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Global Transcriptional Response to Spermine, a Component of the Intramacrophage Environment, Reveals Regulation of *Francisella* Gene Expression through Insertion Sequence Elements▽†

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Tularemia is caused by the category A biodefense agent *Francisella tularensis*. This bacterium is associated with diverse environments and a plethora of arthropod and mammalian hosts. How *F. tularensis* adapts to these different conditions, particularly the eukaryotic intracellular environment in which it replicates, is poorly understood. Here, we demonstrate that the polyamines spermine and spermidine are environmental signals that alter bacterial stimulation of host cells. Genomewide analysis showed that *F. tularensis* LVS undergoes considerable changes in gene expression in response to spermine. Unexpectedly, analysis of gene expression showed that multiple members of two classes of *Francisella* insertion sequence (IS) elements, IS*Ftu1* and IS*Ftu2*, and the genes adjacent to these elements were induced by spermine. Spermine was sufficient to activate transcription of these IS elements and of nearby genes in broth culture and in macrophages. Importantly, the virulent strain of *F. tularensis*, Schu S4, exhibited similar phenotypes of cytokine induction and gene regulation in response to spermine. Distinctions in gene expression changes between Schu S4 and LVS at one orthologous locus, however, correlated with differences in IS element location. Our results indicate that spermine and spermidine are novel triggers to alert *F. tularensis* of its eukaryotic host environment. The results reported here also identify an unexpected mechanism of gene regulation controlled by a spermine-responsive promoter contained within IS elements. Different arrangements of these mobile genetic elements among *Francisella* strains may contribute to virulence by conveying new expression patterns for genes from different strains.

Francisella tularensis is a formidable pathogen, leading to its designation as a category A biodefense agent (9). Among its many characteristics is the ability to replicate in macrophages (12). Upon infection of these cells, *F. tularensis* encounters molecular cues that alert this pathogen to its new environment. The ability of *F. tularensis* to detect and respond to environmental signals contributes to this organism's success as a pathogen. For example, conditions encountered within a mammalian host, including iron restriction, temperature change, and oxidative stress, induce the expression of genes critical to establishing a successful infection (8, 13, 16, 17, 22). Some of these cues can be mimicked in broth culture by eliciting protein expression patterns similar to those observed in vivo (15). Understanding the mechanisms by which *F. tularensis* adapts to its environment may provide insight into the pathogenicity of this organism.

An effective immune response to *F. tularensis* requires innate immunity. Proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α) and gamma interferon, play a major role in controlling *Francisella* infections (10). Both T-cell-deficient and *scid* mice exhibit heightened sensitivity to the *F. tularensis* live vaccine strain (LVS) when depleted of these cytokines (11). *Francisella* inhibits the production of proinflammatory cytokines by macrophages, presumably as a means of evading innate immune defenses (4, 35, 36).

We have previously shown that the growth conditions for *F. tularensis* LVS affect both the production of a known virulence factor and the ability of the bacterium to inhibit macrophage cytokine production (4). The growth of LVS in a chemically defined medium (CDM) (5) elicits an inhibitory phenotype, while bacteria grown in Mueller-Hinton (MH) broth induce cytokine responses from macrophages (4). The chemical signals in CDM and MH broth responsible for these bacterial phenotypes are poorly understood.

Polyamines, among which are spermidine and spermine, are cationic compounds found in all living organisms. These molecules reach millimolar levels in both prokaryotic and eukaryotic cells, and spermine is produced only by eukaryotes (19). Polyamines are associated with DNA synthesis, transcription, translation, and enzyme activity (33, 40). These molecules are known to induce global gene regulation in *Escherichia coli*, leading to optimal growth (39). Polyamines are also essential for biofilm formation in both *Yersinia pestis* and *Vibrio cholerae* (20, 26). Likewise, they contribute to *Streptococcus pneumoniae* virulence through an unknown mechanism (37).

Insertion sequence (IS) elements are small (<2.5-kb) mobile genetic elements that can enter into multiple sites within a given DNA molecule (24). These elements typically encode the

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transposase proteins required for their mobility in regions flanked by small inverted repeats (24). In *Francisella* species, there are several classes of IS elements, designated IS*Ftu1* to IS*Ftu6* (28). Of these classes, IS*Ftu1* and IS*Ftu2* are the most frequent IS elements in each of the *F. tularensis* genomes.

In this work, we have investigated the molecular mechanisms responsible for differential patterns of cytokine induction by *F. tularensis* (4). We have identified an environmental signal, the polyamines spermine and spermidine, that alters LVS behavior and have characterized the bacterial response to this signal. Using a genomewide transcriptome analysis, we found that transcription of the *Francisella* IS elements IS*Ftu1* and IS*Ftu2* increased in response to spermine. The IS elements were also sufficient to drive the expression of downstream genes in response to extracellular spermine. Further, we have shown that this response occurs in bacteria residing inside of macrophages, a relevant environment encountered by the bacterium during infection. In addition, we have found that the fully virulent strain Schu S4 exhibits similar responses. These results define a novel mechanism of gene regulation in this category A biodefense agent and have implications for other pathogens.

MATERIALS AND METHODS

***Francisella* strains and cultivation.** *F. tularensis* LVS was kindly provided by Karen Elkins (Food and Drug Administration). Schu S4 was obtained from the Biodefense and Emerging Infections Research Repository (Manassas, VA). For macrophage infections, bacteria were grown on chocolate II agar for 1 to 3 days at 37°C and 5% CO_2 prior to being inoculated into overnight broth cultures. MH broth supplemented with 0.1% glucose, 0.025% ferric pyrophosphate (Sigma), and IsoVitaleX (Becton Dickinson) was used for these cultures. For experiments including spermine, cultures were supplemented with 200 μM spermine (Sigma), an amount equal to that used in a CDM for the cultivation of *F. tularensis*, unless otherwise indicated (5). Spermidine, putrescine, and agmatine (all from Sigma) were dissolved according to the manufacturer's instructions, and cultures were supplemented with these compounds at 200 μM. Following overnight growth, bacteria were centrifuged, washed twice in Dulbecco's modified Eagle medium, and suspended in infection medium (Dulbecco's modified Eagle medium supplemented with 1% human serum, 25 mM HEPES [Invitrogen], and 1× Glutamax [Invitrogen]). Bacteria were then diluted to an appropriate multiplicity of infection (MOI) prior to infection. All MOIs were confirmed by plating to enumerate viable CFU. All work involving Schu S4 was performed under biosafety level 3 conditions with approval from the Centers for Disease Control and Prevention Select Agent Program.

Macrophage culture and infection. Macrophages were cultured as described previously (4). Unless otherwise indicated, macrophages were cocultured with bacteria at an MOI of approximately 10 for 24 h before supernatants were collected for analysis of cytokine levels. For fluorescence microscopy, macrophages were infected at an MOI of 100 for 1 h at 37°C. The high MOI was used to increase the percentage of infected macrophages (to >80%) following the short incubation. Macrophages were then treated with gentamicin (20 μg/ml) and washed three times with Hanks balanced salt solution to remove extracellular bacteria. All use of human-derived cells was approved by the University of Pittsburgh Institutional Review Board.

For infections of RAW 264.7 cells, the cells were grown to about 80% confluence in 75-cm^2 flasks (approximately 2×10^7 cells/flask) prior to infection. LVS was grown in MH broth overnight, washed, and suspended in macrophage infection medium. A portion of the washed bacteria was harvested at this point to obtain RNA for comparison to bacterial RNA recovered from the RAW 264.7 cells. RAW 264.7 cells were infected with LVS at an MOI of 500 for 2 h to establish a high infection rate. After this 2-h incubation, the cultures were treated with gentamicin (20 μg/ml) and washed three times with Hanks balanced salt solution to remove extracellular bacteria. After an additional 46 h, extracellular bacteria were washed away again using phosphate-buffered saline and RNA was isolated from the remaining RAW 264.7 cells.

ELISA analysis. Macrophage supernatants, harvested 24 h following the introduction of bacteria, were subjected to enzyme-linked immunosorbent assay (ELISA) analysis. TNF-α production was measured using a matched antibody pair and interleukin-12 (IL-12) p40 and IL-1β were measured using a DuoSet system (both from R&D Systems). Following the addition of tetramethylbenzidine substrate solution (Dako) and the measurement of optical density (OD) by using a Molecular Dynamics M2 plate reader, cytokine levels were calculated from a standard curve. The limit of detection for both ELISAs was 15 pg/ml. Levels of cytokines from macrophages exposed to bacteria grown in MH broth or MH broth with spermine were compared using Student's *t* test.

RNA harvest and analysis. RNA was harvested from broth cultures by adding 5 ml of a 16-h culture directly to 15 ml TriReagent LS (Molecular Research Center). For RAW 264.7 cultures, 15 ml of TriReagent was added to the tissue culture flask after washing. RNA extraction continued with the addition of a one-fifth volume of chloroform, and then the aqueous phase was separated by centrifugation in a Phase Lock Heavy tube (Eppendorf). RNA was precipitated with isopropanol and subjected to centrifugation. Pellets were washed with 80% ethanol and resuspended in nuclease-free water. Fifty micrograms of the RNA-containing mixture was treated with DNase (Turbo DNA-*free*; Ambion) and subjected to ammonium acetate precipitation. The RNA quantity was measured by spectrophotometry, and RNA quality was measured with an Agilent bioanalyzer.

Microarray design. Custom *Francisella* microarrays (described previously [17]) were designed using Agilent's eArray framework. All open reading frames (ORFs), including pseudogenes, from *F. tularensis* subsp. *tularensis* (Schu S4), *F. tularensis* subsp. *holarctica* LVS, *F. tularensis* subsp. *holarctica* OSU18, *F. novicida* U112, and the *Francisella* plasmids pOM1 and pFNL10 were included. Each gene from Schu S4, LVS, and OSU18 was spotted in duplicate onto the array, while the others were included as single copies.

Microarray target preparation and hybridization. Synthesis of labeled target cDNA was performed using a mixture of 10 μg of total RNA, 0.5 μg of random hexamers (Invitrogen), and Moloney murine leukemia virus (Agilent). Target cDNA was labeled with Alexa Fluor 555-conjugated dUTP according to the protocol of the manufacturer (Invitrogen). Reaction mixtures were incubated at 40°C for 3 h. Following cDNA synthesis, the remaining RNA was hydrolyzed with 10 μl 1 N NaOH and 10 μl 0.5 M EDTA. The pH was then neutralized with 10 μl 1 M HCl, and the labeled cDNA was precipitated using ammonium acetate and isopropanol and washed with 80% ethanol. Samples of 0.5 μg of labeled cDNA were hybridized to custom Agilent microarrays with an 8 by 15,000 slide format according to the manufacturer's protocol, and the arrays were incubated at 60°C for 18 h in a rotary oven. Following hybridization, arrays were washed with Agilent wash buffers before being scanned on an Agilent microarray scanner (accession no. E-MEXP-2327).

Microarray data analysis. Analysis of the microarray data was carried out using the Gene Expression Data Analysis tool (http://bioinformatics.upmc.edu/GE2/GEDA.html) (27). Briefly, this online software package was used for the normalization of data from individual arrays according to median intensity levels, followed by $\log_2$ transformation of the data. Data were grouped into categories, those for cultures with and without spermine, and subjected to J5 statistical analysis, which is designed for data sets with limited numbers of replicates and reduces the chances of false positives (27). Changes in gene expression were considered to be statistically significant if genes had a J5 score greater than 2. Tables S1 and S2 in the supplemental material report both the J5 values and degrees of change (*n*-fold) for genes with expression levels that changed significantly. Values for genes meeting these requirements were then entered into GenePattern (14), and the data are presented as a hierarchical clustering (Pearson correlation) of $\log_2$-transformed intensity values normalized with respect to medians for individual arrays. The microarray data are available in the ArrayExpress database.

Q-PCR. cDNA synthesis was performed using SuperScript III (Invitrogen) and 1 μg of total RNA. Real-time reactions were performed with a 1:5,000 final dilution of template cDNA. Primer sets were designed using Primer3 (29), and reactions were carried out on a Bio-Rad IQ5 real-time machine using SYBR green (Bio-Rad). The bacterial 50S ribosomal protein L18 gene (FTL_0252) was used as the internal reference, as it was observed to have no change in expression according to microarray data (data not shown). The quantitative PCR (Q-PCR) data are presented as $\log_2$-transformed values expressing degrees of change (*n*-fold) between cultures in MH broth with spermine and those in MH broth alone.

Construction of IS*Ftu* reporter constructs. Constructs were designed using a red fluorescent protein, tdTomato (32), as a reporter to measure gene expression. IS*Ftu1* and IS*Ftu2* sequences were amplified from the LVS genome, along with 30- and 300-bp upstream sequences, respectively, by PCR. Upstream sequences that were included were chosen based on sequence homology among 10 representatives of each transposase element. IS*Ftu* sequences were ligated into

pRSETB-tdtomato using either XbaI (IS*Ftu1*) or NheI (IS*Ftu2*) sites and BamHI sites. The IS*Ftu*-tdTomato fragment was then subcloned into pFNLTP8 by using either XbaI (IS*Ftu1*) or NheI (IS*Ftu2*) sites and EcoRI sites. Plasmids were introduced into *F. tularensis* by electroporation as reported previously (2). Briefly, *F. tularensis* LVS was grown to an OD_{600} of approximately 0.4 in 50 ml of Trypticase soy broth supplemented with 0.1% cysteine, washed three times with 500 mM sucrose, and resuspended in 4 ml of 500 mM sucrose. For electroporation, 1 μl (approximately 1 μg) of plasmid DNA was mixed with 50 μl of electrocompetent cells and the mixture was pulsed in a 0.2-cm-gap cuvette at 2.5 kV, 150 Ω, and 25 μF. Bacteria were immediately resuspended in 1 ml Trypticase soy broth supplemented with 0.1% cysteine and grown at 37°C for 4 h before selection on chocolate II agar plates supplemented with kanamycin (10 μg/ml). All vectors and primers used to create the reporter constructs are listed in Table S1 in the supplemental material. Individual colony transformants were patched onto chocolate agar plates containing kanamycin. These patches were used to inoculate overnight cultures in MH broth either with or without supplemental spermine. Cultures were diluted twofold before the measurement of fluorescence and OD_{600} with a multilabel plate reader (Molecular Devices).

RESULTS

Human macrophage response to *Francisella* LVS following cultivation in the presence of spermine. Previously, we have shown that *F. tularensis* LVS induces different macrophage responses depending on the medium used to cultivate the bacteria (4). Growth to high density in MH broth induces a bacterial phenotype that stimulates macrophages to produce high levels of cytokines (4). Alternately, high-density growth in CDM restores the parental LVS phenotype, which actively inhibits cytokine production by macrophages (4). One major difference between these two media is the presence of high levels of spermine in the CDM (5). Since polyamines have been shown to have significant effects on various processes in other bacteria (20, 26, 37, 39), we wanted to determine if the spermine component of CDM contributed to the LVS phenotype that inhibits macrophage activation. LVS was grown in CDM, MH broth, or MH broth supplemented with spermine at a concentration equal to that in CDM (200 μM), and bacteria from these cultures were tested for their ability to stimulate human monocyte-derived macrophages. Macrophages infected with bacteria that had been grown in MH broth alone produced more of the proinflammatory cytokines TNF-α (Fig. 1A) and IL-12 p40 (Fig. 1B) than those infected with bacteria grown in other media, while macrophages infected with bacteria grown in CDM produced substantially less of these cytokines, consistent with our previous observations (4). Macrophages infected with bacteria grown in MH broth supplemented with spermine produced significantly less TNF-α and IL-12 and also IL-1β (see Fig. S1 in the supplemental material) than those infected with bacteria grown in MH broth alone ($P < 0.01$ for all cytokines tested). In a corollary experiment, the growth of LVS in CDM lacking spermine resulted in a significant increase in TNF-α induction compared to that by bacteria grown in complete CDM (see Fig. S2 in the supplemental material). Importantly, the differences in cytokine production could not be attributed to defects in intracellular growth or bacterial viability because these factors did not differ if LVS was grown with or without spermine (data not shown).

To determine if spermine itself was acting directly with macrophages to affect cytokine production, the tissue culture medium was supplemented with this polyamine at the time of infection. We found that similar amounts of TNF-α were produced by macrophages in response to LVS grown in MH broth, whether or not spermine was added to the tissue culture medium (see Fig. S3 in the supplemental material). These data confirmed that the low proinflammatory cytokine production from macrophages infected with bacteria cultivated in spermine was due to a bacterial response to this polyamine.

To characterize the amount of spermine effective in inducing the observed phenotypic changes, cultures with several different spermine concentrations were set up. Bacteria were grown in MH broth supplemented with a range of spermine concentrations from 200 to 0 μM prior to the infection of macrophages. Reduced cytokine production was observed in response to bacteria grown in concentrations of spermine as low as 20 μM (Fig. 1C). These data indicate that LVS responds to spermine concentrations approximately 2 orders of magnitude lower than the free spermine concentrations in eukaryotic cells (19).

Spermine and spermidine are the end products of polyamine biosynthetic pathways, while agmatine and the diamine putrescine are precursors in these pathways (34). Therefore, we sought to determine if other compounds from these pathways could also induce the low-cytokine-induction phenotype in LVS. Bacteria were grown in MH broth without additives or MH broth supplemented with 200 μM agmatine, putrescine, spermidine, or spermine. As seen previously, the growth of LVS in MH broth led to the highest levels of cytokine induction, while the growth of bacteria in MH broth with spermine failed to elicit the same response (Fig. 1D). Similar to bacteria grown in the presence of spermine, bacteria cultivated with spermidine elicited significantly lower levels of TNF-α than those grown in MH broth alone. LVS bacteria grown with agmatine or putrescine behaved more like bacteria grown in MH broth only and elicited TNF-α production following macrophage infection (Fig. 1D). Therefore, spermine and spermidine were sufficient to trigger a change in *Francisella* that altered host cell activation. Although bacteria are capable of synthesizing spermidine, we observed that exogenous spermine or spermidine added to the bacterial growth medium elicited this phenotype. Because of the similarity of responses to spermine and spermidine, spermine was used as a representative of these polyamines in subsequent experimentation.

***Francisella* gene expression changes in response to spermine.** The change in cytokine induction suggested that substantial alterations occurred in LVS in response to spermine. Therefore, we defined the bacterial response to spermine by global measurements of gene expression. RNA was isolated from LVS grown in MH broth with or without supplemental spermine (200 μM). The RNA was then used to produce labeled target cDNA that was hybridized to a custom *Francisella* microarray. Significant differences in gene expression between LVS cultures grown with and without spermine were identified using a J5 statistical test, which was chosen to limit false positives (27). Data for those genes whose expression was significantly altered in response to spermine were put into GenePattern (14) for hierarchical clustering (Fig. 2A). The clustered data demonstrate a clear pattern of transcriptional regulation in response to extracellular spermine. Expression levels in LVS increased for 187 genes (see Table S1 in the supplemental material) and decreased for 75 genes (see Table S2 in the supplemental material) in response to spermine. This

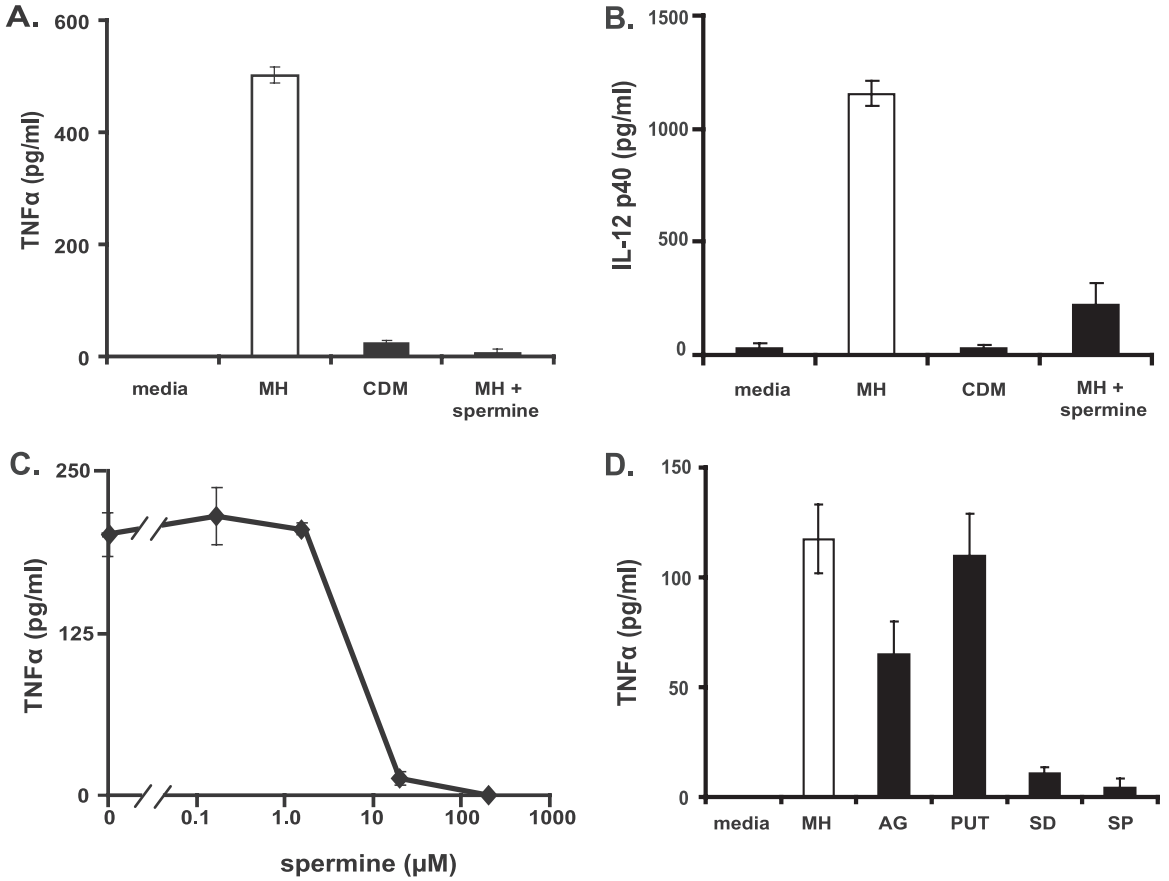

FIG. 1. Inhibition of macrophage cytokine response by *Francisella* grown in the presence of spermine. (A and B) TNF-α and IL-12 p40 production by human monocyte-derived macrophages following 24 h of culture with *F. tularensis* LVS grown in the indicated media. Uninfected control wells contained macrophages with no bacteria added (media). Other wells contained macrophages cultured with LVS (MOI, 10) grown in various bacterial media as indicated. CDM contains 200 μM spermine; MH broth with spermine was supplemented with 200 μM spermine. Data are representative of results from five independent experiments using different blood donors. (C) Titration of spermine levels. *F. tularensis* LVS was grown overnight in MH broth supplemented with various concentrations of spermine (0, 0.2, 2, 20, and 200 μM) before being inoculated into macrophage cultures. Data are representative of results from two independent experiments using different blood donors. (D) Macrophage response to LVS grown in the presence of different polyamines. LVS was grown in MH broth alone or in MH broth supplemented with 200 μM agmatine (AG), putrescine (PUT), spermidine (SD), or spermine diphosphate (SP). Macrophages were exposed to bacteria grown in the indicated media at an MOI of 10 for 24 h. Data are representative of results for two independent cultures from two independent experiments. For all panels, TNF-α levels were measured in supernatants by ELISA and data are presented as the means ± standard deviations of results for triplicate wells within one experiment.

finding indicates that a substantial portion of the *Francisella* transcriptome changes in response to this environmental cue.

To examine the differentially regulated genes more closely, we categorized them based on their genomic annotation (21, 28). The categories chosen were as follows: annotated genes, those with known and annotated functions; hypothetical genes, those with unknown functions; pseudogenes, those annotated as pseudogenes for any reason; and transposase genes, those encoding any of the transposases found in the 109 IS elements located throughout the LVS genome (28). Upregulated genes included 44 annotated and 50 hypothetical genes, 54 pseudogenes, and 39 copies of the IS*Ftu2* transposase gene (Fig. 2B, red bars). Conversely, genes with decreased expression included 51 annotated and 21 hypothetical genes, only 3 pseudogenes, and no transposases (Fig. 2B, blue bars). This analysis was remarkable because relatively high numbers of pseudogenes and transposase genes were expressed in response to spermine. In addition, many of the upregulated genes were in proximity to IS elements in the chromosome. Ten unique probes were designed by using Agilent software to analyze all *F. tularensis* LVS IS elements in the genome. Since the sequences of all members of a class of IS elements are identical, it was not possible to generate unique oligonucleotides for every individual member. Nevertheless, the IS elements are likely coordinately regulated because of their sequence identity.

To confirm the microarray results, eight genes were chosen to represent both upregulated and downregulated transcripts for analysis by real-time Q-PCR. A list of these genes and their orthologs in the *F. tularensis* Schu S4 genome is given in Table 1. Among the downregulated genes, a component of the polyamine transport (*pot*) system (*potG*), a predicted spermidine synthase gene (*speE*), and *vacJ* were chosen for validation. The transposase gene in IS*Ftu2*, a gene encoding a hypothetical

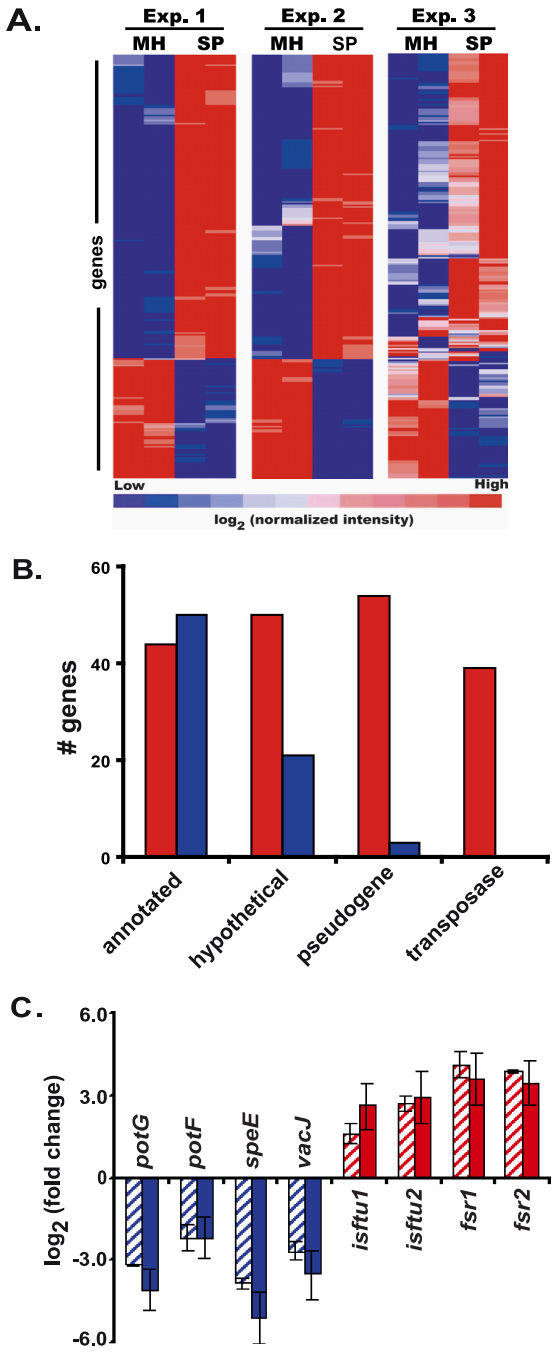

FIG. 2. *F. tularensis* LVS gene expression changes induced by spermine. (A) Hierarchical clustering of genes identified as statistically significant by the J5 test across three independent microarray experiments (experiments 1 to 3 [exp. 1 to exp. 3]). Fluorescence intensities across each array were normalized by dividing by the median intensity for that array and subjecting the data to $\log_2$ transformation. The values from each experiment were input into GenePattern individually and clustered using the Pearson correlation. The 185 induced genes and 75 repressed genes clustered together in GenePattern, validating the statistical analysis by the Gene Expression Data Analysis tool. MH, MH broth alone; SP, MH broth supplemented with spermine. (B) Categorical summary of spermine-induced changes in gene expression. Genes exhibiting increased (red bars) or decreased (blue bars) transcription levels were grouped into functional categories based on their NCBI annotation. Category definitions were as follows: annotated genes, those with known or predicted functions; hypothetical genes, those with unknown functions; pseudogenes, genes annotated as pseudogenes due to mutation, insertion of an IS element, or an unspecified reason; and transposase genes, including any of the 109 annotated transposase-encoding IS elements found in the LVS genome. Data are presented as total numbers of genes per category and are derived from the three independent microarray experiments described in the legend to panel A. (C) Gene expression changes measured by Q-PCR. RNA from two of the microarray experiments described in the legend to panel A and two independent experiments were tested for specific genes using real-time PCR. The results of Q-PCR are represented by solid bars, while corresponding values from the microarray experiments ($n = 3$) are represented by striped bars. Data are presented as means ± standard errors (SEM) of the means of $\log_2$-transformed change (n-fold) values, where the level of change (n-fold) is the ratio of expression in MH broth plus spermine to expression in MH broth alone.

TABLE 1. Gene orthologs[a] in *F. tularensis* strains

Gene or element name	Predicted product	LVS locus(i)	Schu S4 locus(i)
potG	Polyamine transporter	FTL_0681	FTT0562
potF	Putrescine-binding periplasmic protein	FTL_1582	FTT0481
speE	Spermidine synthase	FTL_0500	FTT0431
vacJ	VacJ lipoprotein	FTL_0765	None[b]
IS*Ftu1*	Transposase	ML[c]	ML
IS*Ftu2*	Transposase	ML	ML
fsr1[d]	Hypothetical protein	FTL_1401	FTT1480c
fsr2	Hypothetical membrane protein	FTL_0791	FTT1163c
fsr3	Secretion protein	FTL_1844	FTT0018

[a] Orthologs were defined using BioHealthBase (http://www.biohealthbase.org).
[b] No gene ortholog is present in the Schu S4 genome.
[c] ML, multiple loci (element found at multiple loci in the genome).
[d] *fsr*, *Francisella* spermine response gene.

protein with homology to the product of *lpxF* from *F. novicida* (referred to herein as *fsr1* [F*rancisella s*permine *r*esponse gene *1*]), and an ORF encoding a hypothetical protein specific to *Francisella* (*fsr2*) were chosen from the list of induced genes. Two other genes of interest with J5 scores just below our cutoff were also examined by Q-PCR. These were another member of the annotated *pot* system, *potF*, and the transposase gene in the other predominant class of IS elements, IS*Ftu1*. All of the genes tested by Q-PCR exhibited patterns of expression similar those seen in the microarray experiments (Fig. 2C), validating the previous microarray results. These results also independently confirmed increased transcription of the transposase genes in the IS elements IS*Ftu1* and IS*Ftu2*.

Role of *Francisella* IS elements in the response to spermine. Since the expression of a large number of transposases increased in response to spermine, we investigated transcriptional regulation by the IS elements. Reporter constructs were created in which the sequence of a single copy of either IS*Ftu1* or IS*Ftu2* was cloned upstream of a promoterless allele for the red fluorescent protein tdTomato (32). The IS sequences used for these experiments included the annotated transposase ORF and the upstream sequence that was conserved among multiple IS*Ftu1* copies (30 bp) or IS*Ftu2* copies (300 bp) in the LVS genome (Fig. 3A). The IS*Ftu*-tdTomato gene fusions

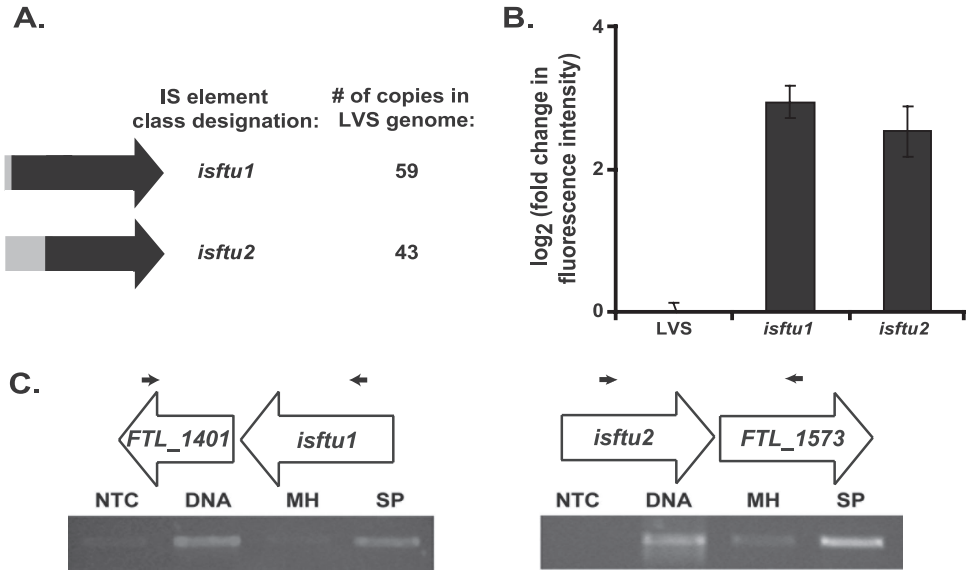

FIG. 3. *F. tularensis* LVS IS elements contain spermine-responsive promoters. (A) Schematic representation of the two predominant classes of *F. tularensis* IS elements. Transposase ORFs are indicated by arrows. Light gray boxes represent the conserved sequence found upstream of 10 copies of each element randomly sampled from the genome: IS*Ftu1*, 30 bp; IS*Ftu2*, 300 bp. (B) Spermine induces expression of tdTomato using IS*Ftu1* or IS*Ftu2* as the promoter element. *F. tularensis* LVS carrying either IS*Ftu1* or IS*Ftu2* (i.e., the transposase gene and 5′-end conserved sequence depicted in panel A) cloned in front of tdTomato was grown in either MH broth or MH broth supplemented with 200 μM spermine. Data are presented as means ± SEM of $\log_2$-transformed values (normalized fluorescence intensity for culture in MH broth with spermine/normalized fluorescence intensity for culture in MH broth alone) for six independent transformants read in triplicate (18 total measurements per experiment). These data are representative of over 25 *F. tularensis* LVS transformants harboring each reporter construct. (C) Identification of polycistronic mRNA containing IS elements and downstream genes. Upper panels depict the genomic organization of the IS elements and downstream genes used in this study. Black arrows illustrate the locations of primers used for RT-PCRs. Primers were designed to generate amplicons spanning the IS elements and ORFs in the 3′ direction from the IS elements. (Lower panels) Agarose gels stained with ethidium bromide showing amplicons from RT-PCRs. RNA was derived from LVS following growth in broth culture. NTC, no-template control; DNA, positive control using LVS genomic DNA as a template; SP, MH broth with 200 μM spermine. No bands were observed in control samples run without RT.

were cloned into pFNLTP8 and transferred into LVS (25). Fluorescence levels were then examined following cultivation in MH broth with or without spermine. When *F. tularensis* LVS carrying these reporter constructs was grown in the presence of spermine, a six- to eightfold induction of fluorescence was observed (Fig. 3B). Reporter constructs driven by either IS*Ftu1* or IS*Ftu2* behaved similarly, as predicted by the results of the gene expression experiments. In contrast, neither LVS alone (Fig. 3B) nor LVS carrying a promoterless control vector (data not shown) was fluorescent after incubation with spermine.

While the reporter constructs confirmed transcriptional activation of the IS element and a downstream red fluorescent protein allele, it was unclear if the IS elements directly regulated the expression of endogenous genes in the LVS chromosome. One possible mechanism for such an event would be the production of a polycistronic message emanating from the IS element that includes the downstream gene. To examine this, we performed reverse transcriptase PCR (RT-PCR) analyses of regions spanning the sequences of two genes shown to be regulated by spermine, FTL_1401 and FTL_1573, and the IS elements (IS*Ftu1* and IS*Ftu2*, respectively) located directly upstream in the *F. tularensis* LVS genome (Fig. 3C). RT-PCR analyses of these genes and their respective IS elements produced bands of the predicted size (~850 bp) (Fig. 3C). In addition to showing that FTL_1401 and FTL_1573 were cotranscribed with their upstream IS elements, this semiquantitative technique showed increased levels of the polycistronic messages in LVS bacteria grown in the presence of spermine. Controls without a template (Fig. 3C) and without RT (data not shown) confirmed that the RT-PCR amplicons were generated from RNA templates. Together with the microarray and Q-PCR data, these results show that the *F. tularensis* IS elements harbor spermine-responsive promoters.

Spermine-responsive genes are regulated during intramacrophage growth. Because spermine is present at millimolar levels in the cytosol of eukaryotic cells (19), we hypothesized that the macrophage environment would activate transcription of spermine-responsive genes. To test this hypothesis, we utilized the murine cell line RAW 264.7 because the available number of primary human macrophages was inadequate to yield sufficient amounts of LVS RNA. LVS was grown within RAW 264.7 cells for 48 h as described in Materials and Methods. Then RNA was isolated and LVS gene expression levels were measured by Q-PCR. Patterns of gene expression changes induced by intramacrophage growth (Fig. 4A) closely resembled those observed upon bacterial growth in MH broth supplemented with spermine (Fig. 2). While the magnitudes of gene expression changes differed slightly in some cases, the overall trends were identical to that observed for LVS cultured in MH broth plus spermine (Fig. 2C and 4A). Although still induced, *fsr2* exhibited a smaller increase in gene expression in macrophages than in broth medium supplemented with sperm-

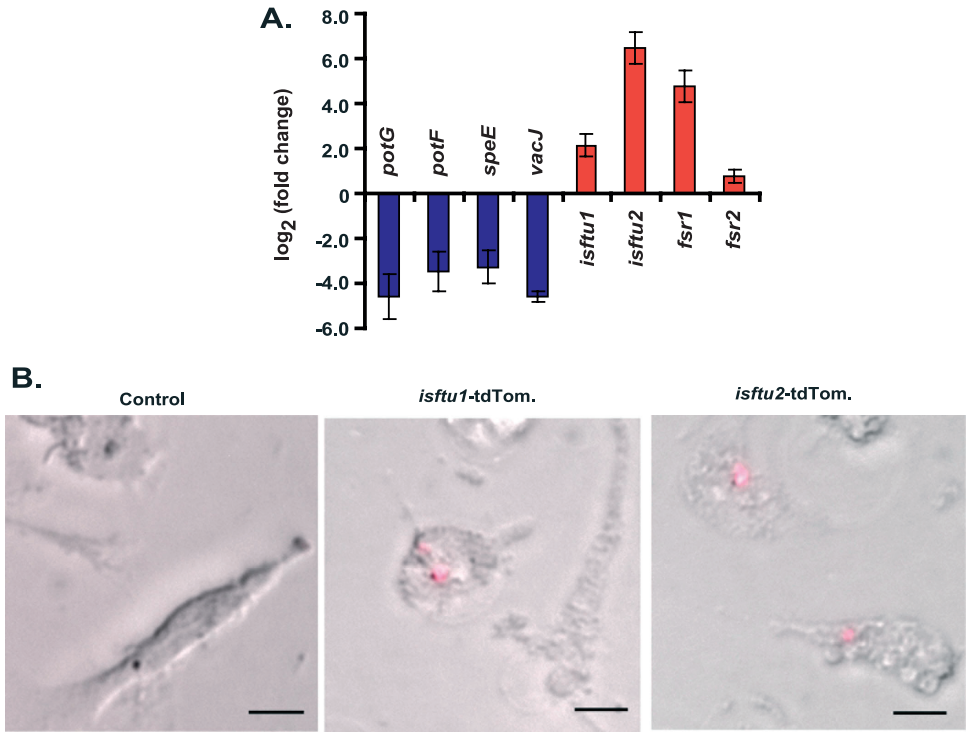

FIG. 4. Regulation of spermine-responsive genes by LVS during intracellular growth. (A) LVS gene expression changes induced by growth in RAW 264.7 cells were examined using Q-PCR. The data are presented as $\log_2$-transformed change (*n*-fold) values comparing LVS bacteria grown intracellularly to bacteria grown in MH broth. Data are the means ± SEM of results from three independent experiments. (B) IS element reporter constructs are activated inside macrophages. LVS strains without (control) or with the IS element-tdTomato reporter constructs were grown in MH broth and used to infect macrophages at an MOI of 100 for 2 h, after which the macrophages were washed and treated with gentamicin. Macrophages were examined for tdTomato expression at 48 h postinfection by using fluorescence microscopy. Scale bars represent 10 μm. Similar results were obtained from four separate experiments.

ine. This finding may be the result of converging signals present in the macrophage cytosol, including reduced levels of iron and glucose, two cues that have been shown to affect *Francisella* gene expression (8, 18). Nevertheless, these results show that gene expression changes observed in response to spermine also occur during intramacrophage infection.

We next used the reporter constructs described above (Fig. 3) to investigate transcription from *Francisella* IS elements in human cells and validate the results obtained with RAW 264.7 cells. For these experiments, human macrophages were infected with LVS or strains bearing these reporter constructs and visualized by fluorescence microscopy. Induction of tdTomato expression was observed when the bacteria were located inside macrophages but not when the bacteria were extracellular (Fig. 4B). Similar to bacteria in broth cultures, neither LVS alone nor LVS with a promoterless tdTomato construct exhibited fluorescence during macrophage infections (Fig. 4B and data not shown). These results are consistent with the possibility that the LVS response to spermine, specifically the activation of a cryptic promoter located within the IS elements, occurs during infection of human macrophages.

Response of virulent *F. tularensis* to spermine. Since our experiments focused on LVS, it was unknown if similar phenotypes would be seen with a fully virulent strain of *F. tularensis.* We investigated the role of the spermine response in virulent *Francisella* by using the Schu S4 strain. We first examined macrophage cytokine production stimulated by Schu S4 following growth with or without spermine. As shown previously (Fig. 1), LVS bacteria grown in MH broth with spermine elicited a minimal TNF-α response while LVS bacteria grown in MH broth alone stimulated significantly higher cytokine production as measured by ELISA (Fig. 5A). Schu S4 demonstrated similar behavior. When grown in the presence of spermine, Schu S4 stimulated substantially less TNF-α from the host macrophages than Schu S4 grown in MH broth alone (Fig. 5A). Similar to the effects for LVS, the effects for Schu S4 were not limited to TNF-α since Schu S4 grown without spermine stimulated significantly more IL-1β production than Schu S4 grown with spermine (see Fig. S1 in the supplemental material).

To further examine the spermine response in the virulent strain, we measured gene expression changes in Schu S4 bacteria grown in the presence and absence of spermine. RNA was isolated from Schu S4 cultures grown with or without 200 μM spermine, and the expression levels of several Schu S4 genes whose orthologs were identified in our microarray analysis using LVS (Fig. 2) were tested by Q-PCR. Schu S4 exhibited patterns of gene expression changes very similar to those in LVS in response to spermine. The Schu S4 orthologs of *potG*, *potF*, and *speE* were downregulated following growth in spermine, while *fsr1* and *fsr2* were upregulated (Fig. 5B). Moreover, the expression of transposases encoded by the Schu S4 IS elements also increased following growth in spermine. In side-by-side cultures, we found that the levels of transposase

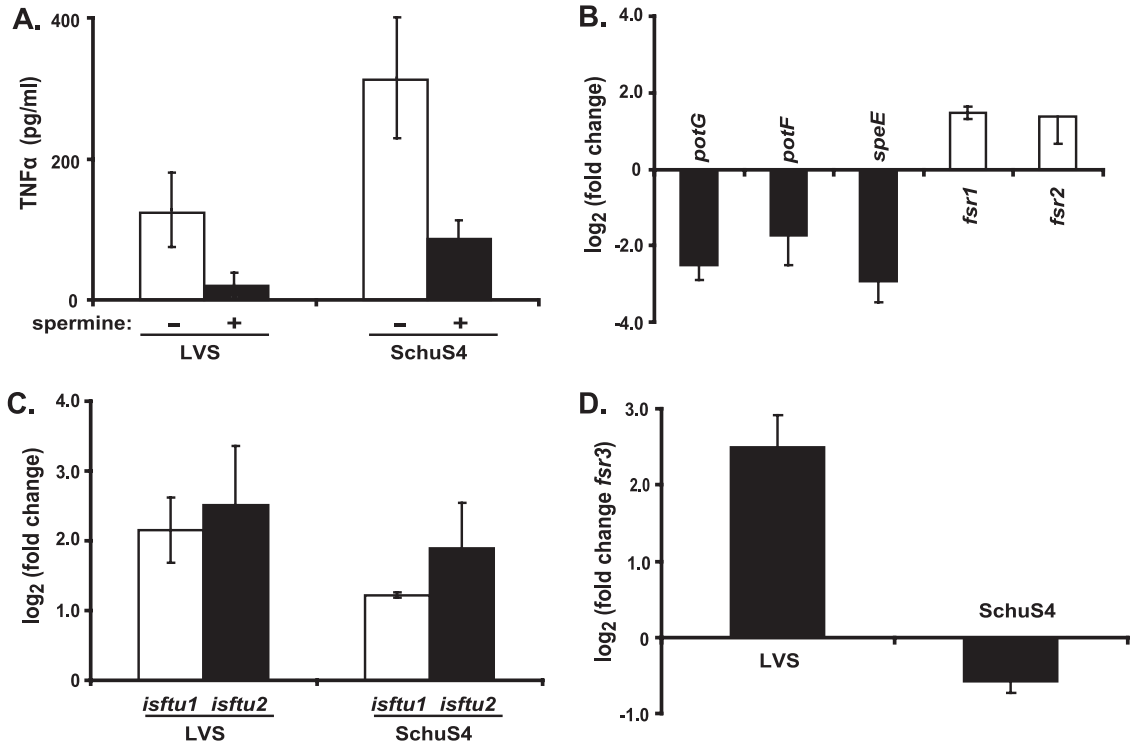

FIG. 5. Response of virulent *F. tularensis* Schu S4 to spermine. (A) Comparison of levels of TNF-α production by human monocyte-derived macrophages following exposure to either *F. tularensis* LVS or *F. tularensis* Schu S4 bacteria grown in the indicated media at an MOI of 10 for 24 h. White bars represent results for the indicated bacteria grown in MH broth; black bars represent results for bacteria grown in MH broth with 200 μM spermine. Data are presented as means ± SEM of results from four independent experiment using different blood donors. (B) Schu S4 gene expression changes in response to spermine. RNA was generated from Schu S4 cultures grown in MH broth with or without 200 μM spermine. The Q-PCR data are presented as means ± SEM of $\log_2$-transformed change (*n*-fold) values comparing bacteria grown with spermine to those grown without ($n = 3$ experiments). (C) Patterns of expression of different IS elements in response to spermine are similar in both LVS and Schu S4. The expression of both transposase gene elements, IS*Ftu1* (white bars) and IS*Ftu2* (black bars), increased in response to extracellular spermine. The Q-PCR data are presented as means ± SEM of $\log_2$-transformed change (*n*-fold) values comparing bacteria grown with spermine to those grown without ($n = 3$ experiments). (D) Strain-specific gene expression controlled by spermine and the presence of an IS element. The expression of *fsr3* was measured in LVS (which contains an IS*Ftu2* IS element upstream of *fsr3*) and Schu S4 (which contains no upstream IS element) by Q-PCR. The data are presented as means ± SEM of $\log_2$-transformed change (*n*-fold) values comparing bacteria grown with spermine to those grown in MH broth only ($n = 4$ experiments).

mRNAs from IS*Ftu1* (Fig. 5B, white bars) and IS*Ftu2* (Fig. 5B, black bars) increased in both LVS and SchuS4 after growth in medium containing spermine (Fig. 5C). Together, these results illustrate the commonality of spermine-induced responses between these two *Francisella* strains.

Since the IS elements appear to regulate transcription in response to spermine, we hypothesized that there would be differential gene expression patterns based on the locations of IS elements in different bacterial chromosomes. To investigate this possibility, we studied the expression of a spermine-regulated gene in LVS identified as *fsr3* (Table 1). This gene was identified by microarray analysis as having higher transcript levels in LVS after spermine treatment. In addition, this gene is downstream of an IS*Ftu2* element in the LVS genome. The expression of genes near *fsr3* in LVS was also induced by spermine, though these changes did not reach statistical significance in the microarray analysis. In contrast, the *fsr3* ortholog in Schu S4 is not downstream of an IS element. As expected, culturing LVS with spermine increased the expression of *fsr3* as measured by Q-PCR (Fig. 5D). Schu S4, however, failed to increase the expression of its *fsr3* ortholog, in fact showing a slight decline in this gene's expression level. Taken together, these data show that spermine responsiveness is conserved across these *Francisella* strains. In addition, *F. tularensis* IS elements contain promoter sequences that influence the transcription of downstream genes in response to this signal.

DISCUSSION

Although *F. tularensis* is a pathogen known to infect many cell types, the mechanisms involved in bacterial adaptation to its intracellular lifestyle have not been elucidated. Here, we have reported that spermine and spermidine, polyamines found in abundance in eukaryotic cells (19), dramatically alter cytokine induction by *F. tularensis*. Additional studies showed significant changes in the LVS transcriptome in response to spermine. We also present evidence that similar transcriptional changes occur during infection of macrophages, supporting the idea that *Francisella* recognizes polyamines as a signal of the intracellular environment.

Polyamine recognition is likely a component of a sophisti-

cated system that integrates multiple environmental signals and regulates gene expression in *Francisella* (8, 17, 18). Wehrly et al. observed significant changes in gene expression during the growth of *F. tularensis* in macrophages, including multiphasic expression changes in genes within the pathogenicity island (38). Hazlett et al. demonstrated similar protein profiles when bacteria were grown in macrophages and in brain heart infusion broth (15). These conditions were associated with higher levels of intracellular growth locus proteins B and C (IglB and IglC), among others, but growth in MH broth was not. The bacterial response to spermine alters macrophage cytokine production and may be a component of the bacterial response to brain heart infusion broth, in which a similar cytokine phenotype was observed (15). The spermine response alone, however, does not account for the cumulative bacterial response to the macrophage cytoplasm. For example, our microarray data indicate that genes encoding IglA, IglC, and IglD are repressed by spermine (see Table S2 in the supplemental material). This finding is consistent with the early cytoplasmic phase defined by microarray analysis (38) but does not reflect the levels of IglC protein, for example (15, 38). Therefore, the gene expression changes we have defined in response to spermine are likely to be combined with responses to other signals that optimize mRNA levels, protein levels, and ultimately the replication of *Francisella* within host cells.

Global gene expression analysis using a comprehensive *Francisella* microarray provided key insights in our studies. One of the more unexpected findings was induction of the expression of transposase genes in the IS elements, specifically IS*Ftu1* and IS*Ftu2*, by spermine (Fig. 2 and 4; see also Table S1 in the supplemental material). In addition, many of the nontransposase genes induced by spermine treatment were adjacent to the IS elements in the genome. This relationship led to the hypothesis that the IS elements carry a spermine-responsive promoter. The results of further experimentation using reporter constructs supported this hypothesis (Fig. 3). Although IS elements are typically associated with genetic rearrangements, other functions have been reported, including service as promoter elements for nearby genes (24). There are, however, no reports of the gene regulation in *Francisella* observed in the present study occurring on a global scale (23, 24, 30). Moreover, this is the first report of polyamines regulating transcription from IS elements.

The availability of different *F. tularensis* strains afforded us the opportunity to test important features of our system. The genome sequences of various *Francisella* subspecies contain over 100 IS elements; however, their genomic localization varies among subspecies (28). Both strains in our study, LVS and Schu S4, stimulated much less cytokine production from human macrophages when grown in the presence of spermine than in the absence of this polyamine, and these strains had similar patterns of gene expression when grown with spermine. Therefore, a mechanism by which *F. tularensis* disrupts innate immune cell activation is regulated by the spermine response and appears to be shared between these two strains. Studying the responses shared between LVS and Schu S4 will provide insight into this disruption of innate immune cell function. In at least one instance, however, gene expression patterns in LVS and Schu S4 following exposure to spermine were dissimilar (Fig. 5D). This discrepancy indicates that gene regulation in response to spermine differs among various francisellae, in part due to IS element location. Unique IS element locations and the resulting changes in gene regulation may, therefore, contribute to the wide range of virulence levels exhibited by different *Francisella* strains in vivo. Global gene expression studies that compare spermine responses in LVS and Schu S4 will help delineate both the similarities and differences that characterize these strains.

We hypothesized that genes annotated as ABC transporter genes for polyamine uptake, *potFGHI*, controlled the spermine response that we have characterized. Transcription of these genes decreased following growth with supplemental spermine (Fig. 2; see also Table S2 in the supplemental material). This result was expected since less transport machinery would be required when extracellular spermine was abundant. To test the role of this putative polyamine transporter system, disruption mutations were created in the genes encoding the periplasmic binding protein (*potF* [FTL_1582]), the cytoplasmic ATP-binding protein (*potG* [FTL_0681]), and one of the two membrane proteins (*potH* [FTL_0682]). All of the mutants, however, retained a wild-type phenotype of cytokine induction or repression when grown in the presence or absence of spermine, respectively (data not shown). This finding suggests that spermine regulates *Francisella* through another mechanism, which is currently under rigorous investigation.

Polyamine effects on bacterial virulence are an emerging theme in host-pathogen interactions. As mentioned above, polyamines regulate biofilm formation in *Vibrio* and *Yersinia* (20, 26). It is also of interest that host polyamine synthesis pathways are activated by pathogens. The infection of mice with *F. tularensis* subsp. *tularensis* (1) and murine macrophages with *Bacillus anthracis* (3) increases the expression of host ornithine decarboxylase. This enzyme catalyzes a key early step in polyamine synthesis and is likely to result in spermine accumulation, potentially enhancing pathogen fitness in this environment. In anthrax infections, increased host ornithine decarboxylase activity is associated with the suppression of apoptosis of infected macrophages, thereby preserving the pathogen's niche (3). These observations, along with those presented in our study, suggest that pathogen responses to polyamines may be a common regulator of virulence and a potential target for therapeutics.

Our results suggest a model in which spermine and spermidine found in the macrophage cytosol elicit substantial changes in *Francisella* gene expression that culminate in little macrophage stimulation and low cytokine production. *Francisella* is known to be taken up into phagocytic vesicles (7), only to escape into the cytoplasm within a few hours (6, 7, 31). In our model, the bacterium encounters spermine and spermidine once it escapes from a phagosome and reaches the cytoplasm. Cytoplasmic polyamines may then trigger *F. tularensis* to alter its gene expression profile under the control, in part, of the many IS elements present in the *Francisella* genome. Though the mechanism by which the bacterium responds to polyamines and subsequently alters transcription remains to be determined, the result likely enables the bacterium to adapt to its new environment. Taken together, these data define a complex system of gene regulation in response to an intracellular environment that contributes to the overall success of this pathogen. Because other intracellular pathogens are likely to en-

counter elevated levels of polyamines within host cells, the adaptive response we have characterized for *F. tularensis* may be broadly relevant for bacterial pathogenesis.

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