A *Francisella tularensis* Locus Required for Spermine Responsiveness Is Necessary for Virulence[▽]

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Tularemia is a debilitating febrile illness caused by the category A biodefense agent *Francisella tularensis*. This pathogen infects over 250 different hosts, has a low infectious dose, and causes high morbidity and mortality. Our understanding of the mechanisms by which *F. tularensis* senses and adapts to host environments is incomplete. Polyamines, including spermine, regulate the interactions of *F. tularensis* with host cells. However, it is not known whether responsiveness to polyamines is necessary for the virulence of the organism. Through transposon mutagenesis of *F. tularensis* subsp. *holarctica* live vaccine strain (LVS), we identified FTL_0883 as a gene important for spermine responsiveness. In-frame deletion mutants of FTL_0883 and FTT_0615c, the homologue of FTL_0883 in *F. tularensis* subsp. *tularensis* Schu S4 (Schu S4), elicited higher levels of cytokines from human and murine macrophages compared to wild-type strains. Although deletion of FTL_0883 attenuated LVS replication within macrophages *in vitro*, the Schu S4 mutant with a deletion in FTT_0615c replicated similarly to wild-type Schu S4. Nevertheless, both the LVS and the Schu S4 mutants were significantly attenuated *in vivo*. Growth and dissemination of the Schu S4 mutant was severely reduced in the murine model of pneumonic tularemia. This attenuation depended on host responses to elevated levels of proinflammatory cytokines. These data associate responsiveness to polyamines with tularemia pathogenesis and define FTL_0883/FTT_0615c as an *F. tularensis* gene important for virulence and evasion of the host immune response.

Francisella tularensis, the causative agent of tularemia, is a small, nonmotile, Gram-negative bacterium (40). The Centers for Disease Control and Prevention classified *F. tularensis* as a category A biodefense agent because of the severity of tularemia and the ease of preparation and dissemination in an intentional release. There are two clinically relevant subspecies of *F. tularensis*: *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B) (49). Type A *Francisella* is capable of infecting a diverse host range and is highly infectious; as few as 10 bacteria can cause fatal disease in humans if left untreated (15, 40). Type B *Francisella* results in a milder disease in humans and is rarely fatal (49). The live vaccine strain (LVS) was generated from a type B isolate, which has become an important model for *F. tularensis* pathogenesis and biology (49).

F. tularensis is a facultative intracellular pathogen, and mutations preventing invasion and intracellular replication typically result in severely attenuated strains (2, 10, 51, 52). Once within the host, *F. tularensis* infects a variety of cell types, including phagocytes, neutrophils, alveolar epithelial cells, hepatocytes, and fibroblasts (1, 6, 12, 25, 38). Phagocytes, par-

ticularly macrophages, are thought to be an important replicative niche for *F. tularensis* *in vivo*. *F. tularensis* is detectable within host macrophages and dendritic cells within 1 h after infection (4, 14, 25). The proinflammatory response within these cells is blocked by *F. tularensis*, which prevents the production of cytokines and chemokines (4, 6, 59). Reduced levels of cytokines early after infection have been correlated with an inability of the host to control the replication of the bacterium (18, 36). The mechanism(s) by which *F. tularensis* manipulates the host immune response is poorly understood. In order to understand how the bacterium interacts with the host, it is critical to define the mechanisms of host immune evasion.

Successful evasion of the host immune response may be partly due to an adaptation of *F. tularensis* to the intracellular environment (7, 34). Loegering et al. demonstrated that after replication within macrophages *F. tularensis* is less stimulatory in subsequent macrophage infections compared to *F. tularensis* cultured in bacterial growth media (34). These authors concluded that *F. tularensis* is able to adapt to the host environment to evade the immune response. However, the mechanism(s) enabling *F. tularensis* to sense and adapt to the host environment must be delineated. Among possible signals, polyamines, temperature, and amino acid concentration are known to be important environmental cues that alter bacterial virulence (7, 26, 28).

Polyamines are small polycationic molecules and are found ubiquitously in the cytosol of both prokaryotes and eukaryotes at millimolar concentrations (64). These compounds have im-

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portant roles in a variety of biological processes such as regulating transcription and translation, altering enzyme activity, and binding to DNA to neutralize its negative charge (58). For *F. tularensis*, polyamines signal the bacterium when it is within the host cytosol (7). Macrophages infected with *F. tularensis* previously cultured in the presence of polyamines, including spermine, produce significantly less proinflammatory cytokines than macrophages infected with *F. tularensis* previously cultured in medium alone (7). The *F. tularensis* proteins required to respond to spermine and adapt to the intracellular environment remain largely unknown. Moreover, the importance of this response remains undefined.

To understand the contribution of the spermine response to pathogenesis, a screen was developed in LVS to identify mutants unable to respond to extracellular spermine. We identified a gene in *F. tularensis* LVS, FTL_0883, which is necessary for spermine responsiveness. Mutants in FTL_0883 or its homolog in the type A strain Schu S4, FTT_0615c, elicit increased levels of cytokines from macrophages, and are attenuated *in vivo*. Our results indicate that FTL_0883 and FTT_0615c are required for *F. tularensis* evasion of host defenses and virulence.

MATERIALS AND METHODS

Bacterial strains. *F. tularensis* subsp. *holarctica* LVS (a gift from Karen Elkins) and *F. tularensis* subsp. *tularensis* Schu S4 (obtained through the National Institutes of Health [NIH] Biodefense and Emerging Infections Research Resources Repository, National Institute of Allergy and Infectious Disease [NIAID]; strain FSC237, catalog number NR-643) were streaked onto chocolate II agar plates and cultured between 1 and 3 days at 37°C with 5% CO₂. Liquid cultures of *F. tularensis* were grown at 37°C at 250 rpm in either Trypticase soy broth supplemented with cysteine (TSB-C), Mueller-Hinton broth (MHB) supplemented with 0.1% glucose, 0.025% ferric pyrophosphate, and IsoVital X, or Chamberlain's defined media (CDM) (8). *Escherichia coli* strain EC100D was used for routine cloning and was cultured in Luria broth or on Luria agar. When antibiotics were required, kanamycin was used at 10 µg/ml for *Francisella* and at 35 µg/ml for *E. coli*, and hygromycin was used at 200 µg/ml for LVS and *E. coli* and at 400 µg/ml was used for Schu S4. All work with Schu S4 and strains generated from Schu S4 was performed in BSL3 containment at the University of Pittsburgh with approval from the Centers for Disease Control and Prevention Select Agent Program.

Transposon screen for spermine response genes. To identify genes involved in responsiveness to spermine, a genetic screen was conducted. Plasmid pSD26 (a gift from Eric Rubin and Simon Dillon) was electroporated into LVS as previously described (28, 30). pSD26 encodes a C9 transposase and a Himar1 transposon with a kanamycin resistance marker driven by the *F. tularensis* *groE* promoter (53). After recovery in TSB-C, the bacteria were plated on cysteine heart broth with 5% defibrinated rabbit blood containing kanamycin. A total of 5,000 colonies were screened first for an inability to utilize extracellular spermine by failing to grow in CDM in the presence of a spermidine synthesis inhibitor, dicyclohexylamine (3, 9, 27, 37, 43). A positive result in the primary screen was determined by an optical density at 600 nm (OD₆₀₀) of <0.06 at 48 h. To confirm that mutants from the primary screen were not CDM auxotrophs, the positive mutants were cultured in CDM without dicyclohexylamine. Mutants with an OD₆₀₀ of <0.06 were considered auxotrophic and removed from the pool of positive mutants. A secondary screen tested the production of tumor necrosis factor alpha (TNF-α) from host cells. Mutants were cultured in MHB alone or MHB supplemented with 200 µM spermine. Human macrophages were infected as described below, and the production of TNF-α was measured by using an enzyme-linked immunosorbent assay (ELISA).

Cloning and generation of in-frame deletion mutants. In-frame deletions of FTL_0883 and FTT_0615c were performed using allelic replacement as described previously (29, 30). The site 1 kb upstream and downstream of each gene was amplified by PCR using two primer pairs, primer 1 (GATCGCATGCAAC TAGGTGATGCTTATTATATACTCC) and primer 2 (GATCCTGCAGTTAA ATTTAATTTTAGTCGAAAAATTAAGAAATAATGAAGT) for the upstream region and primer 3 (GATCCTGCAGTTTATAAAAGGATTATTAT

CTGCCATTTTG) and primer 4 (GATCCCCGGGGCAGCTTATGAGAAAG GCG) for the downstream region. The 1-kb amplicons were cloned into pJH1 individually and then subcloned adjacent to each other into a single pJH1 vector to generate pJH1-ΔFTL_0883 and pJH1-ΔFTT_0615c. Because the genomic sequences of FTL_0883 and FTT_0615c loci and surrounding DNA are 99% similar based on sequence alignment on BLAST, we were able to use the same primers for Schu S4 and LVS for generating the deletion constructs. Confirmation of the deletion of either FTL_0883 (ΔFTL_0883) or FTT_0615c (ΔFTT_0615c) was performed by sequencing from amplicons from PCRs using genomic DNA as the template (Agencourt).

A *cis*-complementing construct was generated utilizing 1 kb upstream of either FTL_0883 or FTT_0615c as the native promoter and to enable the vector to integrate into the genome. Amplicons were generated using primer 4 (described above) and primer 5 (CATGCTGCAGTTATTTCTTAAATTTTTTCGACTAA AATTTTAATAATTTTTC) to include the entire open reading frame. The PCR amplicon was digested with PstI and XmaI and ligated into pJH1 that had been digested with these same enzymes to generate pJH1-FTL_0883 or pJH1-FTT_0615c. To generate a vector control, 1 kb of DNA upstream of FTL_0883 or FTT_0615c was amplified using primers 3 and 4. This amplicon and pJH1 were digested with PstI and XmaI and ligated together to generate pJH1-vector. The *E. coli* strains harboring the *cis*-complementing or vector controls were mobilized to *F. tularensis* ΔFTL_0883 and ΔFTT_0615c by conjugation as performed previously (30). This generated the complementing (ΔFTL_0883/FTL_0883, "complement") and empty vector ("vector") strains in LVS and the complementing (ΔFTT_0615c/FTT_0615c, "complement") and empty vector ("vector") strains in Schu S4.

Determination of growth rates. LVS or ΔFTL_0883 were cultured overnight in either MHB or TSB-C. The cultures were diluted to an OD₆₀₀ of <0.1 in the same media as that used for the overnight culture. A growth curve was generated by measuring the OD₆₀₀ of the culture over a 24-h period using a M2 plate reader (Molecular Devices). The growth rates were calculated using measurements during the exponential phase of growth.

Generation of bone marrow-derived macrophages (BMDM). Bone marrow was obtained from healthy C57BL/6J mice (Jackson Laboratory) that were 6 to 8 weeks old. The femurs and tibias were flushed with 10 ml of cold BMDM medium (Dulbecco modified Eagle medium [DMEM] supplemented with 25 mM HEPES, 25% L-cell medium, 1% sodium pyruvate, 10% fetal bovine serum [FBS], 1% GlutaMAX, 1% nonessential amino acids). The cells were counted and seeded into 100-mm petri dishes at 7 × 10⁶ cells in 10 ml of BMDM medium. Bone marrow cells were allowed to differentiate into macrophages for 7 days. The macrophages were used between 7 and 21 days of culture. The use of bone marrow was approved by the University of Pittsburgh's Department of Environmental Health and Safety and followed the protocol for tissue transfer of the Institutional Animal Care and Use Committee.

Generation of human monocyte-derived macrophages. Human macrophages were differentiated from human mononuclear cells as described previously (6). Peripheral blood mononuclear cells were isolated from buffy coats of human blood donations (Central Blood Bank, Pittsburgh, PA) using Ficoll gradients (GE Healthcare), and then monocytes were isolated using OptiPrep gradients (Sigma). Finally, monocytes were further purified by panning. The resulting monocyte population is >95% pure (6). Purified monocytes were then differentiated into macrophages in DMEM supplemented with 20% FBS, 10% human sera AB (Complement-Replete Gem Cell; Gemini Bio-Products), 25 mM HEPES, and 1% GlutaMAX. The macrophages were cultured for 7 days and used on day 9. All use of human cells was approved by the Institutional Review Board of the University of Pittsburgh.

Infection of macrophages. Macrophages were removed from culture dishes with phosphate-buffered saline containing 8.5 mM lidocaine and 5 mM EDTA, resuspended in infection media (1% human sera AB, 25 mM HEPES, and 1% GlutaMAX in DMEM), seeded into the wells of a 96-well Primaria-coated culture dishes (BD Biosciences) at 5 × 10⁴ cells/well, and infected with bacteria grown in MHB or TSB-C. The bacterial cultures were standardized to an OD₆₀₀ of 0.3, and the actual bacteria concentrations were determined by plating on chocolate agar plates. For experiments measuring cytokine production, bacteria were incubated with the macrophages at a multiplicity of infection (MOI) of 10 for 24 h in 5% CO₂ at 37°C. For experiments measuring the growth of *Francisella* within macrophages, a gentamicin protection assay was performed as described previously (6). Briefly, macrophages were incubated with bacteria at an MOI of 500 for 2 h and then washed with Hanks balanced salt solution (HBSS) containing 20 µg of gentamicin/ml for 20 min to kill extracellular bacteria. Wells were washed again with warm HBSS and then incubated at 37°C with 5% CO₂ in infection medium. At 2 or 24 h postinfection, the macrophages were lysed with 0.02% sodium dodecyl sulfate (SDS). Serial dilutions of the lysates were plated

on chocolate II agar plates for enumeration of viable bacteria. Macrophage integrity was not overtly altered based on phase-contrast microscopy. The fold change in growth was calculated by dividing the number of viable bacteria at 24 h by the number of viable bacteria at 2 h.

Animal infections. Unless otherwise specified, female 6- to 8-week-old C57BL/6J mice (Jackson Laboratory) were used. Experiments assessing the role of proinflammatory cytokines used 6- to 12-week-old female B6129S-Tnfrsf1a^{tm1lmx}/J (TNFR1/IL-1R1 knockout [KO]) and B6129SF2/J (control for KO mice) mice (Jackson Laboratory). All mice were housed in ABSL-3 facilities at the University of Pittsburgh. The mice were infected intratracheally by oropharyngeal instillation as described previously (29). A bacterial inoculum was deposited in the base of the oropharynx of anesthetized mice, allowing it to be aspirated. The mice were infected with approximately 10,000 CFU of LVS or 100 CFU of Schu S4. The mice were monitored twice a day after infection to assess morbidity, which was determined by a scale that incorporated the activity, appearance, and posture of the mice. Once a predetermined score was achieved, the mice were euthanized.

For experiments requiring serial harvest, lungs, livers, spleens, and blood were removed from anesthetized mice. Blood was removed by cardiac puncture by using a heparin-coated needle and syringe. The lungs were homogenized in RPMI supplemented with 10% FBS, and the livers and spleens were homogenized in TSB-C. The samples were diluted and plated onto chocolate II agar plates. The process of homogenization, diluting, and plating occurred in less than 30 min, which is less than the doubling time of the bacteria (~2 h). The bacteria were incubated at 37°C with 5% CO₂ for 72 h, and individual colonies were counted to determine the CFU. All animal infection studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

ELISA. The supernatants were harvested at 24 h postinfection, and the cytokine levels were measured by ELISA. Murine TNF- α was measured by using a matched antibody pair (eBiosciences). Human TNF- α , interleukin-12 p40 (IL-12p40), IL-6, and IL-1 β were measured by using DuoSets (R&D Systems). Cytokine concentrations were determined using a Molecular Dynamics M2 plate reader after the addition of TMB substrate (Dako). The limit of detection was 15 pg/ml for murine and human TNF- α . The limits of detection of human IL-12p40, IL-6, and IL-1 β were 15, 9, and 4 pg/ml, respectively.

Real-time PCR. Bacteria were cultured for 16 to 18 h in MHB alone or in MHB containing 200 μ M spermine. RNA was harvested using TRI-Reagent RT liquid samples (Molecular Research Center) according to the manufacturer's instructions and suspended in nuclease-free water. The samples were treated with DNase (Turbo DNA-free; Ambion), and the RNA quantity was measured by spectrophotometry. Quantitative real time-PCR was performed as previously described (7). The data are depicted as the log₂ fold change of transcripts levels in bacteria cultured in MHB with spermine divided by the transcript level in bacteria cultured in MHB alone using the $\Delta\Delta C_T$ method.

Measurement of NF- κ B activation. RAW264.7 cells stably transfected with a NF- κ B-GFP reporter were seeded into a 96-well plate as described for macrophage infections. The reporter contains green fluorescent protein (GFP) driven by the E-selectin promoter containing a NF- κ B binding site (57). The cells were infected with LVS at an MOI of 10. At 24 h postinfection, the cells were washed with HBSS and visualized on a Zeiss Axiovert 200M microscope. Images were collected by using the Zeiss Axiovision software, and the brightness and contrast were adjusted consistently for all images in Adobe Photoshop. For NF- κ B immunoblotting, BMDM were cultured with LVS strains at an MOI of 500 for 2 h. Macrophages were lysed using a lysis buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl [pH 7.4]). The protein samples were separated by polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes (Millipore), blocked with 5% milk, and probed for total NF- κ B p65 and phospho-p65 (antibody catalog numbers 3034 and 3033, respectively; Cell Signaling). Membranes were washed and incubated with donkey anti-rabbit antibody coupled to horseradish peroxidase (A6154; Sigma). After another wash, the signal was measured with SuperSignal West Femto chemiluminescent substrate (Pierce) and exposure to film. The relative amount of phospho-p65 was calculated by dividing the phospho-p65 levels by the total p65 levels using densitometry analysis performed with ImageJ 1.44P software (NIH).

Luminex. Lung homogenates from infected mice were centrifuged at 20,000 $\times$ g to remove cells and tissue debris. The resulting supernatants were filtered through a 0.2- μ m-pore-size filter and treated with gentamicin (100 μ g/ml). Blood was collected from mice by cardiac puncture with heparinized syringes, and plasma generated by centrifugation at 20,000 $\times$ g was then treated with gentamicin (300 μ g/ml) and ciprofloxacin (25 μ g/ml). These samples were tested using a Milliplex 32-plex mouse cytokine/chemokine panel (Millipore) on a Bio-Plex 200 system (Bio-Rad Laboratories). Analyte concentrations were calculated against the standards using Milliplex Analyst software (version 3.5; Millipore).

Statistics. For statistical analysis involving the wild type and mutant only, we used a two-tailed Student *t* test with an alpha value of 0.05. When more than two strains were compared, a one-way analysis of variance (ANOVA) was used to determine experimental significance and a Tukey post hoc test was used to make pairwise comparisons. Statistical differences in survival were determined by a log-rank test. GraphPad Prism5 (GraphPad Software) was used for all statistical analysis.

RESULTS

Spermine responsiveness in *F. tularensis* requires the presence of FTL_0883. Polyamines, particularly spermine, act as intracellular cues for *F. tularensis* (7). A transposon screen was performed in LVS to identify mutants that were unable to respond to extracellular spermine. Bacteria can either make their own polyamines (putrescine and spermidine) or import polyamines (spermidine, putrescine, and spermine) from the extracellular environment to grow (64). To identify mutants that were unable to utilize extracellular polyamines, we used an inhibitor of spermidine synthase, dicyclohexylamine (3, 9, 27, 37, 43). Isolates were selected from a library of mutants made with a Mariner-based transposon (50) that were unable to replicate in the presence of both the inhibitor and spermine in CDM. Of the nearly 5,000 transposon mutants screened, 257 mutants were initially identified in the primary screen as unable to replicate in CDM with the inhibitor. Of the 257 mutants, 32 were unable to replicate in the presence of spermine and the inhibitor and were not simply auxotrophs in CDM (data not shown). These isolates were then subjected to a secondary screen for their ability to stimulate cytokine production from macrophages when they were cultured in the presence or absence of spermine. Of the 32 mutants identified from the primary screen, two mutants stimulated at least equivalent cytokines from macrophages whether they were cultured in the presence or absence of spermine. One of the transposon mutants, 13B47 contained a transposon inserted in FTL_0883 and was selected for further investigation because its response to spermine was different from wild-type LVS, and it had been previously attributed to virulence in a negative selection screen in mice to identify mutants defective for dissemination to the spleen (62). As previously described, wild-type LVS grown in MHB with spermine stimulates significantly less TNF- α than when it is grown in MHB alone (Fig. 1A). Strain 13B47 stimulated more cytokines than did wild-type LVS whether or not spermine was present in the MHB (Fig. 1A).

An in-frame deletion of FTL_0883, Δ FTL_0883, was generated for further study. Similar to the transposon mutant, Δ FTL_0883 grown in MHB or MHB plus spermine stimulated more TNF- α production than did the wild type ($P < 0.01$; Fig. 1A and B). Culturing 13B47 in MHB with spermine resulted in a significant increase in the induction of TNF- α in some experiments. In contrast, the deletion mutant elicited equivalent amounts of cytokines from the macrophages whether it was cultured in the presence or absence of spermine (Fig. 1A). Due to the possibility of polar effects in the transposon mutant, subsequent experiments were performed with the in-frame deletion mutant, Δ FTL_0883. To confirm the responses were the result of the deletion, genetic complementation studies were performed to establish the role of FTL_0883 in these phenotypes. Δ FTL_0883 containing a *cis*-complementing construct cocultured with macrophages stimulated TNF- α levels compa-

FIG. 1. FTL_0883 is required for response to extracellular spermine. (A) Cytokine response to bacteria grown with or without spermine. Bacterial strains were cultured in either MHB or MHB supplemented with spermine overnight. Human macrophages were cocultured with the strains at an MOI of 10. At 24 h postinfection, the supernatants were harvested and TNF- α was measured by ELISA. Macrophages incubated with medium alone produced TNF- α levels that were below detectable limits. Statistical significance was determined by using a Student *t* test comparing the amount of TNF- α elicited from a strain cultured in MHB alone or supplemented with spermine. ***, $P < 0.001$. (B) Genetic complementation restores wild-type phenotypes. Bacteria were cultured in MHB supplemented with spermine. Human macrophages were infected at an MOI of 10, and TNF- α was measured from supernatants collected 24 h postinfection by ELISA. Macrophages in control wells only with medium produced undetectable levels of TNF- α . The data are presented as the means $\pm$ the standard deviations (SD) of triplicate wells within one experiment representative of two independent experiments using cells from different donors. Statistical significance was determined by using a one-way ANOVA, followed by a Tukey post hoc test. **, $P < 0.01$; ***, $P < 0.001$ (compared to wild-type LVS). (C) Real-time PCR analysis of transcriptional response to spermine. LVS or Δ FTL_0883 were cultured in MHB with or without spermine overnight prior to RNA harvest. The data are presented as the mean log₂ fold changes (MHB+spermine/MHB) in transcript levels $\pm$ the standard errors of the mean (SEM) of three independent experiments.

able to wild type when they were grown in the presence of spermine (Fig. 1B). Integration of an empty vector did not alter the response of the macrophages to the mutant when cultured in the presence of spermine (Fig. 1B). Therefore, FTL_0883 is needed for *F. tularensis* to become less stimulatory when exposed to extracellular spermine. The lack of a spermine response in Δ FTL_0883 was not simply the result of a general growth defect. Similar growth rates of LVS and Δ FTL_0883 were observed in the media used for our studies: MHB (LVS, 2.63 ± 0.41 h; Δ FTL_0883, 2.81 ± 0.32 h) and TSB-C (LVS, 2.28 ± 0.05 h; Δ FTL_0883, 2.23 ± 0.08 h).

The cultivation of *F. tularensis* in the presence of spermine changes the transcriptional profile of the bacteria compared to bacteria grown without spermine (7). As an additional test of spermine responsiveness in the deletion mutant, the expression of three genes of the spermine regulon in *Francisella* (7)—FTL_0500, FTL_1401, and FTL_0681—was analyzed. As expected for wild-type *Francisella*, there was increased transcription of FTL_1401 and decreased transcription of FTL_0500 and FTL_0681 when LVS was cultured in MHB with spermine compared to MHB alone (Fig. 1C). The transcriptional changes by Δ FTL_0883, however, were substantially blunted compared to the wild type (Fig. 1C), demonstrating that the mutant had a diminished transcriptional response to spermine. Although the effects of spermine were not abolished, most likely because of multiple levels of transcriptional regulation, these observations demonstrate that FTL_0883 contributes to the transcriptional response to spermine in *F. tularensis*.

Macrophages are activated by strains with mutations in FTL_0883 or FTT_0615c. We extended our initial observations with the Δ FTL_0883 mutant by investigating other host cells and a virulent type A strain of *F. tularensis*, Schu S4. An in-frame deletion in the Schu S4 homologue of FTL_0883, FTT_0615c (Δ FTT_0615c), was generated. Human macrophages and murine BMDM were infected at an MOI of 10 with wild-type bacteria, deletion mutants, or mutants that received their cognate gene or an empty vector in *cis*. After 24 h, the supernatants were collected to analyze the amount of cytokines

in the media. Significantly more TNF- α was produced by human and murine macrophages infected with the Δ FTL_0883 mutant compared to the wild type grown in TSB-C ($P < 0.001$; Fig. 2A and B), which was similar to results obtained with MHB supplemented with spermine (Fig. 1B). Restoration of FTL_0883 with the complementation construct, but not an empty vector, restored TNF- α levels close to those observed with the wild type (Fig. 2A and B). Similar results were also obtained when RAW264.7 cells were used (data not shown). A comparable pattern of cytokine production was observed with strains in the Schu S4 background. The Δ FTT_0615c mutant stimulated significantly more TNF- α from both human and murine macrophages than did the wild type ($P < 0.001$; Fig. 2C and D). Strains of Δ FTT_0615c that received the complementing construct, but not the vector control, behaved similarly to the wild type by stimulating lower levels of TNF- α than the deletion mutant.

Although TNF- α was used as a prototypical proinflammatory cytokine, similar trends in the production of other proinflammatory cytokines were observed. More IL-6, IL-12p40, and IL-1 β was produced by human macrophages cultured with Δ FTL_0883 ($P < 0.01$ for all cytokines; Fig. 3A), and more IL-6 and IL-1 β was produced when human macrophages were cocultured Δ FTT_0615c ($P < 0.01$ for both cytokines; Fig. 3B). Similar to TNF- α (Fig. 2), stimulation of macrophages was reduced when FTL_0883 or FTT_0615c were returned in *cis*, but there was no effect when an empty vector was used (Fig. 3A and B). These results show that FTL_0883 and FTT_0615c have a general role in limiting proinflammatory cytokine production by macrophages infected with *Francisella*.

F. tularensis actively inhibits intracellular signaling in macrophages, including NF- κ B activation, thereby limiting proinflammatory cytokine production (60). We hypothesized that the heightened stimulation of macrophages by Δ FTL_0883 could correlate with an enhanced NF- κ B activation. As a functional test of NF- κ B activation, LVS strains were cocultured with RAW264.7 cells bearing a NF- κ B GFP reporter in which the expression of GFP is under the regulation an NF- κ B-

FIG. 2. LVS Δ FTL_0883 and Schu S4 Δ FTT_0615c stimulate TNF- α . Bacteria cultivated overnight in TSB-C were used to infect either human macrophages or murine BMDM at an MOI of 10. After 24 h, the supernatants were removed, and an ELISA was performed to quantify the levels of TNF- α . The data are presented as the means $\pm$ the SD of triplicate wells within one experiment representative of at least three independent experiments. (A) Human macrophages infected with LVS strains; (B) BMDM infected with LVS strains; (C) human macrophages infected with Schu S4 strains; (D) BMDM infected with Schu S4 strains. TNF- α levels were compared by a one-way ANOVA, followed by a Tukey post hoc test to allow for pairwise comparisons. ***, $P < 0.001$ compared to wild-type strains. ND, not detected.

FIG. 3. Δ FTL_0883 and Δ FTT_0615c stimulate proinflammatory cytokine production. Human macrophages were infected at an MOI of 10 for 24 h. After 24 h, the supernatants were removed, and ELISAs were performed to quantify the levels of IL-6, IL-12p40, and IL-1 β . (A) Human macrophages infected with LVS strains; (B) human macrophages infected with Schu S4 strains. IL-12p40 was not detected in these samples. ND, not detected for values that were below the limit of detection for the ELISA. The data are presented as the means $\pm$ the SD of triplicate wells within one experiment representative of two or three independent experiments. The induction of cytokines by mutant strains was compared to the wild type with a one-way ANOVA, followed by a Tukey post hoc test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

dependent promoter (57). The NF- κ B reporter cells showed minimal GFP expression when cultured with wild-type LVS, similar to the low levels observed with the media control (Fig. 4A). In contrast, infection with the Δ FTL_0883 strain greatly enhanced GFP expression compared to LVS (Fig. 4A). Similar to the results seen with TNF- α production (Fig. 2), complementing the Δ FTL_0883 mutant with a wild-type copy of the gene, but not the empty vector, reduced the level of GFP expression (Fig. 4A). Because the NF- κ B reporter cells were insufficiently sensitive to detect changes at earlier time points and to assess activation on a molecular level, the activation of NF- κ B was assessed by immunoblot (Fig. 4B). Whole-cell lysates were generated from BMDM 2 h after infection to measure the phosphorylation of the p65 subunit of NF- κ B (Fig. 4B). Consistent with the results obtained with the NF- κ B reporter strains, there was a 4-fold increase in the phosphorylation of p65 in cells infected with Δ FTL_0883 compared to cells infected with LVS (Fig. 4B). This early NF- κ B activation was reduced when the gene was restored in *cis* such that phosphorylated p65 was 2.5-fold higher in BMDM cultured with vector compared to complement (Fig. 4B). These data corroborate the results obtained by ELISA and demonstrate that macrophages are more activated by Δ FTL_0883. These findings indicate that FTL_0883 is needed by *Francisella* to minimize the activation of macrophages.

Induction of TNF- α by Schu S4 does not correlate with replication within macrophages. Activated macrophages control *F. tularensis* replication *in vitro* (16). Since we observed enhanced activation of macrophages infected with the deletion mutants as measured by proinflammatory cytokine production, we hypothesized that growth of these mutants within macrophages would be restricted. To test this, gentamicin protection

FIG. 4. LVS Δ FTL_0883 stimulates activation of NF- κ B. (A) RAW264.7 cells containing GFP driven by a promoter whose activity is regulated by activation of NF- κ B were infected at an MOI of 10 with *F. tularensis* grown overnight in TSB-C. After 24 h, the cells were examined by fluorescence microscopy to assess the relative expression of GFP within the cells. Exposure times were constant across all fluorescent images. Images are from one experiment representative of three independent experiments. Scale bar, 100 μ m. (B) NF- κ B is activated early by Δ FTL_0883. BMDM were infected at an MOI of 500 for 2 h, and whole-cell lysates were tested for NF- κ B p65 and phospho-p65 as described in Materials and Methods. Phospho-p65 levels were normalized to total p65 levels, with the ratios as follows: media, 0.3; LVS, 1.2; Δ FTL_0883, 4.2; complement, 2.4; and vector, 5.8. Similar results were seen in two independent experiments.

assays were performed using human monocyte-derived macrophages and murine BMDM infected with the LVS strains. Wild-type LVS replicated well in either host macrophage, increasing 10- to 100-fold over 24 h (Fig. 5A and B). Consistent with our hypothesis, the Δ FTL_0883 mutant was attenuated for growth in both human macrophages and BMDM, growing at least 10-fold less than the wild type (human macrophages $P < 0.01$, BMDM $P < 0.001$; Fig. 5A and B). As expected, returning the gene in *cis*, but not the empty vector, nearly restored growth to wild-type levels. The differences in growth rates were not attributable to differences of invasion between the mutant and wild-type as the number of intracellular bacteria 2 h after infection differed by <2 -fold between Δ FTL_0883 and LVS. The reduced growth of the Δ FTL_0883 mutant was also not the result of an increased sensitivity to SDS that was used to lyse the macrophages because the viability of the mutant and the wild type were unaffected by this concentration of SDS (data not shown). These results show FTL_0883 is necessary for intracellular growth of LVS in macrophages.

In contrast, the mutation in the Schu S4 background yielded unexpected results. Schu S4 and the Δ FTT_0615c mutant grew similarly in both human and murine macrophages (Fig. 5C and D). Growth of the complemented strain or the empty vector strain was also indistinguishable. Again, similar rates of invasion were observed at 2 h postinfection for Schu S4 and Δ FTT_0615c. Although FTL_0883 was needed for wild-type rates of replication of LVS within macrophages, FTT_0615c was dispensable for replication of Schu S4 within macrophages.

These results were unexpected, but they are not unprecedented, since there are established differences in the ability of Schu S4 and LVS to withstand host defenses such as oxidative stress (33), which could restrict replication of Δ FTL_0883 in activated macrophages but permit wild-type replication of Δ FTT_0615c.

Although attenuated for growth in macrophages, the LVS mutant strain showed wild-type growth in nonmacrophages. Wild-type LVS, Δ FTL_0883, complement, and vector all grew similarly in nonmacrophage HEK293 cells; there was no significant difference in replication among the strains (Fig. 5E). These results confirm observations in broth culture that Δ FTL_0883 does not suffer from a generalized growth defect.

Replication within a macrophage does not correlate with virulence *in vivo*. The macrophage is believed to be an important, intracellular niche for *F. tularensis* within the host. Moreover, replication in macrophages has typically been associated with virulence (5, 21, 24, 32, 41, 45, 48). Based on the growth patterns in macrophages described above, we hypothesized that the Δ FTL_0883 mutant would be attenuated but the Δ FTT_0615c mutant would retain virulence *in vivo*.

Virulence of LVS and Δ FTL_0883 was assessed first. Mice infected with wild-type LVS lost a significant amount of weight, a measure of morbidity, approaching 25% of their starting body weight by day 7 (Fig. 6A). In contrast, mice infected with Δ FTL_0883 lost $<5\%$ of their body weight over the course of the experiment, which was significantly different from the wild type from day 4 until day 12 ($P < 0.05$; Fig. 6A). The weights of all of the mice returned to their preinfection

FIG. 5. FTL_0883 but not FTT_0615c is needed for the replication of *F. tularensis* within macrophages *in vitro*. Bacteria cultivated overnight in TSB-C were used to infect human macrophages or BMDM at an MOI of 500, followed by gentamicin treatment 2 h postinfection. At 2 and 24 h, the macrophages were lysed and *F. tularensis* was diluted and plated to enumerate CFU. The data are presented as the mean fold change in CFU (CFU at 24 h/CFU at 2 h) $\pm$ the SD of one experiment representative of at least three independent experiments. (A) Human macrophages infected with LVS strains; (B) BMDM infected with LVS strains; (C) human macrophages infected with Schu S4 strains; (D) BMDM infected with Schu S4 strains; (E) HEK293 cells infected with LVS strains. To assess differences in the fold change in CFU, a one-way ANOVA was performed, followed by a Tukey's test to make pairwise comparisons. *, $P < 0.05$; **, $P < 0.01$. ***, $P < 0.001$ (compared to wild-type strains).

values, and no mice died with the dose administered. Nevertheless, there was a significant decrease in morbidity for mice infected with Δ FTL_0883 compared to mice infected with LVS. This is consistent with the hypothesis that failed growth in macrophages *in vitro* predicts attenuation *in vivo*.

Once again, the Δ FTT_0615c mutant yielded unexpected results. We anticipated that the mutant in the Schu S4 background would be fully virulent in mice because it replicated efficiently in macrophages *in vitro*. Surprisingly, there was a significant delay in time to death for mice that were infected with the Δ FTT_0615c mutant compared to wild-type Schu S4 (Fig. 6B). The mean time to death was 6 days and 15 days for the wild type and mutant, respectively, and one-third of the mice infected with the mutant survived until the end of the study 22 days postinfection ($P < 0.01$ [log-rank test]). Therefore, Δ FTT_0615c was attenuated *in vivo* compared to Schu S4, even though the mutant was able to replicate at wild-type rates in macrophages *in vitro*. These results contradict the hypothesis that replication in macrophages *in vitro* is a predictor of virulence and suggest that the proinflammatory response *in vitro* is a better indicator of attenuation *in vivo*.

The kinetics of bacterial replication were next investigated in mice infected with the Δ FTT_0615c and Schu S4 strains. We

hypothesized that since there was an increase in the mean time to death for mice infected with Δ FTT_0615c, there would be reduced bacterial burden. Mice were sacrificed on day 2 or 4 postinfection, and homogenates of their lungs, livers, spleens, and blood were tested for CFU. On day 2, lower bacterial burdens were measured in all organs of mice infected with Δ FTT_0615c compared to Schu S4-infected mice, although there was only a statistically significant difference in the blood where the mutant could not be detected ($P < 0.05$; Fig. 7). On day 4, however, there were statistically significant differences of viable bacteria in all organs ($P < 0.05$ to 0.001 ; Fig. 7). There were 2 to 4 logs fewer CFU were recovered from Δ FTT_0615c-infected organs compared to Schu S4-infected organs (Fig. 7). Similar to day 2, bacteria were not detected in the blood of mice infected with Δ FTT_0615c on day 4. These data demonstrate that FTT_0615c is important for *F. tularensis* replication *in vivo*, and its presence enhances dissemination to secondary sites of infection.

Cytokine and chemokine responses to Δ FTT_0615c *in vivo*.

We next tested whether the enhanced cytokine stimulation we observed with the Δ FTT_0615c mutant *in vitro* was recapitulated *in vivo*. The concentrations of cytokines and chemokines in lung homogenates and plasma were analyzed by Luminex on

FIG. 6. FTL_0883 and FTT_0615c are required for virulence *in vivo*. (A) Mice were infected intratracheally with 10,000 CFU of LVS or Δ FTL_0883 ($n = 4$ per group). The weights of the mice were monitored for 2 weeks postinfection. The data are presented as the means $\pm$ the SD from two representative experiments performed similarly. The data under the line indicate days the weight of the mice infected with the mutant were statistically different from the wild-type-infected mice. *, $P < 0.05$ (Student t test). (B) Mice were infected intratracheally with 100 CFU of either Schu S4 or Δ FTT_0615c. The data are combined from two independent experiments ($n = 9$ total per group). $P < 0.01$ (log-rank test).

days 2 and 4 postinfection. There was no difference in the concentration of any of the 32 analytes tested in the lungs of mice infected with Schu S4 or Δ FTT_0615c on day 2 (Fig. 8 and data not shown). On day 4, however, significantly higher

concentrations of TNF- α , IL-1 α , and IL-1 β were present in the lungs of mice infected with the mutant ($P < 0.01$; Fig. 8A). In contrast, significantly greater concentrations of granulocyte colony-stimulating factor (G-CSF; CSF3), monocyte chemoattractant protein 1 (MCP-1; CCL2), and lipopolysaccharide-induced CXC chemokine (LIX; CXCL5) were found in the lungs of mice infected with Schu S4 ($P < 0.001$ to 0.05; Fig. 8B). Interestingly, other analytes were not elevated in mice infected with the Δ FTT_0615c strain, including gamma interferon (IFN- γ ; data not shown). This was unexpected since IFN- γ is typically associated with effective host defenses against *F. tularensis* (16, 17, 35). Nevertheless, pulmonary infection by Δ FTT_0615c was associated with elevated levels of prototypical proinflammatory cytokines, which correlated with fewer viable bacteria.

The striking lack of bacteremia after pulmonary infection by the Δ FTT_0615c strain suggested high concentrations of cytokines in the blood might contribute to restricting the growth of the mutant. Using Luminex technology, we measured cytokines in the blood of the same mice described above on days 2 and 4 postinfection. In contrast to the lung measurements, mice infected with Δ FTT_0615c had significantly lower plasma concentrations of TNF- α and IL-1 β than mice infected with Schu S4 ($P < 0.001$ to 0.05; Fig. 9A). The plasma concentrations of IL-1 α were also 20-fold lower than those in the lungs of mice infected with the mutant and were indistinguishable from plasma of mice infected with Schu S4 (Fig. 8A and 9A). Mice infected with wild-type Schu S4 had significantly greater plasma concentrations of G-CSF and MCP-1 than mice infected with Δ FTT_0615c, which was 2- to 5-fold higher than the levels in lung homogenates ($P < 0.001$ to 0.01; Fig. 8B and 9B). Therefore, the levels of proinflammatory cytokines found in the blood were not elevated in mice infected with Δ FTT_0615c. Rather, the systemic cytokine levels were directly correlated with the bacterial burden of mice infected with the Δ FTT_0615c and wild-type strains.

Although the enhanced cytokine production in the lungs of mice correlated with the attenuation of the mutant, it was unclear whether these cytokines prolonged the survival of the mice infected with Δ FTT_0615c compared to mice infected

FIG. 7. Mice infected with FTT_0615c have reduced bacterial burdens. Mice were infected with 100 CFU of either Schu S4 or Δ FTT_0615c. On days 2 and 4 postinfection, mice were sacrificed, and the CFU was determined as described in Materials and Methods. The limit of detection for spleens and lungs was 100 CFU, for blood it was 100 CFU/ml, and for livers it was 200 CFU. In total, 11 Δ FTT_0615c-infected mice (three independent experiments) and 8 Schu S4-infected mice (two independent experiments) were sacrificed on day 2, and 11 Δ FTT_0615c-infected mice (three independent experiments) and 6 Schu S4-infected mice (two independent experiments) were sacrificed on day 4. The data are presented as means $\pm$ the SD. The bacterial burdens for mice infected with Schu S4 or Δ FTT_0615c were compared for each organ on each day, and the differences were determined to be significant by using a Student t test. ND, not detected (values that are below the detectable limit); *, $P < 0.05$; ***, $P < 0.001$.

FIG. 8. Wild-type and Δ FTT_0615c Schu S4 strains elicit different cytokine and chemokine profiles within the lungs *in vivo*. (A) Analytes were significantly increased in the lungs of mice infected with Δ FTT_0615c compared to Schu S4-infected mice. (B) Analytes were significantly increased in the lungs of mice infected with Schu S4 compared to mice infected with Δ FTT_0615c. Supernatants from lung homogenates were assayed by using Luminex, as described in Materials and Methods. A total of 11 samples (three independent experiments) from both Schu S4 and Δ FTT_0615c were tested on day 2. A total of 11 samples (three independent experiments) were tested on day 4 for Δ FTT_0615c, and a total of 7 samples (two independent experiments) were tested for Schu S4. The data are presented as the means $\pm$ the SD. Student *t* tests were used to determine statistically significant differences in cytokine production between the wild-type and mutant strains on a particular day. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

with Schu S4. To investigate this possibility, mice lacking both TNF- α type 1 receptor and IL-1 β type 1 receptor (TNFR1/IL-1R KO) or control mice were infected with the Δ FTT_0615c strain. Mice were significantly more susceptible to the infection when lacking the TNF- α and IL-1 β receptors than were the wild-type mice ($P < 0.01$; Fig. 10). The median time to death was 10 days for the knockout mice compared to 15 days for the wild-type mice, with two of the five wild-type mice surviving the infection until the end of the study on day

20. These results define a role for TNF- α and IL-1 signaling in prolonging the survival of the mice infected with Δ FTT_0615c.

DISCUSSION

The pathogenesis of *F. tularensis* is associated with its ability to evade immune detection (11, 13, 22, 59). Adaptation to the intracellular mammalian environment is likely a mechanism contributing to the stealth nature of this pathogen. Polyamines

FIG. 9. Mice infected with Δ FTT_0615c have lower proinflammatory responses in plasma. (A) Plasma concentrations of the analytes depicted in Fig. 8A. (B) Plasma concentrations of the analytes depicted in Fig. 8B. Plasma was collected from heparinized blood and tested by using Luminex as described in Materials and Methods. A total of 11 samples (three independent experiments) from both Schu S4 and Δ FTT_0615c were tested on day 2. A total of 11 samples (three independent experiments) were tested on day 4 for Δ FTT_0615c, and a total of 7 samples (two independent experiments) were tested for Schu S4. The data are presented as the means $\pm$ the SD. Student *t* tests were used to determine statistically significant differences in cytokine production between the wild-type and mutant strains on a particular day. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

FIG. 10. TNF- α receptor and IL-1 β receptor contribute to prolonged survival of mice infected with Δ FTT_0615c. Control (B6129SF2/J) or knockout (TNFR1/IL-1R1 KO) mice were infected intratracheally with 80 CFU of Δ FTT_0615c, and survival was monitored over time. The experiment was performed with five animals in each group. $P < 0.01$ (log-rank test).

are abundant in the intracellular environment and cue *Francisella* to the host's cytosol (7). The objectives of the present study were to identify genes important for the polyamine response in *F. tularensis* and to determine whether the genes contribute to the virulence of the organism. By performing a transposon screen within LVS, we identified FTL_0883 as a gene important for the spermine response. Unlike LVS, the FTL_0883 mutants stimulated high levels of cytokines from macrophages even if they were cultured in the presence of extracellular spermine (Fig. 1A and B). The loss of spermine response in Δ FTL_0883 is not simply the result of stimulating higher amounts of cytokines than the wild type. Other transposon mutants from this screen and the Δ pyrF mutant in LVS (29) stimulate more cytokines from macrophages than did the wild type. Nevertheless, they stimulate less cytokine production from macrophages after growing in MHB with spermine than MHB (data not shown). Further, FTL_0883 is needed for an optimal transcriptional response to spermine (Fig. 1C). Therefore, FTL_0883 contributes to the response of *F. tularensis* to extracellular spermine.

Based on our data, an adequate response to spermine minimizes the activation of macrophages. Previously, we proposed that a response to spermine could be an important determinant that enables host immune evasion (7). Consistent with this proposal, we observed a significant increase in the production of proinflammatory cytokines by macrophages infected with Δ FTL_0883 and Δ FTT_0615c compared to wild-type strains (Fig. 2 and 3). We also observed enhanced NF- κ B activation and increased production of proinflammatory cytokines in macrophages infected with Δ FTL_0883, findings which both independently demonstrate that the macrophage is in an activated state (Fig. 2 to 4). Similar to the *in vitro* observations, Δ FTT_0615c stimulated more proinflammatory cytokines within the lung *in vivo* (Fig. 8). The enhanced immune response within the lung correlated with a decrease in systemic bacterial numbers in mice infected with Δ FTT_0615c. Published data indicate that greater activation of the immune response controls the replication of *F. tularensis* Schu S4 and that the control correlated with increases in proinflammatory

cytokine production (20). Similarly, our results demonstrate that there is a more robust proinflammatory response in the lungs of mice infected with Δ FTT_0615c compared to Schu S4, which correlated with control of the mutant. Moreover, the TNFR1/IL-1R KO mice are more susceptible to infection. This demonstrated that activation of immune responses by Δ FTT_0615c, leading to the production of the proinflammatory cytokines TNF- α and IL-1 β , is required to control the infection.

Our results provide a significant step forward from previous studies that suggested a role for Δ FTL_0883 and Δ FTT_0615c in *Francisella* virulence. A negative selection screen *in vivo* identified the homologue in *F. novicida* was important for dissemination to the spleen in mice, and another negative selection screen showed that the homologue in *F. novicida* was important for survival in *Drosophila* (44, 62). In addition, the presence of a functional FTT_0615c correlated with virulence within type A strains, although a spontaneous deletion in a gene in the pathogenicity island, *pdpC*, confounded this study (56). Using gene specific deletions and complementation studies, our results provided direct evidence that FTL_0883 and FTT_0615c are important mediators of immune evasion in *F. tularensis*. To our knowledge, the present study is the first to describe a *Francisella* gene important for sensing the host environment and its role during *in vivo* infections.

The present study further questions the paradigm that intramacrophage replication is a hallmark of *Francisella* pathogenesis (54). There have been multiple reports correlating the ability of mutants to replicate within macrophages to their fitness *in vivo* (5, 21, 24, 32, 41, 45, 48). Although this correlation was true for Δ FTL_0883, it was not true for Δ FTT_0615c. The Δ FTT_0615c strain replicated in macrophages *in vitro* at rates similar to that of Schu S4. Unexpectedly, however, we found that there was a substantial increase in the mean time to death for mice that were infected with Δ FTT_0615c compared to wild-type Schu S4. These results suggest that replication within a macrophage *in vitro* is not sufficient for virulence *in vivo*. We have also recently published that a Schu S4 *pyrF* mutant is unable to replicate in macrophages; it is, however, essentially fully virulent within a mouse (29). Therefore, experiments with the *pyrF* mutant demonstrated that replication within a macrophage is not necessary for the virulence of *Francisella* (29). In conjunction with the present study, intramacrophage replication appears neither necessary nor sufficient to predict Schu S4 virulence in a mouse model of tularemia. However, these findings do not diminish the potential role(s) of the macrophage as a component of host defenses since macrophage activation correlates with reduced virulence among the Schu S4 strains (29; the present study).

The function of the proteins encoded by FTL_0883 and FTT_0615c remains undefined. Homologues were originally identified in *Salmonella* during a screen conducted to identify mutants that were resistant to cobalt (23). From the screen, four mutants were identified that had increased resistance to cobalt, the *corA* to *corD* mutants (23). *corA*, a magnesium transport protein, was demonstrated to be important for the virulence of *Salmonella* and other bacteria (46, 50, 65). *corC* has not been studied in any significant detail beyond the initial screen. Based on homology searches, there is a CorC domain and two tandem CBS domains in FTL_0883 and FTT_0615c

proteins. CBS domains bind to adenine derivatives and magnesium; they are proposed to function as intracellular switches, which are sensitive to the energy and ion concentrations within the cell (31, 55). The function of the CorC domain within the protein remains unknown. It is possible that within *Salmonella* where there is a functional CorA, CorC functions in conjunction with CorA to mediate divalent cation efflux (23). However, a function in cation efflux within *F. tularensis* is unlikely since its genome does not encode a functional *corA*. Previous work with CorC has been performed in systems that contain a CorA protein. This is the first system to describe the importance of a putative CorC homologue that is independent of CorA. *Francisella* provides a useful model for studying the functions of proteins encoded by FTL_0883/FTL_0615c that are independent of the activities of the CorA protein, which likely occur in a large number of bacteria as half of sequenced bacterial genomes lack a functional CorA protein (42).

Based on available information, the proteins encoded by the FTL_0883/FTL_0615c loci could modulate the *Francisella* spermine response in one of three ways. First, CBS domains can regulate the activity of ABC transport systems, such as CIC channels (19). In *Francisella*, it is plausible that the FTL_0883/FTL_0615c proteins interact with other ABC transport systems, such as the polyamine transport system, to regulate the transport of polyamines. Altered polyamine transport in Δ FTL_0883/ Δ FTL_0615c strains could affect the mutants' ability to respond to extracellular spermine. Second, bioinformatics analysis (psortb) localized the protein products of FTL_0883/FTL_0615c to the cytoplasmic membrane, and it was identified in the membrane fraction of *F. tularensis* subsp. *tularensis* FSC033 (61). The localization of the FTL_0883/FTL_0615c gene products to the membrane could enable it to function as a signal transducer, connecting the identification of extracellular spermine at the surface to transcriptional responses inside the bacteria. Third, the transcriptional response to spermine may result in an up- or downregulation of proteins that interact with FTL_0883/FTL_0615c proteins within the membrane, resulting in bacteria that are less stimulatory for macrophages. The molecular function(s) of the FTL_0883/FTL_0615c gene products is currently under active investigation.

The spermine response by *F. tularensis* enables the bacteria to limit the proinflammatory cytokines produced by the immune cells. This response is critical for the rapid pathogenesis and the disease severity associated with *F. tularensis*. Bacterial adaptation in response to extracellular polyamines is not limited to *Francisella* and is a more common theme in microbiology. The development of biofilms by *Vibrio cholerae* and *Yersinia pestis* is regulated by the concentration of extracellular polyamines (39, 47, 63). In addition, efflux pump expression and quorum sensing in *Burkholderia pseudomallei* are regulated by the presence of spermidine (9). FTL_0883 is conserved among *Burkholderia*, *Vibrio*, and *Yersinia* species based on a BLAST homology search, which suggests that FTL_0883 could serve as a model for the function of the homologous proteins. It is possible the homologues within these bacteria play important roles both in regulating their responses to polyamines specifically and their virulence more generally.

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