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Hfq, a Novel Pleiotropic Regulator of Virulence-Associated Genes in *Francisella tularensis*▽

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Francisella tularensis **is a highly infectious pathogen that infects animals and humans, causing tularemia. The ability to replicate within macrophages is central for virulence and relies on expression of genes located in the** ***Francisella*** **pathogenicity island (FPI), as well as expression of other genes. Regulation of FPI-encoded virulence gene expression in** ***F. tularensis*** **involves at least four regulatory proteins and is not fully understood. Here we studied the RNA-binding protein Hfq in** ***F. tularensis*** **and particularly the role that it plays as a global regulator of gene expression in stress tolerance and pathogenesis. We demonstrate that Hfq promotes resistance to several cellular stresses (including osmotic and membrane stresses). Furthermore, we show that Hfq is important for the ability of the** ***F. tularensis*** **vaccine strain LVS to induce disease and persist in organs of infected mice. We also demonstrate that Hfq is important for stress tolerance and full virulence in a virulent clinical isolate of** ***F. tularensis*****, FSC200. Finally, microarray analyses revealed that Hfq regulates expression of numerous genes, including genes located in the FPI. Strikingly, Hfq negatively regulates only one of two divergently expressed putative operons in the FPI, in contrast to the other known regulators, which regulate the entire FPI. Hfq thus appears to be a new pleiotropic regulator of virulence in** ***F. tularensis*****, acting mostly as a repressor, in contrast to the other regulators identified so far. Moreover, the results obtained suggest a novel regulatory mechanism for a subset of FPI genes.**

Regulation of gene expression by noncoding or small RNAs (sRNAs) is prevalent in both prokaryotes and eukaryotes, and recent studies have identified numerous sRNAs in different organisms. Most sRNAs encoded by bacterial chromosomes act by base pairing with mRNA targets that are encoded in *trans*, and these sRNAs commonly require Hfq in order to function (47). Hfq is a bacterial RNA-binding protein that was initially recognized as a host factor for replication of the Qβ RNA phage in *Escherichia coli* (17). The Hfq protein is very abundant, and it belongs to the eukaryotic and archaeal families of Sm and Sm-like proteins, respectively, that form homohexameric structures (for reviews, see references 5 and 53). The importance of Hfq became clear when an *E. coli hfq* null mutant was created. This mutant had pleiotropic phenotypes, such as a decreased growth rate, increased sensitivity to cellular stresses, and increased cell length (51). Hfq is a posttranscriptional regulator that binds sRNAs and mRNA and facilitates RNA-RNA interaction (1, 18, 23, 32, 35, 55). For the most part, sRNA-mRNA interactions result in mRNA degradation and/or inhibition of translation, but they can also increase translation (for reviews, see references 1, 20, and 48). As might be expected from the pleiotropic phenotypes of an *hfq* mutant, deletion of *hfq* has been correlated with considerable changes in gene expression observed at the mRNA or protein level (14, 15, 21, 42, 46).

In the last few years, the role of Hfq in the pathogenesis of several bacterial species has been examined (11, 14, 30, 37, 39, 41, 42, 45). A *Salmonella enterica* serovar Typhimurium *hfq* mutant was found to be severely attenuated for intracellular replication in vitro and for virulence in mice (42). Similarly, decreased virulence was observed for *hfq* mutants of *Brucella abortus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Shigella flexneri*, and *Legionella pneumophila* (11, 14, 30, 39, 41, 45). In many cases, the *hfq* mutation also rendered the bacteria more sensitive to cellular stress. In striking contrast, deletion of *hfq* in three different *Staphylococcus aureus* strain backgrounds did not result in any detectable phenotype (4).

Bacteria have developed numerous regulatory mechanisms so that they are capable of a rapid molecular response with coordinated gene expression in order to survive in a variety of hostile environments. A macrophage is probably one of the most stressful environments that facultative intracellular bacterial pathogens encounter. The aim of the present work was to understand how Hfq contributes to the stress response and virulence of *Francisella tularensis*, a gram-negative facultative intracellular pathogen that causes tularemia in humans and a large number of animal species. Three *F. tularensis* subspecies are capable of inducing disease in humans: *F. tularensis* subsp. *tularensis* (type A), *F. tularensis* subsp. *holarctica* (type B), and *F. tularensis* subsp. *mediasiatica*. The fourth subspecies of *F. tularensis* ("*F. tularensis* subsp. *novicida*") causes disease in

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mice, but it is rarely pathogenic in humans. In humans, an *F. tularensis* infection can be acquired by ingestion of contaminated water or food, through arthropod bites, from contaminated animal carcasses, or by inhalation of bacteria. Due to its high infectivity and the severity of the disease, *F. tularensis* has been classified by the Centers for Disease Control and Prevention as a potential biological weapon (for reviews, see references 29 and 43).

The pathogenicity of *F. tularensis* is believed to be dependent largely on its ability to replicate inside host cells, particularly macrophages. Several factors important for virulence have been identified, but the molecular mechanisms underlying disease are unclear. Several of the genes located in the *Francisella* pathogenicity island (FPI) (38), such as *iglC*, *iglD*, *iglA*, *pdpA*, *pdpB*, and *pdpD*, have been demonstrated to be required for virulence in at least one subspecies (12, 19, 24, 27, 38, 40, 49, 54).

There are only three known regulators of virulence in *F. tularensis*, MglA, SspA, and PmrA, all of which control expression of FPI genes (3, 7, 10, 25, 34). Each of these proteins also regulates expression of genes outside the FPI; however, whereas MglA and SspA regulate the same set of genes outside the FPI, PmrA controls a separate group of genes (7, 10, 34). Recently, a new protein regulator of FPI gene expression was identified (6). This protein, FevR, controls the genes in the MglA-SspA regulon and seems to act in parallel with MglA (6).

The genome of *F. tularensis* does not encode any complete regulatory two-component systems (34) and encodes only one putative alternative sigma factor, σ^{32} (31), suggesting that this organism has an exceptionally limited repertoire of pleiotropic regulatory mechanisms. Expression of the alternative sigma factors σ^{S} and/or σ^{E} is controlled by the regulatory protein Hfq in several gram-negative bacteria, such as *E. coli*, *S.* Typhimurium, *P. aeruginosa*, and *V. cholerae*, and the sigma factors play an integral role in the Hfq regulatory network in these species (8, 14, 21, 36, 45). As the *F. tularensis* genome does not encode either σ^{S} or σ^{E}, the Hfq regulatory network could be expected to be different from the regulatory networks in other bacterial species.

This prompted us to address the role of Hfq in stress resistance and virulence in *F. tularensis* subsp. *holarctica*. For this, we created an *hfq* deletion mutant of the live vaccine strain, strain LVS, and demonstrated that it has pleiotropic phenotypes, such as slower growth and increased sensitivity to cellular stress, and is severely attenuated for virulence in mice. A similar mutant of the virulent clinical isolate *F. tularensis* subsp. *holarctica* FSC200 was also investigated. Using this mutant, we could verify that Hfq is also important for growth, stress tolerance, and full virulence in a human-pathogenic *Francisella* strain. Finally, we show that Hfq is a global regulator in *F. tularensis* LVS and that it controls expression of a subset of genes located in the FPI. Thus, Hfq is a novel pleiotropic regulator of virulence in *F. tularensis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *F. tularensis* LVS and a Δ*hfq* derivative were routinely grown in liquid Schaedler medium containing vitamin K3 (Schaedler K3 medium) (Biomerieux), in Chamberlain medium (9), on premade chocolate agar plates (Biomerieux), or on chocolate agar plates prepared from Difco GC medium supplemented with hemoglobin and IsoVitaleX enrichment (BD Diagnostics). *F. tularensis* FSC200 and an Δ*hfq* derivative (FSC768) were routinely grown in Chamberlain medium or on McLeod agar plates. *E. coli* strains were grown in Luria-Bertani medium or on Luria-Bertani agar plates. When required, the medium was supplemented with kanamycin (10 to 20 μg/ml for *F. tularensis* and 50 μg/ml for *E. coli*), chloramphenicol (1.75 μg/ml for LVS and 10 μg/ml for *E. coli*), or polymyxin B (100 μg/ml).

Construction of *hfq* mutant strains. The counterselectable plasmid pPV-hfq used to generate a nonpolar *hfq* deletion mutant of strain LVS was constructed by overlap PCR. Primers Hfq-AF and Hfq2 amplify the 912-bp region upstream of position 13, and primers Hfq3 and Hfq-BR amplify the 1,021-bp region downstream of position 210 (relative to the *hfq* translation start site). Primers Hfq3 and Hfq2 have 23 nucleotides of overlapping sequence, resulting in fusion of *hfq* codon 4 to *hfq* codon 71 after crossover PCR. PCR with primers Hfq-AF and Hfq2 and with primers Hfq3 and Hfq-BR were performed with Mango *Taq* polymerase (Bioline, Massachusetts), and the products were purified using a QIAquick PCR purification kit (Qiagen, California). Two microliters of each purified product was included in a PCR mixture without primers and subjected to 20 cycles of PCR (94°C for 40 s, 48°C for 40 s, and 72°C for 90 s) to anneal and extend the crossover product. Next, 2 μl of the extended crossover product was used as a template for PCR using primers Hfq-AF and Hfq-BR. The gel-purified fragment was digested with NheI and SalI and cloned into XbaI-SalI-digested pPV (19). The plasmid was introduced into *F. tularensis* LVS by conjugation from *E. coli* strain S17-1. S17-1/pPV-hfq and LVS were grown to exponential phase, mixed (ratio, approximately 1:20) in 0.9% NaCl, and spotted onto Schaedler medium agar plates (Bio-Rad) supplemented with MEM vitamins (Sigma-Aldrich). The plates were incubated at 25°C for 16 h, and bacterial material was recovered in Schaedler K3 medium (Biomerieux) supplemented with 100 μg/ml polymyxin B (Sigma). After 3 h of incubation at 37°C (to eliminate the majority of the *E. coli* cells), bacteria were spread on chocolate agar plates containing 100 μg/ml polymyxin B and 1.75 μg/ml chloramphenicol. Colonies appeared after 5 to 6 days of incubation at 37°C and were subsequently passaged once on plates with selection, followed by one passage in liquid medium without selection (to allow recombination to occur). Next, bacteria were passaged once on agar plates containing 5% sucrose. Isolated colonies were checked for loss of the wild-type *hfq* gene by analyzing the size of the fragment obtained after PCR using primers 5up-hfqA and 3down-hfqB (which annealed to the regions outside the fragment cloned in pPV-hfq). Furthermore, colonies were verified to be *F. tularensis* colonies using primers TUL4-435 and TUL4-863 (44). One colony harboring an *hfq* gene that was smaller, as determined by PCR analysis, was used for further study. Genomic DNA was isolated and used as a template in a PCR with the 5up-hfqA/3down-hfqB primer pair. The PCR product was directly sequenced using primers Hfq-AF, Hfq-BR, and miaA-F870 to verify the presence of an *hfq* gene with an in-frame deletion of codons 5 to 70. As a control, genomic DNA from the LVS wild-type strain was used in the same PCR, and the product was sequenced. Sequencing confirmed the deletion of Hfq amino acids 5 to 70 and revealed that a single nucleotide substitution resulted in an amino acid substitution (Gly→Cys) in the remaining peptide in the mutant clone.

The *hfq* mutant of the FSC200 strain was constructed essentially as previously described (19). Briefly, a deletion construct was amplified by PCR using primer pairs Hfq_{200}-A/Hfq_{200}-B and Hfq_{200}-C/Hfq_{200}-D with strain FSC200 genomic DNA as the template. The resulting overlap PCR fragment, the Hfq_{200}-A/Hfq_{200}-D fragment, was cloned into the pCR4TOPO cloning vector (Invitrogen) and subsequently cloned as a 2.4-kb PmeI-NotI fragment into SmaI-NotI-digested suicide mutagenesis vector pDMK2 (K. Kadzhaev, unpublished). pDMK2-hfq, carrying the deletion fragment, was introduced into recipient strain FSC200 by conjugal mating with *E. coli* S17-1λ*pir* as the donor strain. Single-crossover allelic exchange of the suicide plasmid was selected by plating bacteria on McLeod agar plates containing kanamycin and polymyxin B. To complete the allelic exchange, a second recombination event was selected for utilizing *sacB*-dependent sucrose sensitivity as described previously (33). The resulting mutant strain (FSC768) was verified by PCR using primers Hfq_{200}-E_For and Hfq_{200}-F_Rev and by loss of kanamycin resistance.

Determination of the transcription start site of *hfq*. The 5′ end of the *hfq* mRNA was determined by 5′ rapid amplification of cDNA ends (5′-RACE) using a 5′/3′ RACE kit from Roche Applied Science. One microgram of RNA isolated from LVS grown in Schaedler K3 medium at exponential phase was used as the template for cDNA synthesis using the gene-specific primer hfq_R194. The cDNA was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA), a poly(A) tail was added at the 3′ end, and the cDNA was PCR amplified using the gene-specific primer hfq_R133 and the poly(A) tail-specific primer oligo dT-anchor. An aliquot of the PCR product was used as the template

TABLE 1. Plasmids, strains, and primers used in this study

Plasmid, strain, or primer	Genotype, description, or sequence	Reference, source, or comment
Plasmids		
pFNLTP6 *gro-gfp*	Shuttle plasmid with LVS *groE* operon promoter upstream of *gfp*, Km^r Ap^r	28
p6-*gro*	Shuttle plasmid derivative with *groE* promoter, Km^r Ap^r	This study
p6-*gro-hfq*	Shuttle plasmid derivative with *groE* promoter upstream of *hfq*, Km^r Ap^r	This study
pPV	*oriT sacB*, Ap^r Cm^r, *Francisella* suicide plasmid	19
pPV-hfq	pPV carrying *hfq* deletion fragment for mutagenesis of LVS	This study
pDMK2	mob_{RP4} ori_{R6K} *sacB*, Km^r, *Francisella* suicide vector	K. Kadzhaev, unpublished
pDMK-hfq	pDMK2 carrying *hfq* deletion fragment for mutagenesis of FCS200	This study
pCR4TOPO	PCR cloning vector, Amp^r Km^r	Invitrogen
pCR2.1TOPO	PCR cloning vector, Amp^r Km^r	Invitrogen
Strains		
F. tularensis LVS	*F. tularensis* subsp. *holarctica* live vaccine strain, Pm^r	A. Sjöstedt
F. tularensis Δ*hfq* mutant	LVS strain with deletion of codons 5 to 71 of *hfq*	This study
F. tularensis FSC200	*F. tularensis* subsp. *holarctica*, clinical isolate	Human ulcer, Sweden
F. tularensis FSC768	FSC200 with deletion of codons 9 to 113 of *hfq*	This study
E. coli DH5α	F^- ϕ80*lacZ*ΔM15 *endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1* (*lacZYA-argF*)*U169*	Invitrogen
E. coli TOP10	(F^-) *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* (Δ*ara-leu*)*7697 galU galK rpsL* (Sm^r) *endA1 nupG*	Invitrogen
E. coli S17-1	(F^-) RP4-2-Tc::Mu *aph*::Tn*7 recA*, Sm^r	Lab collection
E. coli S17-1λ*pir*	(F^-) RP4-2-Tc::Mu *aph*::Tn*7 recA* λ*pir* lysogen, Sm^r	Lab collection
Primers		
Hfq-AF	5′- TGGGTCGACTACAGCCAAGCCAACTTTACAAG[a]	Mutant construction
Hfq2	5′-TATTCTTGACATTATCTCACTTC	Mutant construction
Hfq-BR	5′-CGGCTAGCAAGATCAGACTCTATAGCCTCTTC[b]	Mutant construction
Hfq3	5′-GAAGTGAGATAATGTCAAGAATAGTTTATAGCTCTTTTAATCCATA[c]	Mutant construction
Hfq_FNdeI	5′-GGGAATTCCATATGTTGCTAGAATATACGCGAAG[d]	Complementing plasmid
Hfq_RBamHI	5′-CGCGGATCCTTACTCGTGAATATTACCTTC[e]	Complementing plasmid
5up-HfqA	5′-AATTAATGCAGAAATTATTAGTG	Mutant verification
3down-HfqB	5′-CTTTTTCAACCTGCTCAATGTAGC	Mutant verification
Hfq-F22	5′-CAAGACCCGTTCTTAAATGCT	RT-PCR
Hfq-R79	5′-CTAGATATACAGATACGCTAAC	RT-PCR
Hfq-R133	5′-CGATACAGAATTGGTCAAAAGC	RT-PCR/5′ RACE
Hfq-R194	5′-GCTGGAACTATAGTAGAAATAG	RT-PCR, 5′ RACE
miaA-F870	5′-GCTATGGAGAACGAAACTAAAGA	RT-PCR
HflX-R170	5′-GATATCTGGCTCAGGATGG	RT-PCR
Hfq_{200}-A_for	5′-AACAGTCCAGGGTGACTAAAATCGAGCCAAT	Mutant construction
Hfq_{200}-B_rev	5′-ATATTACTCGTGTGACATTATCTCACTTCCTTTTA	Mutant construction
Hfq_{200}-C_for	5′-GAGATAATGTCACACGAGTAATATATCTTTTCTTT[f]	Mutant construction
Hfq_{200}-D_rev	5′-TAACGTCGACTAACACCAAGCTCATACATCT	Mutant construction
Hfq_{200}-E_For	5′-GCGGAAAGAAGATAGATAAGA	Mutant verification
Hfq_{200}-F_Rev	5′-TATCTTCGACTCCTGTTATTTC	Mutant verification
TUL4–435	5′-GCTGTATCATCATTTAATAAACTGCTG	44
TUL4–863	5′-TTGGGAAGCTTGTATCATGGCACT	44
hel_F	5′-GGGATGTCGCCTTTTGATTTTC	qRT-PCR[g]
hel_R	5′-CTCTTTTGTCCCTTGTGCTTGC	qRT-PCR[g]
iglC_F	5′-TGGGTGTTTCAAAAGATGGAATG	qRT-PCR
iglC_R	5′-TGCGCAACATACTGGCAAGC	qRT-PCR
FTL_0994_F	5′-CGCTAATGCAATCGTTGATGG	qRT-PCR
FTL_0994_R	5′-CCGGTGACATGATCAACCTCAT	qRT-PCR
FTL_0120_F	5′-CAAGCACCTCATTTGCTTCGAT	qRT-PCR
FTL_0120_R	5′-TTCTATTGCTTGAGCATCATCTTTTTG	qRT-PCR
pdpB_F	5′-TGAATCCGAAACCAATTCATCT	qRT-PCR
pdpB_R	5′-GGCGCCCACAATGACTTATC	qRT-PCR
FTL_0317_F	5′-GTCGTGCGGACCAAACAAAA	qRT-PCR
FTL_0317_R	5′-CGCAGCATCAGTTGTGAGATTG	qRT-PCR
katG_F	5′-ATACGCCGCAACACAAATGG	qRT-PCR
katG_R	5′-CATCCCCATAGCTCGGAAGG	qRT-PCR
iglA_F	5′-TCAAAAGGAAGATCTGTGGATGC	qRT-PCR
iglA_R	5′-CAAAGTTTGGTGCCTCAAAATCA	qRT-PCR
iglB_F	5′-GGAATGGGTATGGTGGCAAA	qRT-PCR
iglB_R	5′-GGATGCTCAAGCAAAGCTTCAA	qRT-PCR

[a] The SalI site is underlined.
[b] The NheI site is underlined.
[c] The sequence overlapping Hfq2 is underlined.
[d] The NdeI site is underlined.
[e] The BamHI site is underlined.
[f] The sequence that overlaps part of Hfq_{200}-B_rev is underlined.
[g] See reference 7.

in a second PCR with primers hfq_R133 and PCR-anchor, and the fragment obtained was directly sequenced. Additionally, the PCR product was ligated into pCR2.1-TOPO and several clone sequences. This confirmed that in five clones the transcription start site was at position −65 relative to the ATG start codon, as determined by direct sequencing. Three clones had 5′ ends at positions −29, +22, and +110, indicating that these clones were degraded transcripts.

Hfq antiserum and immunoblotting. The *hfq* gene of LVS was cloned into the pET-28a expression vector (Novagen) to create a protein with a histidine tag at the N terminus. This protein was expressed in *E. coli* strain BL21(DE3), and a gel piece containing the overexpressed protein was used for production of polyclonal antisera in rabbits (Centre de Production Animale, Olivet, France). Antiserum was purified by incubation with an *F. tularensis* Δ*hfq* bacterial lysate before it was used for immunoblotting. Aliquots of *F. tularensis* LVS (and the Δ*hfq* strain as a control) were harvested at different cell densities, and equal amounts of cell lysate (corresponding to 2×10^8 bacteria per well) were deposited on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane using a semidry Transblot apparatus (Bio-Rad) and incubated with the Hfq antiserum. After washing, horseradish peroxidase-linked secondary antibody was added, and binding was detected using an ECL Plus kit (Amersham).

Construction of complementing plasmid p6*gro-hfq*. Plasmid pFNLTP6-*gro-gfp* (28) was digested with BamHI, which excised the *gfp* gene, and was subsequently self-ligated, yielding plasmid p6-*gro*. The *hfq* gene was PCR amplified using primers Hfq_FNdeI and Hfq_RBamHI and the Expand high-fidelity PCR system (Roche Applied Science). The product was digested with NdeI and BamHI and ligated into NdeI-BamHI-digested p6-*gro*, producing p6-*gro-hfq*. This construct was verified by sequencing and introduced into *F. tularensis* LVS by electroporation (28).

Stress resistance. *F. tularensis* LVS and its Δ*hfq* derivative were grown overnight in Schaedler K3 medium. For experiments with liquid media, the cultures were diluted 10-fold in fresh medium containing either 1% or 2% NaCl and incubated at 37°C with agitation. Growth was assessed by measuring the optical density at 600 nm (OD_{600}). For heat resistance tests, overnight cultures were diluted in fivefold steps, and 3 μl of each dilution was spotted onto chocolate agar plates and incubated at 37°C or 40°C. For experiments with the complemented mutant strain, wild-type and mutant bacteria containing p6*gro* or p6*gro-hfq* were grown overnight in Schaedler K3 medium supplemented with kanamycin. The resulting cultures were diluted in 10-fold steps, and 3 μl of each dilution was spotted onto chocolate agar containing kanamycin and either 2% NaCl, 0.2% SDS, or water (as a control). *F. tularensis* FSC200 and FSC768 were grown on McLeod agar plates and suspended in Chamberlain medium. Each suspension was used to inoculate Chamberlain medium containing 1 or 2% NaCl and to prepare fivefold serial dilutions and was incubated at 37°C without agitation. NaCl resistance and heat resistance were assessed as described above. All the experiments described above were performed at least twice, and results of a single representative experiment are presented.

Multiplication in macrophages. J774 cells were propagated in RPMI medium or Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells were seeded at a concentration of $\sim 2 \times 10^5$ cells per well in 12-well (for experiments with LVS strains) or 24-well (for experiments with FSC200 strains) tissue plates (Falcon), and monolayers were used 24 h after seeding. Bone marrow-derived macrophages (BMM) from BALB/c mice were obtained and cultured as described previously (13). RAW macrophages were propagated in RPMI medium. J774 macrophage monolayers were incubated for 90 min (LVS) or 120 min (FSC200), RAW macrophages were incubated for 90 min, and BMM were incubated for 60 min at 37°C with the bacterial suspensions (multiplicities of infection, approximately 100) to allow the bacteria to enter. After washing (time zero of the kinetic analysis), the cells were incubated in fresh culture medium containing gentamicin (10 μg/ml) to kill extracellular bacteria. At several time points, cells were washed three times in RPMI medium or phosphate-buffered saline (PBS) and processed for counting of surviving intracellular bacteria. For this, bacteria were recovered by lysis of macrophages with distilled water (LVS) or 0.1% sodium deoxycholate in PBS (FSC200). The titer of viable bacteria released from the cells was determined by spreading preparations on agar plates. For each strain and time in an experiment, the assay was performed in triplicate. Each experiment was independently repeated at least two times, and the data presented below are the averages of two experiments (six wells).

Infection of mice and virulence assays. Bacterial stocks of LVS and the LVS Δ*hfq* mutant (in 16 to 20% glycerol) stored at −80°C were thawed and diluted to obtain the appropriate concentration (CFU/ml) in 0.15 M NaCl. Six- to 8-week-old female BALB/c mice (Janvier, Le Genest St. Isle, France) were inoculated intraperitoneally (i.p.) with 200 μl of a bacterial suspension. Groups of five mice were inoculated with various doses of bacteria, and the mortality was followed for 9 days. The actual number of viable bacteria was determined by plating dilutions of a bacterial suspension on chocolate plates. Animal experiments were performed according to the INSERM guidelines for laboratory animal husbandry.

For experiments examining the kinetics of infection, BALB/c mice were infected with $\sim 3 \times 10^4$ CFU of LVS or *hfq* mutant bacteria. At 2, 3, 4, and 7 days after infection, groups of five mice were sacrificed, and the numbers of bacteria in the liver and spleen were determined by plating homogenized samples on chocolate agar plates. Four mice infected with LVS died before day 7.

Infection with *F. tularensis* strain FSC200 and the FSC200 Δ*hfq* mutant was performed approximately as described above, except that the mice were infected i.p. or subcutaneously with 100 μl of the appropriate bacterial suspension. For the competitive index (CI) infection studies, mice in groups of five animals were infected subcutaneously with approximately 100 bacteria using a 1:1 mixture of the wild-type and mutant strains (FSC200 and FSC768). Five days after infection mice were sacrificed, and spleens were isolated and homogenized in PBS. The homogenates were serial diluted and spread on McLeod agar plates, and colonies were analyzed by using PCR to distinguish the mutant from the wild type. The output value after infection was determined by dividing the number of mutant CFU by the number of wild-type CFU. This ratio was divided by the initial ratio of the inoculum (verified by viable counting) to obtain a final CI. C57Black/6 female mice (Scanbur BK AB, Sollentuna, Sweden) were housed under conventional conditions, given food and water ad libitum, and allowed to acclimatize for at least 7 days before infection. The study was approved by the Local Ethics Committee on Laboratory Animals in Umeå, Sweden.

Transcriptional profiling using DNA microarrays. RNA was isolated from LVS and the LVS Δ*hfq* mutant grown to exponential phase in Schaedler K3 medium (OD_{600}, ~0.25) or Chamberlain medium (OD_{600}, ~0.35) and from colonies grown on chocolate agar using Trizol reagents (Invitrogen), followed by purification of the aqueous phase on RNeasy columns (Qiagen). RNA samples were treated with DNase I (Ambion), and 3 μg of each sample was used in a reverse transcription (RT) reaction. Cy3-labeled cDNA (LVS) and Cy5-labeled cDNA (LVS Δ*hfq* mutant) were prepared using the protocol for aminoallyl labeling of microbial RNA (The Institute for Genomic Research [TIGR]; http://pfgrc.jcvi.org/index.php/microarray/protocols.html). The labeled samples were combined and hybridized as described at the TIGR website (http://pfgrc.jcvi.org/index.php/microarray/protocols.html) with an *F. tularensis* microarray provided by The Pathogen Funtional Genomic Resource Center (Rockville, MD). This microarray contains 2,331 oligonucleotides (70 nucleotides) representing open reading frames from strains LVS and Schu S4, as well as plasmids pFNL10 and pOM1, each spotted four times. The experiments were each performed two or three times, and duplicate labeling and hybridization experiments were performed for each set of RNAs. The microarray slides were scanned using a Genepix 4000B microarray scanner (Molecular Devices), and the images were analyzed using the Spotfinder and MIDAS software (TIGR). Statistically significant changes in gene expression under each condition were identified by performing a one-class analysis using the significance analysis of microarray (SAM) program (52) with a 0% false discovery rate. Genes with an average change of at least twofold (calculated using all microarray slides and the four values for each slide) in at least two experiments are shown in Table 2.

Quantitative RT-PCR (qRT-PCR). One microgram of RNA was reverse transcribed using random hexamers and Superscript II reverse transcriptase (Invitrogen) according to the protocol provided by the manufacturer. Real-time PCR was performed with gene-specific primers using an ABI PRISM 7700 and SYBR green PCR master mixture (Applied Biosystems, Foster City, CA). To calculate the amount of transcript, a standard curve was created for each gene-specific primer set using a series of diluted genomic DNA from LVS. To compare the transcript levels of LVS and the *hfq* mutant, the amounts of transcript were normalized using the DNA helicase gene (FTL_1656), as the expression of this gene has been shown to change little during growth (7). The differences between LVS and the *hfq* mutant were calculated using triplicate samples, and the results were expressed as averages ± standard deviations. Student's *t* test was used to determine if the difference between the normalized transcript level of LVS and the normalized transcript level of the *hfq* mutant is significant.

Microarray data accession numbers. The raw and processed microarray data have been submitted to the ArrayExpress database under accession numbers E-MEXP-1884, E-MEXP-1891, and E-MEXP-1892.

RESULTS

Promoter analysis and construction of *hfq* deletion mutant of *F. tularensis* LVS. The *F. tularensis* LVS Hfq protein (en-

TABLE 2. Hfq-regulated genes[a]

Locus	Gene product	Gene	KEGG classification	Change (fold)[b]		
				Defined medium	Agar plates	Broth
Genes significantly downregulated in the *hfq* mutant (n = 16)						
FTL_0194	Cytochrome *o* ubiquinol oxidase subunit IV	*cyoD*	Metabolism	0.21		0.42
FTL_0195	Protoheme IX farnesyltransferase	*cyoE*	Metabolism	0.28		0.32
FTL_0221	Amino acid permease			0.50		0.41
FTL_0317	Hypothetical protein			0.41	0.41	0.36
FTL_0392	Type IV pilus fiber building block protein			0.42	0.47	0.33
FTL_0421	Conserved hypothetical lipoprotein	*lpnA*		0.46	0.31	0.47
FTL_0568	Transposase	*isftu2*		**0.84**	0.48	0.47
FTL_0570	Hypothetical protein			0.34	0.42	0.39
FTL_0898	Host factor I for bacteriophage Qβ replication	*hfq*		0.12	0.04	0.08
FTL_0899	Protease, GTP-binding subunit	*hflX*		0.37	0.47	**0.75**
FTL_1016	Short-chain dehydrogenase			0.31	0.50	0.48
FTL_1394	Galactose-proton symporter, major facilitator superfamily transport protein	*galP2*		0.47		0.49
FTL_1504	Peroxidase/catalase	*katG*	Metabolism	0.37	0.49	**0.64**
FTL_1555	Hypothetical membrane protein			**0.53**	0.49	0.36
FTL_1678	Conserved membrane hypothetical protein			**0.53**	0.47	0.36
FTL_1764	Pseudo				0.13	0.37
Genes significantly upregulated in the *hfq* mutant (n = 88)						
FTL_0045	Orotidine 5′-phosphate decarboxylase	*pyrF*	Metabolism		2.60	3.30
FTL_0046	Dihyroorotate dehydrogenase	*pyrD*	Metabolism		2.58	3.44
FTL_0117/ FTL_1163	Hypothetical protein			4.83	3.92	3.72
FTL_0118/ FTL_1164	Hypothetical protein			3.24	**1.91**	2.28
FTL_0119/ FTL_1165	Hypothetical protein			4.21	3.19	2.68
FTL_0120/ FTL_1166	Hypothetical protein			6.02	5.82	2.46
FTL_0121/ FTL_1167	Hypothetical protein			7.72	4.08	4.55
FTL_0122/ FTL_1168	Conserved hypothetical protein			9.35	6.49	4.94
FTL_0123/ FTL_1169	Hypothetical protein			3.49	4.03	3.53
FTL_0124/ FTL_1170	Hypothetical protein			5.21	5.35	3.64
FTL_0125/ FTL_1171	Hypothetical protein	*pdpB*		10.7	6.00	5.15
FTL_0126/ FTL_1172	Hypothetical protein	*pdpA*		8.19	3.63	3.69
FTL_0237	50S ribosomal protein L4		Genetic information processing	**1.23**	2.61	2.51
FTL_0239	50S ribosomal protein L2	*rplB*	Genetic information processing		2.24	2.77
FTL_0240	30S ribosomal protein S19		Genetic information processing	**1.23**	2.48	3.26
FTL_0242	30S ribosomal protein S3	*rpsC*	Genetic information processing		2.93	2.64
FTL_0261	DNA-directed RNA polymerase, alpha subunit		Metabolism and genetic information processing	**1.25**	2.66	3.30
FTL_0396	Fusion protein PurC/PurD		Metabolism	**1.46**	2.45	2.16
FTL_0414	GTP-binding protein	*engA*		2.75	3.46	**1.53**
FTL_0437	Riboflavin biosynthesis protein RibF	*ribF*	Metabolism	3.27	5.02	2.06
FTL_0460	Hypothetical protein			3.31	2.74	2.71
FTL_0461	Hypothetical protein			**1.88**	2.60	2.22
FTL_0534	1-Deoxy-D-xylulose 5-phosphate reductoisomerase	*dxr*	Metabolism	2.97	3.69	**1.62**
FTL_0535	Outer membrane protein			2.14	5.31	
FTL_0603	O-antigen flippase	*wzx*		2.02	3.43	
FTL_0604	Glycosyltransferase	*wbtK*		**1.67**	2.04	2.01
FTL_0605	Glucose-1-phosphate thymidylyltransferase	*wbtL*	Metabolism		2.95	2.24
FTL_0669	Exodeoxyribonuclease V, beta chain	*recB*	Genetic information processing	**1.75**	2.17	2.23
FTL_0670	Exodeoxyribonuclease V, alpha subunit	*recD*	Genetic information processing	4.16	4.04	**1.77**
FTL_0737	Hypothetical membrane protein			5.81	4.63	**1.79**
FTL_0739	Glucose-inhibited division protein A	*gidA*	Cellular processes	3.18	2.03	
FTL_0788	Glutamine amidotransferase class II family protein			4.09	3.28	**1.94**
FTL_0797	Type IV pili associated protein		Genetic information processing	2.99	2.35	**1.32**
FTL_0798	Type IV pilus glycosylation protein		Genetic information processing	4.06	3.38	**1.81**
FTL_0827	Type IV pilus polytopic inner membrane protein		Genetic information processing	2.32	4.31	2.32
FTL_0882	Apolipoprotein *N*-acyltransferase			6.09	7.01	
FTL_0883	Metal ion transporter protein		Environmental information processing	3.33	3.15	**1.74**
FTL_0884	Hypothetical protein			**1.49**	3.09	2.55
FTL_0885	PhoH-like protein	*phoH*		2.31	4.40	**1.77**
FTL_0893	ATP-dependent protease, ATP-binding subunit	*clpX*		2.22	3.42	**1.81**
FTL_0894	DNA-binding, ATP-dependent protease La	*lon*		2.16	2.76	**1.92**
FTL_0921	Endonuclease III		Genetic information processing	2.78	2.11	**1.75**
FTL_0993	HesA/MoeB/ThiF family protein			3.61	3.38	2.75

Continued on following page

TABLE 2—*Continued*

Locus	Gene product	Gene	KEGG classification	Change (fold)[b]		
				Defined medium	Agar plates	Broth
FTL_0994	Hypothetical protein			5.66	4.91	2.55
FTL_1004	D-Alanyl-D-alanine carboxypeptidase	*vanY*		2.44	2.08	**1.79**
FTL_1005	Hypothetical protein			10.6	4.06	**1.86**
FTL_1043	Hypothetical protein			7.12	5.79	3.75
FTL_1044	Hypothetical protein			6.70	3.46	4.03
FTL_1045	Conserved hypothetical lipoprotein			3.27	3.01	2.27
FTL_1046	D-Alanyl-D-alanine carboxypeptidase (penicillin-binding protein) family protein	*dacB*		11.6	3.70	2.78
FTL_1049	DNA primase	*dnaG*	Genetic information processing		4.16	4.04
FTL_1050	RNA polymerase sigma-70 factor	*rpoD*	Genetic information processing	2.47	3.04	**1.75**
FTL_1064	Hypothetical protein			4.10	4.47	2.10
FTL_1065	ABC transporter, ATP-binding protein	*yhbG*		2.64	7.30	3.39
FTL_1066	Fumarylacetoacetate hydrolase family protein				2.37	2.59
FTL_1067	Hypothetical protein			2.79	5.48	2.32
FTL_1068	tRNA pseudouridine synthase A	*truA*	Genetic information processing	**1.46**	3.27	3.56
FTL_1074	GDSL-like lipase/acylhydrolase family protein			**1.72**	2.54	2.51
FTL_1106	Alanyl-tRNA synthetase	*alaS*	Metabolism and genetic information processing	2.41	2.80	2.06
FTL_1108	Cytosol aminopeptidase family protein			2.49	2.65	**1.65**
FTL_1174	Cystathionine beta-synthase (cysteine synthase)	*cbs*	Metabolism	6.30	3.86	3.20
FTL_1177	tRNA modification GTPase	*trmE*		2.71	2.37	
FTL_1190	Chaperone protein GrpE (heat shock protein family 70 cofactor)	*grpE*		2.65	2.03	3.27
FTL_1228	Hypothetical protein			3.15	**1.43**	2.41
FTL_1229	ABC transporter, ATP-binding protein			4.57	**1.35**	3.29
FTL_1230	Cysteine desulfurase activator complex subunit SufB			2.49	2.63	**1.44**
FTL_1261	Anthranilate synthase component II	*trpG*	Metabolism	6.60	3.05	3.42
FTL_1262	Chorismate binding family protein			10.2	4.00	2.90
FTL_1266	Lipase/esterase			5.90		2.91
FTL_1267	Hypothetical protein			3.19	**1.88**	2.81
FTL_1271	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	*bioA*	Metabolism	2.71		2.03
FTL_1273	8-Amino-7-oxononanoate synthase	*bioF*	Metabolism	2.57	2.00	**1.49**
FTL_1274	Biotin synthesis protein BioC	*bioC*		5.02	3.25	3.12
FTL_1275	Dethiobiotin synthetase	*bioD*	Metabolism	3.23	2.66	3.89
FTL_1276	Bifunctional protein BirA		Metabolism	5.74	2.66	
FTL_1283	Glutamate-1-semialdehyde-2,1-aminomutase	*hemL*	Metabolism	2.02	2.90	**1.76**
FTL_1284	Glutathione synthetase		Metabolism	2.56	4.62	2.20
FTL_1285	Methionyl-tRNA formyltransferase		Metabolism and genetic information processing	5.04	4.87	2.52
FTL_1372	Hypothetical lipoprotein			2.65	3.61	**1.14**
FTL_1396	Galactose-1-phosphate uridylyltransferase	*galT*	Metabolism	2.54	2.03	**1.28**
FTL_1397	Galactokinase	*galK*	Metabolism	3.37	3.93	**1.33**
FTL_1484	Methylase		Metabolism	4.56	4.53	**1.36**
FTL_1530	Pseudo			**1.61**	2.88	2.36
FTL_1539	Penicillin-binding protein (peptidoglycan synthetase)	*ftsI*	Metabolism and cellular processes	2.39	2.65	**1.79**
FTL_1616	Phosphoenolpyruvate carboxykinase	*pckA*	Metabolism	2.33	3.78	
FTL_1639	Hypothetical protein			2.59	2.99	**1.59**
FTL_1806	Major facilitator superfamily (MFS) transport protein			3.37	5.45	**1.22**
FTL_1826	NADH dehydrogenase I, E subunit		Metabolism	2.13	2.18	**1.72**

[a] Genes whose expression is significantly changed (at least twofold, as determined using the SAM program) in LVS and the *hfq* mutant under at least two conditions are included.

[b] The values are average changes between the *hfq* mutant and LVS in all microarrays. Bold type indicates a gene whose expression is significantly changed (as determined using the SAM program), but the change is less than twofold.

coded by FTL_0898) was identified by in silico analysis of the LVS genome. It consists of 109 amino acids and is 100% identical to Hfq of another *F. tularensis* subsp. *holarctica* strain (FSC200) and 98% identical to the protein from *F. tularensis* subsp. *tularensis* strain Schu S4. Furthermore, it is 53% identical (78% similar) to the protein from *E. coli*, and all residues proposed to contribute to binding of RNA are conserved (Fig. 1A) (5). The *hfq* gene is located downstream of the *miaA* gene [encoding tRNA delta(2)-isopentenylpyrophosphate transferase] and upstream of *hflX* (encoding GTP-binding protein HflX) (Fig. 1B), a gene organization that is conserved in many bacterial species. RT-PCR experiments demonstrated that there is cotranscription of *hfq* and *hflX*, but not of *miaA* and *hfq* (Fig. 1B and C). 5′ RACE experiments showed that the start site of the *hfq* transcript is located at position −65 relative to the ATG codon in the 110-bp *miaA-hfq* intergenic region (Fig. 1E). Putative −10 and −35 boxes are similar to the motifs recognized by the σ^{70}-containing RNA polymerase and are separated by 17 nucleotides, indicating that *hfq* is not dependent on σ^{32}.

To study the role of Hfq in *F. tularensis*, we constructed an *hfq* mutant of *F. tularensis* LVS in which there was an in-frame deletion of amino acids 5 to 70. Using RT-PCR, we verified that the deletion in *hfq* had no polar effect on transcription of the downstream gene *hflX* (Fig. 1D). In some bacteria, expression of Hfq has been reported to depend on the growth phase

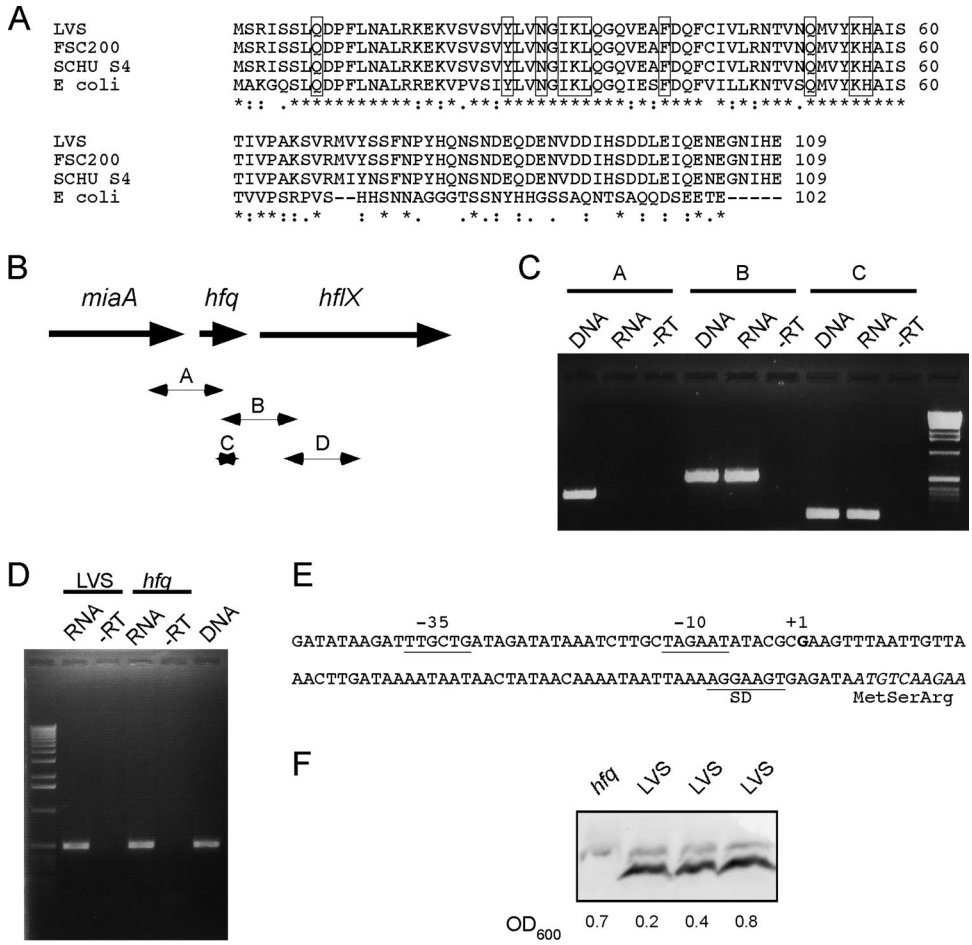

FIG. 1. *Francisella* Hfq protein and *hfq* locus. (A) Alignment of the Hfq proteins of *F. tularensis* strains LVS, FSC200, and Schu S4 and Hfq of *E. coli* performed using the ClustalW program. Residues that are identical in all strains are indicated by asterisks, conserved substitutions are indicated by colons, and semiconserved substitutions are indicated by periods. Amino acids which have been implicated in binding of RNA (5) are enclosed in boxes. (B) Genetic organization of the *hfq* locus in *F. tularensis*. *miaA* encodes tRNA delta(2)-isopentenylpyrophosphate transferase, and *hflX* encodes a GTP-binding protein. Arrows indicate fragments A, B, C, and D amplified in PCR as shown in panels C and D. (C) RT-PCR of the *hfq* locus. PCR to amplify fragments A, B, and C (see panel B) was performed with genomic DNA from LVS (DNA), with RNA from LVS after RT (RNA), or with RNA from LVS without RT (−RT) as a control. (D) Deletion in *hfq* has no polar effect on the downstream gene *hflX*. RT-PCR to amplify fragment D (see panel B) was performed with RNA isolated from either LVS or the *hfq* mutant after RT (RNA) or with RNA without RT (−RT). A control reaction was performed with DNA isolated from LVS (DNA). (E) Sequence of the promoter region of *hfq*. The transcription start site (+1) as determined by 5′ RACE is indicated by bold type, and putative −10 and −35 sequences are underlined. SD, Shine-Delgarno sequence. (F) Immunoblotting of bacterial lysates from strain LVS and the *hfq* mutant using anti-Hfq antiserum. The same amount of bacterial material, based on the optical density of the culture, was deposited in each lane for bacteria at different growth phases, including early exponential phase (OD_{600}, 0.2), exponential phase (OD_{600}, 0.4), and early stationary phase (OD_{600}, 0.8).

or growth rate. In *P. aeruginosa* the level of Hfq is higher in stationary phase (46), whereas in *E. coli* the level of this protein has been reported both to decrease in stationary phase (2, 22) and to increase in stationary phase (50). We assessed, using immunoblotting, the level of the Hfq protein in *F. tularensis* LVS in different growth phases. For this, we constructed a recombinant Hfq protein with an N-terminal histidine tag that was used for production of polyclonal anti-Hfq antibodies in rabbits (see Materials and Methods for details). As shown in Fig. 1F, we did not observe any major differences between the protein levels at the beginning of exponential phase (OD_{600}, 0.2), in mid-exponential phase (OD_{600}, 0.4), and in early stationary phase (OD_{600}, 0.8).

Growth characteristics of the *hfq* mutant. On chocolate agar plates, the colonies formed by the *hfq* mutant are smaller than the colonies formed by the wild-type strain (Fig. 2A). When grown in broth, the mutant exhibited a decreased growth rate and grew to a slightly lower density (Fig. 2B). Growth phenotypes on plates and/or in broth have also been observed for *hfq* mutants of *E. coli*, *S.* Typhimurium, *V. cholerae*, *B. abortus*, *P. aeruginosa*, and *L. pneumophila* (14, 30, 39, 42, 45, 51). We also checked by using transmission electron microscopy whether *hfq* inactivation had an impact on cell division, as previously reported for *E. coli hfq* mutants (51). Under the conditions tested (bacteria scratched from chocolate agar plates), the morphology of the *hfq* mutant was undistinguishable from that

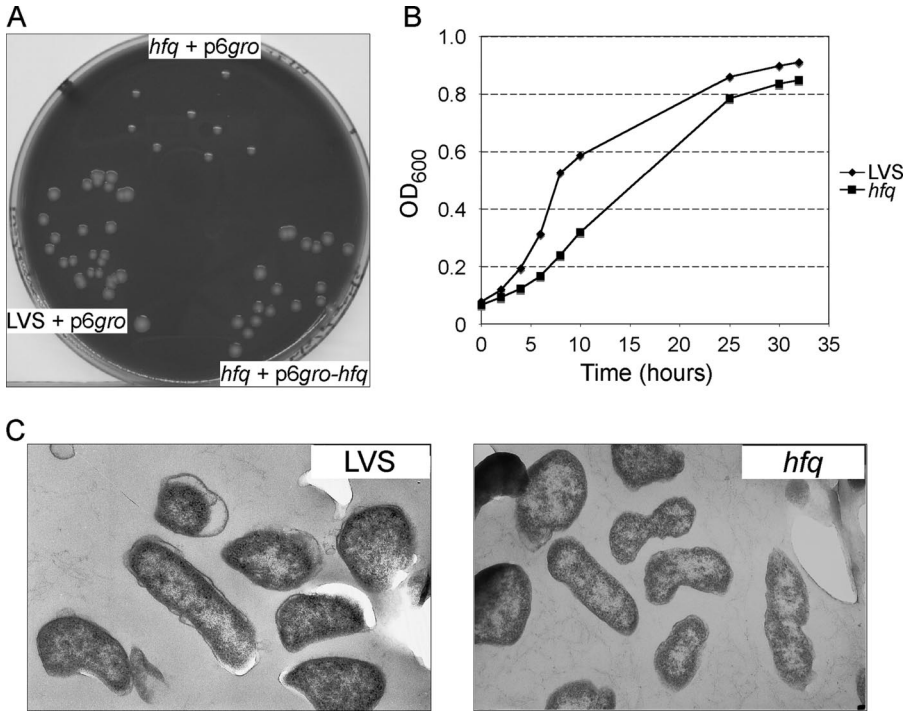

FIG. 2. Growth characteristics of the LVS *hfq* mutant. (A) Colonies of wild-type *F. tularensis* LVS, the *hfq* mutant, and the *hfq* mutant containing a plasmid containing *hfq* on chocolate plates after 72 h of growth at 37°C. (B) Growth of *F. tularensis* LVS and the *hfq* mutant in Schaedler K3 broth at 37°C. (C) Transmission electron micrographs of LVS and the *hfq* mutant after growth on solid medium.

of parental strain LVS, indicating that the lack of Hfq had no effect on bacterial septation (Fig. 2C).

To ensure that the observed growth phenotype resulted from inactivation of *hfq* and not from polar effects on downstream genes, we complemented the mutant strain with a plasmid copy of *hfq*. Introduction of plasmid p6*gro-hfq* restored growth on plates to a level that was the same as the level for the wild-type parent (Fig. 2A), confirming the specific involvement of *hfq*.

Hfq contributes to stress resistance in *F. tularensis*. We next addressed the role of Hfq in stress resistance in *F. tularensis* LVS. Serial dilutions of wild-type and mutant bacterial stationary-phase cultures were spotted onto chocolate agar plates and incubated at 37°C or 40°C to compare the abilities of the organisms to grow at an elevated temperature. At 37°C, we observed a slight difference in growth between the two strains at the highest dilution (78,125-fold) (data not shown) but not at lower dilutions (Fig. 3A). At 40°C, the *hfq* strain grew poorly, and growth ceased completely at dilutions higher than 25-fold (Fig. 3A). In contrast, the growth of the wild-type strain at 40°C was comparable to that at 37°C (Fig. 3A and data not shown). The growth of the two strains was also compared in medium supplemented with NaCl (Fig. 3B). The wild-type strain grew to a higher density in medium with 1% NaCl than in medium without this supplement (Fig. 3B), whereas addition of 1% NaCl had no effect on the growth of the *hfq* strain. In medium containing 2% NaCl, the wild-type strain exhibited slightly enhanced growth compared to the growth in medium without this supplement, but the enhancement was less than that seen with the 1% NaCl supplement. In contrast, the *hfq* strain grew poorly in medium containing 2% NaCl, and the final density was about 50% of the density in regular medium. When the mutant strain was spotted on solid medium containing 2% NaCl, the growth was again severely affected, but complementation with a plasmid containing the *hfq* gene (p6*gro-hfq*) restored growth (Fig. 3C). We further assessed the effects of the membrane-perturbing agent SDS by spotting dilutions of stationary-phase cultures onto solid media containing 0.2% SDS. As observed in the presence of NaCl, growth of the mutant strain was almost not noticeable, whereas only slightly fewer bacteria were observed for the highest dilution on control plates (Fig. 3C). The increased sensitivity of the *hfq* mutant to SDS was alleviated by introduction of plasmid p6*gro-hfq*, further demonstrating that the observed phenotypes were due to a lack of *hfq*.

Hfq does not play a major role in intracellular multiplication in vitro. To examine if Hfq contributes to *Francisella* pathogenesis, we initially compared the abilities of *F. tularensis* LVS and the *hfq* mutant to multiply inside macrophages in vitro (Fig. 4). For this, we infected murine macrophage-like J774 cells, RAW murine macrophages, and BMM. In all three types of macrophages, the wild-type strain grew to significantly higher levels after 24 h ($P < 1 \times 10^{-5}$, Student's *t* test), but at 48 h the difference was insignificant in J774 and BMM and only slightly a higher level was seen in RAW macrophages ($P = 0.005$). The slight growth defect of the *hfq* mutant during the initial stages of intracellular multiplication might be related to the slight growth defect seen in broth.

The *hfq* mutant is attenuated for virulence in mice. To determine if Hfq is important for the ability of *F. tularensis*

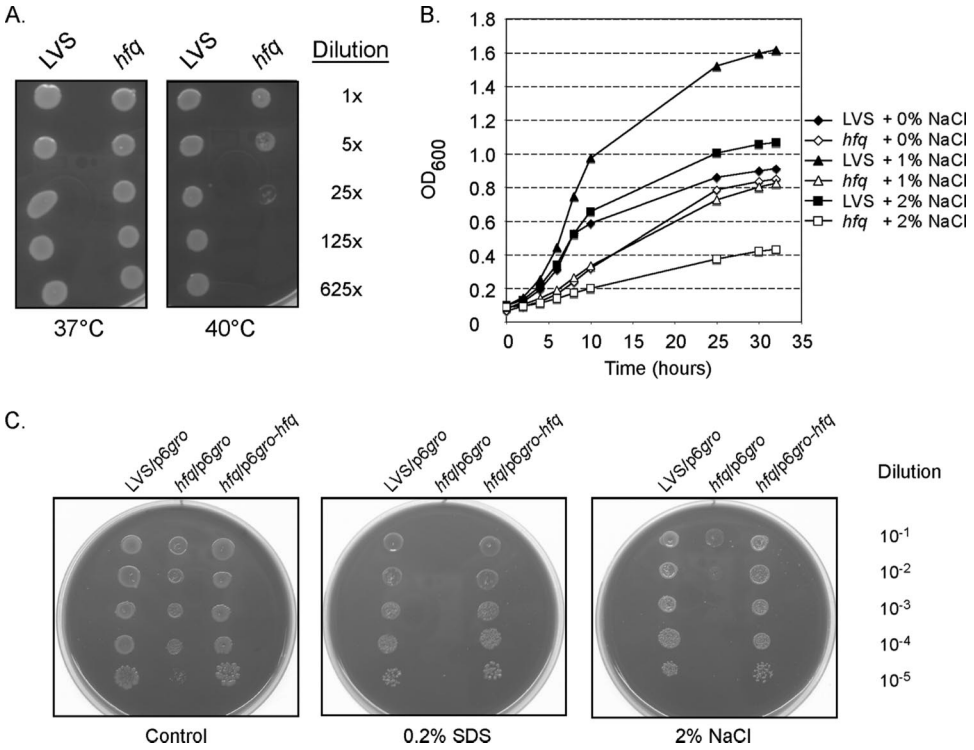

FIG. 3. The *hfq* mutant is more sensitive to stress than the LVS parent strain. (A) Serial fivefold dilutions of stationary-phase cultures were spotted onto chocolate agar plates and incubated at 37°C or 40°C. (B) Growth of LVS and the *hfq* mutant in complex broth supplemented with 0%, 1%, or 2% NaCl. (C) Serial 10-fold dilutions of stationary-phase cultures of LVS, the *hfq* mutant, and the *hfq* mutant containing a plasmid with *hfq* were spotted onto chocolate agar plates containing 0.2% SDS, 2% NaCl, or H_2O (control) and incubated at 37°C.

to cause disease, we infected 6- to 8-week-old BALB/c mice with strain LVS and the *hfq* mutant. Groups of five mice were inoculated by the i.p. route with different numbers of bacteria, and the survival of the mice was followed for 9 days (Fig. 5A). One of the mice infected with LVS at the highest dose ($\sim 3 \times 10^4$ bacteria) survived this infection, whereas all of the mice infected with the lowest dose ($\sim 3 \times 10^1$ bacteria) survived. In contrast, all of the mice survived infection with the lower doses of the *hfq* mutant ($\sim 3 \times 10^2$ to $\sim 3 \times 10^5$ bacteria), and only infection with a high dose of the *hfq* mutant ($\sim 3 \times 10^6$ bacteria) resulted in death. These results demonstrate that virulence is attenuated in the *hfq* mutant.

To investigate the fate of the bacteria inside host tissues, we infected mice with $\sim 3 \times 10^4$ LVS or *hfq* mutant bacteria and followed the kinetics of infection by assessing the number of viable bacteria in the spleen and liver. Groups of five mice were sacrificed 2, 3, 4, and 7 days after infection, and numbers of viable bacteria were determined by plating diluted tissue homogenates (Fig. 5B). The number of LVS bacteria increased from day 2 to day 4, reaching about 10^8 bacteria per organ, and only one of the mice infected with LVS survived until day 7. The *hfq* strain was initially detected in both the spleen and liver at levels similar to the wild-type strain levels, but the numbers of bacteria declined after 2 days and all mice survived. These data indicate that the *hfq* mutant is able to infect mice and to persist for some period of time in the infected organs. How-

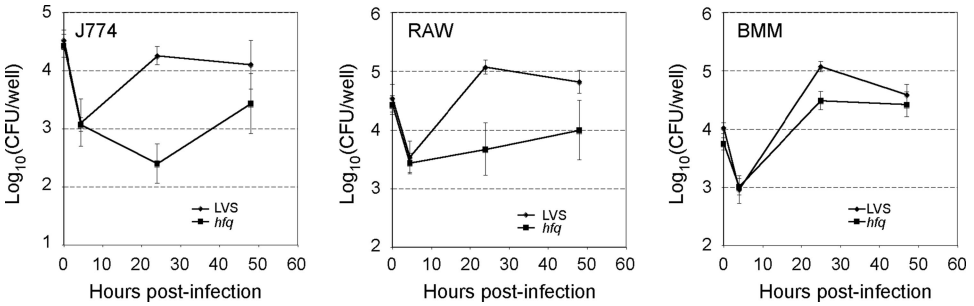

FIG. 4. Intracellular multiplication of LVS and the *hfq* mutant in murine macrophage cell lines J774 and RAW and in murine BMM. Macrophages were infected by LVS or *hfq* mutant bacteria at a multiplicity of infection of ~100, and the number of intracellular bacteria was determined after 0, 4, 24, and 48 h of infection. The results are expressed as average $\log_{10}$ (CFU/well) ± standard deviation for two experiments, each performed in triplicate.

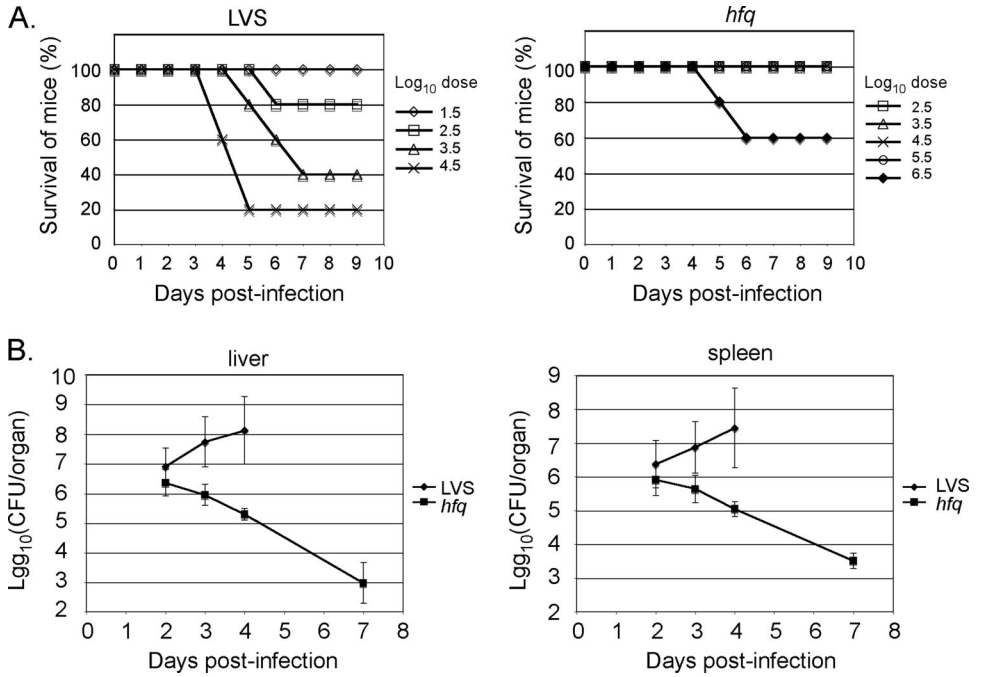

FIG. 5. The LVS *hfq* mutant is attenuated for virulence in mice. (A) Survival of mice after infection with LVS or the *hfq* mutant for 9 days after infection. Groups of five mice were infected by the i.p. route with different numbers of bacteria. The numbers of bacteria (in $\log_{10}$ CFU) used for infection are indicated on the right. (B) Multiplication of LVS and the *hfq* mutant in target organs of mice after i.p. infection with $\sim 3 \times 10^4$ CFU. Five mice were sacrificed after 2, 3, 4, and 7 days of infection, and the bacterial burdens in the spleen and liver were determined. Only one mouse survived after infection by LVS to day 7, and therefore no data for LVS at day 7 are shown.

ever, it is unable to multiply efficiently inside host tissues and cause disease.

Hfq plays a role in the virulent *F. tularensis* clinical isolate FSC200 similar to the role that it plays in LVS. To further study the role of Hfq in *F. tularensis*, we created an *hfq* mutant of *F. tularensis* subsp. *holarctica* clinical isolate FSC200. As observed for the LVS genetic background, the FSC200 *hfq* mutant formed colonies on nutrient agar that were smaller than the wild-type parent colonies and grew to a lower density in broth (Fig. 6A and data not shown). When organisms were grown in broth supplemented with 1% NaCl, the growth of FSC200 and the growth of the FSC200 *hfq* mutant, FSC768, were unaffected (Fig. 6A). However, in broth supplemented with 2% NaCl, the growth of FSC768 decreased compared to the growth in nonsupplemented broth, whereas the growth of FSC200 was unchanged. These results are similar to the results obtained for the *hfq* mutant of the LVS strain (Fig. 3B); likewise, we observed increased sensitivity of the *hfq* mutant to SDS (data not shown). In contrast to our observations for the LVS strain, we did not observe increased sensitivity to growth at an elevated temperature for FSC768 compared to FSC200 (Fig. 6B).

The involvement of Hfq of FSC200 in pathogenesis was addressed by assessing multiplication in macrophages in vitro and by performing virulence experiments with mice. The FSC768 mutant strain multiplied in J774 murine macrophage-

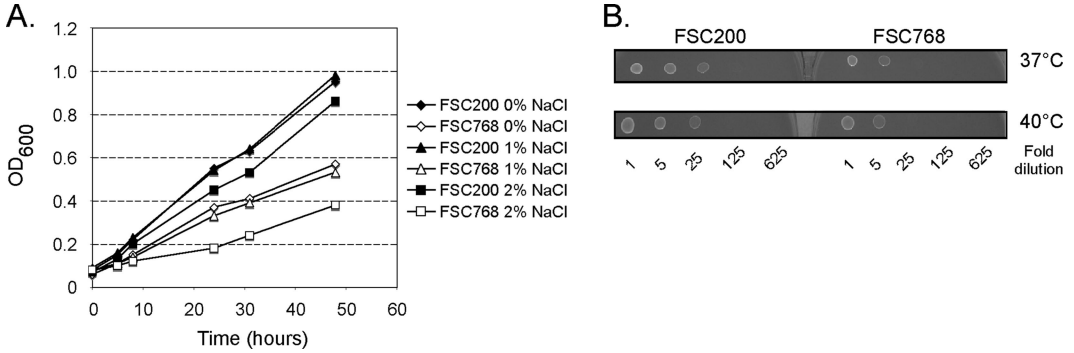

FIG. 6. Growth characteristics and stress resistance of the FSC200 *hfq* mutant. (A) Growth of FSC200 and FSC768 in defined medium with additional 0%, 1%, or 2% NaCl. (B) Serial fivefold dilutions of stationary-phase cultures were spotted onto McLeod agar plates and incubated at 37°C or 40°C.

like cells at levels that resemble the wild-type strain levels, although a slightly lower level was seen after 48 h ($P < 3 \times 10^{-5}$, Student's t test) (Fig. 7A). Mice that were infected via the i.p. route by FSC200 and FSC768 died within 9 days, even when they were infected with the lowest dose (~2 CFU). However, the mice infected with FSC768 survived longer than the mice infected with FSC200 (Fig. 7B). The average time to death was 1 to 2 days longer for the mice infected with the *hfq* mutant than for the mice infected with the wild-type strain at the same dose. Similar results were obtained when mice were infected subcutaneously. The lowest dose of FSC768 resulted in a survival rate of 40%, whereas the same dose of FSC200 killed all mice. At a higher dose ($\sim 2 \times 10^1$ CFU), all mice died, but the time to death was shorter for mice infected with the wild-type strain than for mice infected with the mutant strain. To further establish the role of Hfq in the pathogenesis of the human-pathogenic strain, we performed competition infection experiments with mice. For these experiments, a mixture of FSC200 and FSC768 bacteria (1:1 ratio; 10^2 CFU) was used to infect groups of five mice by the subcutaneous route. After 5 days of infection, the number of viable bacteria was determined by plating dilutions of spleen homogenates, and this was followed by PCR to distinguish wild-type bacteria from mutant bacteria. The average CI (Fig. 7C), defined as the ratio of mutant bacteria to wild-type bacteria, is 0.09, clearly demonstrating that the *hfq* mutant is attenuated for virulence.

Taken together, these results show that Hfq in the clinical isolate of *F. tularensis* subsp. *holarctica*, like Hfq in LVS, contributes to virulence in mice, as well as to growth in vitro and resistance to stress.

Transcriptome analyses demonstrate global changes in gene expression in the *hfq* mutant. Hfq is a global regulatory protein that influences gene expression by affecting translation and/or mRNA stability. To identify genes that are regulated by Hfq, we compared the transcriptome of LVS to that of the *hfq* mutant using DNA microarrrays. The microarrays (supplied by the Pathogen Functional Genomics Resource Center) contain oligonucleotides (four copies of each oligonucleotide) representing the open reading frames of *F. tularensis* strains LVS and Schu S4. To minimize any specific effect of a certain medium or growth condition on the results, we isolated RNA from bacteria grown under three different conditions: after growth on chocolate agar plates, after growth to exponential phase in defined medium (9), and after growth to exponential phase in complex broth (Schaedler K3 broth). All growth experiments were repeated at least twice, and each sample was labeled and hybridized to the microarrays in duplicate. The data obtained were analyzed using the SAM program (52) to identify genes for which there were statistically significant changes in expression under specific growth conditions. Genes with significantly changed expression (≥2-fold) under at least two conditions are shown in Table 2. We found that 16 genes were expressed at a lower level in the *hfq* mutant and 88 genes were expressed at a higher level in the mutant strain, suggesting that Hfq more often acts as a repressor than as an activator of expression in *F. tularensis*. However, we cannot conclude from this analysis which genes Hfq directly regulates and which genes have altered expression as a result of other changes. Among the downregulated genes were, in addition to *hfq* itself, *hflX* located downstream of *hfq*, as well as genes encoding

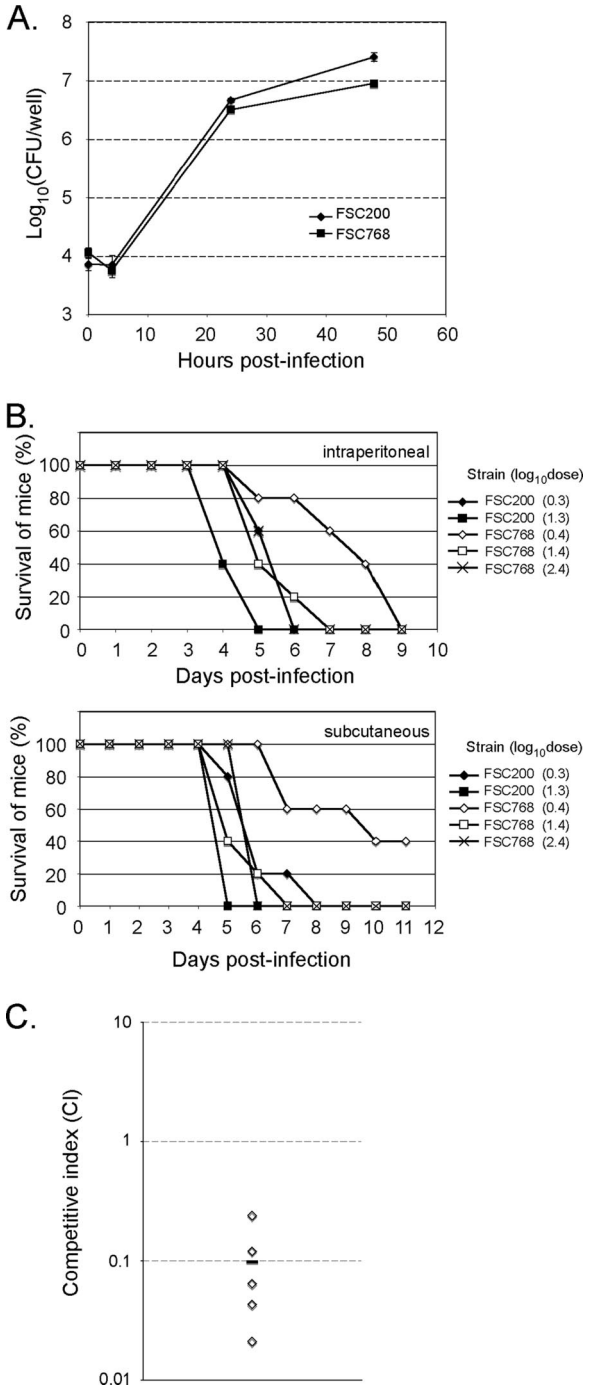

FIG. 7. Virulence of FSC200 is affected by the *hfq* mutation. (A) Intracellular replication in the J774 murine macrophage cell line. (B) Survival of mice after infection with FSC200 or FSC768 for 11 days after infection. Groups of five mice were infected by the i.p. or subcutaneous route with different numbers of bacteria. The numbers of bacteria (in $\log_{10}$ CFU) used for infection are indicated in parentheses after the strain designation. (C) Five mice were inoculated by the subcutaneous route with approximately 100 bacteria from a 1:1 mixture of FSC200 and FSC768. Spleens were homogenized after 5 days of infection, and the numbers of wild-type and mutant bacteria were determined. The results are expressed as the CI, which was calculated using the ratio of mutant bacteria to wild-type bacteria. Each symbol represents data for a single mouse, and the average CI is indicated by a solid line.

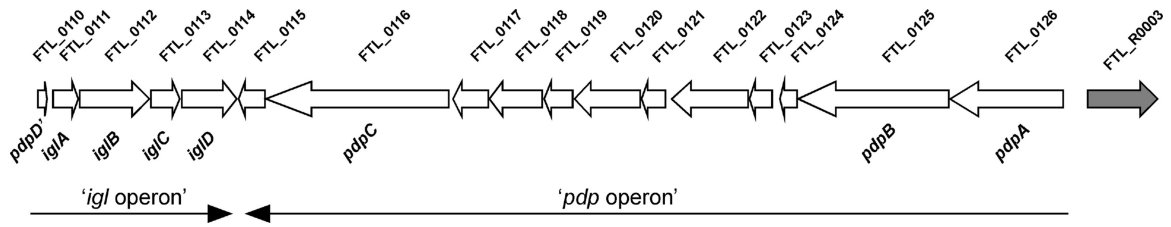

FIG. 8. Schematic diagram of the FPI. Genes are numbered according to the LVS strain nomenclature for one of the two copies of the FPI. The two proposed divergent operons are indicated at the bottom.

putative membrane proteins, lipoproteins, and transport proteins. A number of genes that were upregulated in the *hfq* mutant are involved in metabolic functions (e.g., four genes encoding enzymes of the biotin metabolic pathway). Also, several genes in the "genetic information and processing" category according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg-bin/show_organism?org = ftl) were upregulated. These genes include genes encoding type IV pilus proteins, ribosomal proteins, and nucleases. Most of the genes (48 genes) are not assigned to a KEGG category, and 25 of these genes encode hypothetical proteins and conserved hypothetical proteins. Strikingly, in the group of hypothetical genes are 10 genes that are part of the FPI. These 10 genes are located in the same orientation on the genome in the proposed "*pdp*" operon, while the expression of none of the genes in the "*igl*" operon changes significantly (Fig. 8).

To verify the microarray results, we selected eight genes for qRT-PCR experiments. Two of these genes were downregulated in the *hfq* mutant (FTL_0317 and *katG* [FTL_1504]), three were upregulated in the *hfq* mutant (FTL_0120, *pdpB* [FTL_0125], and FTL_0994), and the expression of three genes did not change (*iglC* [FTL_0113], *iglA* [FTL_0111], and *iglB* [FTL_0112]). Five of the selected genes are part of the FPI (*pdpB*, FTL_0120, *iglA*, *iglB*, and *iglC*). The results obtained with RNA samples prepared from bacteria grown on agar plates or in defined medium are shown in Fig. 9 and, in general, confirm the data obtained in the microarray analysis. We similarly observed increased expression of *pdpB*, FTL_0120, and FTL_0994 and lower expression of *katG* and FTL_0317 in the *hfq* mutant. However, the expression of *iglC* was found to be lower in the *hfq* mutant than in LVS by qRT-PCR, whereas as determined by microarray analysis the expression of this gene was not significantly changed. However, when we performed qRT-PCR with the *iglA* and *iglB* genes, which are part of the "*igl*" operon of the FPI and are adjacent to the *iglC* gene, we did not observe any difference in expression between the wild-type strain and the *hfq* mutant.

DISCUSSION

The Hfq protein contributes to the stress resistance and virulence of several pathogenic bacteria, including extracellular as well as intracellular organisms. The impact of *hfq* inactivation in these bacteria appears to be quite variable in terms of both the extent and the severity. A paradigm for the action of Hfq cannot be established from the data because the amount of data obtained in each case is quite variable and because our knowledge concerning the regulatory mechanisms and pathogenicities of these organisms is also quite heterogeneous.

Here we describe for the first time characterization of *hfq* mutants of two different strains of *F. tularensis* subsp. *holarctica*, the vaccine strain LVS and the human-pathogenic clinical isolate FSC200. We demonstrate that Hfq has a role in stress resistance, as well as in the ability to cause disease in mice. We further show that Hfq regulates, directly or indirectly, the expression of several genes in LVS, acting mainly as a repressor. Strikingly, we find that Hfq regulates expression of a subset of genes located in the FPI, thereby demonstrating that there is differential regulation of the two putative divergent operons in the FPI.

Hfq is involved in virulence. In *F. tularensis* LVS, *hfq* gene expression is likely driven by a sigma 70 promoter, and Hfq production is constant in all growth phases in broth. Similar to what has been observed for several other bacteria, *hfq* inactivation results in a slight growth defect in LVS, both on solid media and in liquid media. The *hfq* mutant appeared to be highly sensitive to heat treatment (40°C) and was also more

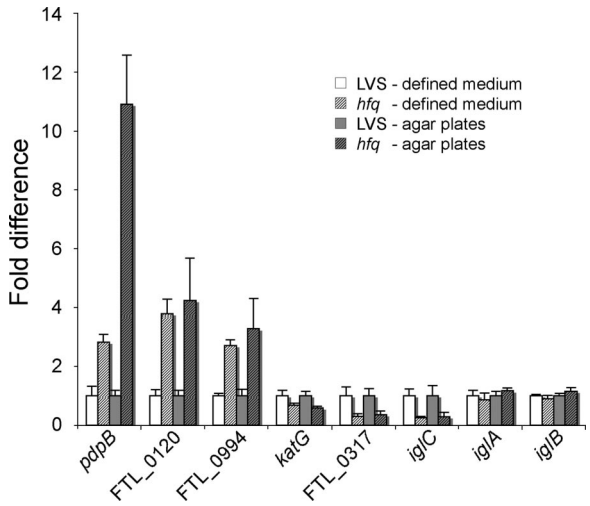

FIG. 9. Quantification of transcription of selected genes by real-time RT-PCR. Transcript levels were normalized to the level of DNA helicase (FTL_1656), and the differences (relative to LVS) and standard deviations are indicated for eight genes. RNAs from strains grown under two different growth conditions (defined medium and solid medium) were used for the analysis. The data are data for triplicate samples analyzed at the same time. The difference between the transcript levels of the LVS and *hfq* strains was significant ($P < 0.05$) for both growth conditions for *pdpB*, FTL_0120, FTL_0994, *katG*, FTL_0317, and *iglC*, as determined using Student's *t* test.

sensitive than the parental LVS strain to other stresses, including SDS and a high NaCl concentration (2%). These experiments demonstrate that Hfq contributes to resistance to various stress conditions in *F. tularensis* LVS.

We examined the contribution of Hfq to *Francisella* intracellular survival by comparing the abilities of the vaccine strain LVS and the isogenic *hfq* mutant to multiply inside J774 and RAW macrophage cell lines and in BMM. In all three types of macrophages, we found that inactivation of *hfq* led to an intracellular growth defect, particularly after 24 h. However, at 48 h the growth differences between the two strains were far less pronounced, suggesting that Hfq has a dominant role during the initial stages of intracellular multiplication.

We also evaluated the importance of Hfq in a mouse infection model of tularemia. Vaccine strain LVS, which is considered avirulent for humans, has significant virulence for mice, and four of five mice infected by the i.p. route with a dose of $\sim 3 \times 10^4$ bacteria succumbed to the infection. In contrast, an infecting dose of $>10^6$ bacteria was required to cause death when mice were infected with the *hfq* mutant by the i.p. route, demonstrating the severe attenuation of virulence of the *hfq* mutant with the LVS background. We also studied the infection kinetics of bacterial multiplication in the liver and spleen after i.p. infection with $\sim 3 \times 10^4$ LVS or *hfq* mutant bacteria. Strikingly, while the number of LVS bacteria was up to 10^8 bacteria per organ after 4 days (ultimately leading to death in most cases), the number of *hfq* mutant bacteria started to decrease after 2 days in both the spleen and liver, and all mice survived. These data indicate that the *hfq* mutant was unable to multiply efficiently inside host tissues and cause disease.

We further evaluated the role of Hfq in *F. tularensis* by creating an *hfq* mutant of the human-pathogenic clinical isolate FSC200. Like the mutant with the LVS genetic background, the FSC200 *hfq* mutant showed a slight growth defect in both solid and liquid media. The FSC768 mutant strain was also more sensitive than its isogenic parent to a high NaCl concentration (2%) or SDS, but it did not show increased sensitivity to an elevated temperature. In J774 macrophages, growth of the mutant was altered very moderately (after 48 h), reflecting the milder impact of the *hfq* deletion on the intracellular survival of FSC200. In mice, the *hfq* mutant also showed some attenuation, but the virulence defect was clearly less pronounced than that in the mutant with the LVS background. However, by performing a more sensitive CI analysis for spleens of animals infected with a 1:1 mixture of the wild-type and mutant strains, we could conclusively demonstrate that the *hfq* mutant was attenuated for virulence and more than 10 times less competitive for establishing an infection than the wild-type strain.

Strain FSC200 is extremely virulent in mice, and less than five bacteria (in this study the typical low doses were two to four bacteria) are required for a lethal infection by either the i.p. or subcutaneous route. Therefore, it is difficult to compare the levels of attenuation for this highly virulent strain and LVS. Importantly, the *hfq* mutant with the virulent FSC200 background was also attenuated compared to parental strain FSC200, both in single-strain infections and in competition infection experiments with the wild-type strain. This strongly supports the conclusion that Hfq is also important for virulence in human-pathogenic *F. tularensis* strains.

The fact that Hfq appeared to be less important for virulence in virulent strain FSC200 than in LVS is likely related to the fact that the mouse is far from the ideal model for tularemia. We have seen similar effects in other studies where we examined the role of a pilin gene, *pilA*, in virulence. In one study, where we used a moderately virulent strain isolated from a hare, we found that PilA mutants were severely attenuated compared to a strain expressing PilA (16). In contrast, when *pilA* mutants of virulent strains like FSC200 and the type A strain Schu S4 were used to infect mice, the level of attenuation was lower (unpublished results). Thus, it appears that the extremely low infection doses for these human-pathogenic strains in mice make it more difficult to establish the role in virulence of genes that do not have a major effect on intracellular multiplication and survival. A major strength of this work is that we have established that Hfq contributes to virulence both in the widely studied model strain LVS and in a clinical isolate.

Hfq, a novel regulator of the FPI. Several studies, including proteomic and microarray analyses, with various bacteria have shown that Hfq controls the expression of numerous genes, including genes encoding virulence factors (14, 15, 26, 42, 46). Here, we compared the transcriptome of LVS to that of an *hfq* mutant using DNA microarrrays. Total RNA was extracted from bacteria grown on solid medium or in liquid culture (complex broth or chemically defined medium). Of the 104 genes with significantly changed expression, only 16 were expressed at a lower level in the *hfq* mutant (and are thus positively regulated by Hfq in a wild-type context), suggesting that Hfq acts more often as a repressor than as a positive regulator of gene expression in *F. tularensis*. However, we expect that some of these genes are not directly regulated by Hfq but the expression is changed indirectly. The 88 genes upregulated in the *hfq* mutant belong to a variety of functional categories, ranging from metabolic functions to type IV pilus biogenesis or ribosomal proteins, but the majority of them (55%) have not been assigned to any putative function. Strikingly, within the group of hypothetical genes, a cluster of 10 contiguous genes that are in the same orientation is part of the FPI. These genes belong to the proposed "*pdp*" operon. Remarkably, none of the genes in the "*igl*" operon of the FPI, which are in the opposite orientation, were expressed at higher levels in the *hfq* mutant (Fig. 9).

Of note, the *iglA-iglD* operon has a G+C content of 30.6% (compared to an average G+C content of 33% for the *Francisella* chromosome), while the *pdpA*-FTL_0115 region ("*pdp*" operon) has a G+C content of 26.6% and is the region with the lowest G+C content in the chromosome. For example, the G+C content of the *pdpA* gene (26.99%) is strikingly different from that of the adjacent rRNA genes (52.07% for FTL_R0003, the first gene of the *rrn* operon in the opposite orientation). The 374-bp intergenic region between FTL_R0003 and *pdpA* (FTL_0126) comprises two distinct halves, one immediately upstream of *pdpA* with a very low G+C content (ca. 20%) and the other with a significantly higher G+C content (>35%), suggesting that there is a putative promoter region immediately upstream of *pdpA*.

The *pdpA* and *pdpB* genes have been identified in at least two different screens of transposon insertion mutant libraries. Inactivation of *pdpA* in *F. tularensis* subsp. *novicida* led to a severe intramacrophage growth defect, and, in vivo, the *pdpA*

mutant was highly attenuated (38, 54). Inactivation of *pdpB* in *F. tularensis* subsp. *novicida* also led to a severe in vitro growth defect in J774 and RAW cells, as well as in primary murine BMM, and to strong attenuation of virulence in the mouse (49, 54).

Four different regulatory proteins have been shown to control expression of the FPI. MglA interacts with the SspA protein, forming a heterodimer that binds RNA polymerase to control virulence gene expression positively (10). MglA and SspA seem to act in parallel with the FevR protein, which controls the same set of genes as these proteins (6). The fourth regulatory protein, PmrA, activates virulence gene expression as well, but it controls another set of genes in addition to the FPI (34). All four proteins affect virulence gene expression positively, and they all seem to regulate the entire FPI (although not every gene was found in each study, each study identified several genes in both of the putative divergent operons).

Our study is the first study in which it was shown that expression of only one part of the FPI is affected by mutation of a regulatory protein. Furthermore, the expression of the virulence genes is increased in the mutant strains, implying that Hfq is a negative regulator of virulence gene expression, in contrast to the other regulators of the FPI.

Even though the virulence genes are expressed at higher levels in the *hfq* mutant, it is possible that the deregulation is the background for the virulence attenuation that we observe in mice, as strict control of the FPI may be a requirement for bacterial survival in host organs. However, the virulence attenuation could be an effect of Hfq regulation of other target genes that impair intracellular multiplication in vivo.

Altogether, the capacity of Hfq to affect various cellular functions, such as metabolism, type IV pilus production, and virulence-related phenotypes, in *F. tularensis* probably reflects the ability of this chaperone to interact with a wide range of different regulatory RNAs, which remain to be identified. Of note, no homologues of sRNAs previously identified in other bacteria could be identified by in silico analysis of *F. tularensis* genomes, suggesting that the sRNAs produced by *F. tularensis* possess unique regulatory properties.

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