (S151) is required for FTN_0818 function. (*A*) All strains were grown in CHB medium for 18 h, and the OD_{600} was measured every hour. (*B*) WT, His-tagged *FTN_0818* (FTN_0818-8xHis), and His-tagged *FTN_0818-S151A* (FTN_0818-S151A-8xHis) were grown in TSB and whole cell lysates (WCL) were resolved by SDS/PAGE and visualized with anti-His antibody. (*C*) FCP escape was quantified at 30 min, 2 h, and 6 h pi in macrophages infected with WT, *FTN_0818-S151A*, or *FTN_0818-S151A* supplemented with biotin. Two hundred bacteria were counted per sample, and colocalization of *F. novicida* with LAMP-1 was used as a marker for phagosomal localization. (*D*) Macrophages were infected with the indicated strains, colony-forming units were quantified 6 h pi, and fold replication was calculated. (*E*) Mice were infected s.c. with a 1:1 mixture of WT with the *FTN_0818-S151A* strain. At 48 h pi, spleens were harvested to quantify bacterial levels, and the CI was calculated. ***P* < 0.001; ****P* < 0.0001.

Table S1. Modified CHB (mCHB)

	CHB, g/L	mCHB, g/L
KH_2PO_4	1	4.096
H_2HPO_4	1	1.707
NaCl	10	10
$MgSO_4*7H_2O$	0.135	0.135
$FeSO_4*7H_2O$	0.002	0.002
Ca-pantothenate	0.002	0.002
Thiamine-HCl	0.004	0.004
Spermine-PO_4	0.04	0.04
Ammonium sulfate		2
Glucose	4	2
Pyruvate		2
His	0.2	0.155
Arg	0.4	0.174
Met	0.4	0.149
Cys	1.2	1.158
Lys	0.4	0.183
Pro	2	0.115
Leu	0.4	0.131
Ile	0.4	0.131
Val	0.4	0.117
Thr	2	0.119
Tyr	0.4	0.181
Asp	0.4	0.133
Glu		0.147
Phe		0.165
Gln		0.146
Asn		0.132
Ala		0.089
Gly		0.075
Trp		0.204
Ser	0.4	0.105
pH	6.2–6.4	5.5

Table S2. Primers used in this study

Primer name	Primer
FTN_0818-Kan insertion mutant	
FTN0818 F1	aaccattagtcagtattgac
FTN0818 R1	accactaaatagtacacaac
FTN0818 arm1-sKAN F	ggagtcatatatcaaaccaaatctctttgggttgtcact
FTN0818 arm1-sKAN R	ggagtcatatatcaaaccaaatctctttgggttgtcact
FTN0818 sKAN-arm2 F	cagaattggttaattggttgttccttattaaatcttgtgtac
FTN0818 sKAN-arm2 R	gtacacaagatttaataaggaacaaccaattaaccaattctg
FTN0818 check F	aaacatcagaggtattggag
FTN0818 check R	attgatatcggagctgaatc
FTN_0818 complement	
818Comp arm1 F	agaattggttaattggttgtatattggtttgatatatgact
818Comp arm1 R	agtcatatatcaaaccaatatacaaccaattaaccaattct
818Comp sKan F	attgagtttataaacaaaagataaatctctttgggttgtcact
818Comp sKan R	agtgacaacccaaagagatttatcttttgtttataaactcaat
FTN_0818 clean deletion	
arm1-FRTsKAN F	ggagtcatatatcaaaccaagaggtcgacggtatcgataa
arm1-FRTsKAN R	ttatcgataccgtcgacctcttggtttgatatatgactcc
FRTsKAN-arm2 F	tatcgatcctgcagctatgcttccttattaaatcttgtgtac
FRTsKAN-arm2 R	gtacacaagatttaataaggaagcatagctgcaggatcgata
FTN_0818 S151A	
FTN_0818 S151A arm1-sCAT F	ggagtcatatatcaaaccaattacgccccgccctgccac
FTN_0818 S151A arm1-sCAT R	gtggcagggcggggcgtaattggtttgatatatgactcc
FTN_0818 S151A sCAT F	aaatacgatgagtgacaacctatttatcttttgtttataaactc
FTN_0818 S151A sCAT R	gagtttataaacaaaagataaataggttgtcactcatcgtattt
FTN_0818 S151A mut F	tccagcagcatcacccatt
FTN_0818 S151A mut R	aatgggtgatgctgctgga
FTN_0818 S151A check R2	tttagatcgagctacaagag
FTN_0818 S151A check R3	tatcttctcgctagaatatg
LVS FTL_1266 disruption	
FTL1266 r1	attgatatcggagctgaatc
FTL1266 f1	gcaccaccaaatccagtacc
FTL_1266 check F1	atttctcaaatagagtcagc
IBS-1	aaaaaagcttataattatccttataggtccgataagtgcgcccagataggtg
EBS1d-1	cagattgtacaaatgtggtgataacagataagtccgataaattaacttacctttctttgt
EBS2-1	tgaacgcaagtttctaatttcggttacctatcgatagaggaaagtgtct
qRT-PCR	
FTN_0818	
FTN_0818 real-time F	acagcaaggaacttatgttga
FTN_0818 real-time R	ccaaggatgcccatgaaaccat
uvrD qRT-PCR	
uvrD real-time F	gggatgtcgccttttgattttc
uvrD real-time R	ctcttttgtcccttgtgcttgc
iglA qRT-PCR	
iglA real-time F	cgccaacaactaggactctg
iglA real-time R	tcccaaattcaaggttgatg
F. tularensis SchuS4 FTT_0941 deletion	
FTT_0941 F2	agggatccatatgtgagtcttggttgctcaggag
FTT_0941 R1-SacI	gcattattagaccctaaaatagctttgaataaggtgattgag
FTT_0941-F1-BamHI	aggagctcaggtattggagcagttatcgcagcag
FTT_0941-R2	aagctattttagggtctaataatgcttctaatgatggattgtatgg

F, forward; mut, mutant; R, reverse.