Identification of *Francisella tularensis* Genes Affected by Iron Limitation†

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Cells of an attenuated live vaccine strain (LVS) of *F. tularensis* grown under iron-restricted conditions were found to contain increased quantities of several proteins relative to cells of this same strain grown under iron-replete conditions. Mass spectrometric analysis identified two of these proteins as IglC and PdpB, both of which are encoded by genes located in a previously identified pathogenicity island in *F. tularensis* LVS. Regions with homology to the consensus Fur box sequence were located immediately in front of the *iglC* and *pdpB* open reading frames (ORFs), and in silico analysis of the *F. tularensis* Schu4 genome detected a number of predicted 5′ untranslated regions that contained putative Fur boxes. The putative Fur box preceding *Francisella* iron-regulated gene A (*figA*) had the highest degree of identity with the consensus Fur box sequence. DNA microarray analysis showed that nearly 80 of the genes in the *F. tularensis* LVS genome were up- or down-regulated at least twofold under iron-restricted growth conditions. When tested for possible siderophore production by means of the Chrome Azurol S assay, a wild-type *F. novicida* strain produced a large reaction zone whereas its *figA* mutant produced very little reactivity in this assay. In addition, a cross-feeding experiment demonstrated that this siderophore-like activity produced by the wild-type *F. novicida* strain could enhance the ability of the *F. novicida figA* mutant to grow under iron-restricted conditions. This study provides the first identification of iron-regulated genes in *F. tularensis* LVS and evidence for the production of a siderophore-like molecule by *F. novicida*.

Francisella tularensis is a gram-negative coccobacillus that causes tularemia in both humans and animals (28, 33, 40). *F. tularensis* subspecies *tularensis* (type A) and *F. tularensis* subspecies *holarctica* (type B) are highly infectious for humans. Inoculation or inhalation of as few as 10 CFU of *F. tularensis* type A (i.e., *F. tularensis* Schu4) may cause disease in humans (37, 38). Because of its extremely high infectivity, this bacterium is listed as a class A biological warfare agent (7, 28). Type B strains of *F. tularensis* are less virulent than type A strains for higher mammals and typically cause nonfatal disease in humans. An attenuated live vaccine strain (LVS) of *F. tularensis* which was derived from a virulent type B strain by passage in vitro (37, 38) has been widely used in studies of *F. tularensis*. *Francisella novicida*, which is closely related to *F. tularensis* (12, 17), is highly virulent in mice but has relatively low virulence for humans (28). *F. novicida* is more easily manipulated genetically and has less fastidious growth requirements than *F. tularensis* strains (13). Recently, *F. novicida* was used to identify and characterize a pathogenicity island involved in intracellular growth (27). In addition, the MglA protein was shown to be involved in the regulation of bacterial gene products necessary for intracellular growth of this pathogen (21, 36). With these few exceptions, however, little is known about the *F. tularensis* gene products involved in virulence.

For several decades, investigators have been aware of the importance of iron in microbial physiology (4). In this context, the human body provides an environment of extreme iron limitation from the standpoint of the bacterium because free iron is not normally present in the bloodstream or tissues (42). As a consequence, successful commensal organisms and pathogens express high-affinity iron uptake systems by which they can obtain iron in vivo. Bacterial genes that encode factors involved in the iron acquisition process in vivo are considered virulence factors and have been subjected to intensive study. However, compared with the well-documented iron acquisition systems of other facultative intracellular pathogens, including *Legionella pneumophila* (8), *Yersinia pestis* (32), and *Mycobacterium* species (34), virtually nothing is known about how *F. tularensis* acquires iron from its mammalian hosts.

In a study of the effect of stress conditions on the virulence of *F. tularensis* LVS, it was reported that a few cell envelope proteins appeared to be up-regulated when iron was limiting (2). However, these proteins were not identified and there have been no subsequent reports on this subject to date. In the present study, we used two different methods to identify *F. tularensis* LVS genes whose expression was up-regulated during growth under iron-restricted conditions. *Francisella* iron-regulated gene A (*figA*) was among those most highly expressed under these conditions and was shown, by mutant analysis and cross-feeding experiments, to encode a protein necessary for the synthesis and/or secretion of a siderophore-like activity by *F. novicida* U112.

MATERIALS AND METHODS

Bacterial strains and culture media. *F. tularensis* LVS no. 11 (11) and *F. novicida* U112 (27) have been described previously. These strains and the others used in this study are listed in Table 1. The basal medium for these studies was Mueller-Hinton agar (Difco, Detroit, MI) supplemented with 2% (vol/vol) IsoVitaleX (Becton-Dickinson, Sparks, MD), 0.1% (wt/vol) glucose, and 1% (vol/vol) fetal bovine serum (FBS); this medium was designated MH−. For routine cultivation of *Francisella* strains, ferric pyrophosphate (Fe-PP$_i$) was added to MH− to a final concentration of 250 μg/ml (330 μM); the resultant medium was designated MH+. For iron-restricted growth, deferoxamine mesylate (DF; Desferal; Novartis, East Hanover, NJ) was added to MH− to a final

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
E. coli strains		
DH5α	Host strain for cloning experiments	34
HB101	Host strain essential for propagating plasmids carrying mutated *Francisella* genes used for electroporation of *Francisella*	34
Francisella tularensis strains		
LVS no. 11	*F. tularensis* LVS	10
LVS *figA::np-kan*	Mutant of LVS with promoterless *kan* cartridge inserted in *figA* gene	This study
LVS *figA::np-kan*(pKD107)	LVS *figA::np-kan* containing pKD107 with *F. novicida* U112 *figA* gene; expresses FigA protein	This study
Francisella novicida strains		
U112	Wild-type strain of *F. novicida*	26
U112 *figA::np-kan*	Mutant of U112 with promoterless *kan* cartridge inserted in *figA* gene	This study
U112 *figA::np-kan*(pKD107)	U112 *figA::np-kan* containing pKD107 with *F. novicida* U112 *figA* gene; expresses FigA protein	This study
U112 *figA::np-kan*(pKD108)	U112 *figA::np-kan* containing pKD108 with *F. tularensis* LVS *figA* gene; expresses FigA protein	This study
U112 *figA::np-kan*(pFNLTP-CAT)	U112 *figA::np-kan* containing vector pFNLTP-CAT	This study
Plasmids		
pACYC184	Broad-host-range cloning vector; Chlor^r Tet^r	New England Biolabs
pKD101	pACYC184 with 2.2-kb fragment containing *figA* flanking regions	This study
pKD101-*np-kan*	pKD101 with promoterless *kan* cartridge inserted into KpnI site	This study
pFNLTP6	Shuttle vector for *Francisella*; Kan^r Amp^r	24
pKD105	pFNLTP6 with *F. tularensis* LVS *figA* gene inserted into BamHI site	This study
pKD108	pKD105 with ΔE*cat* cartridge inserted into XhoI site; this vector backbone containing the ΔE*cat* cartridge is the same as that of pFNLTP-CAT	This study
pFNLTP-CAT	pFNLTP6 with ΔE*cat* cartridge inserted in XhoI site; Kan^r Amp^r Chlor^r	This study
pKD107	pFNLTP-CAT carrying *F. novicida* U112 *figA* gene	This study

concentration of 100 μM; this medium was designated MH−/DF. Broth-based media of all three types lacked FBS. All strains were routinely grown at 37°C in an atmosphere of 95% air–5% CO_2 or in broth at 37°C with aeration.

Growth of *F. tularensis* LVS under iron-replete and iron-restricted conditions. *F. tularensis* LVS was streaked from a frozen stock onto MH+ agar and grown at 37°C for 24 h. For iron-replete growth, bacteria from this agar plate were inoculated into 1 ml of MH+ broth and grown at 37°C overnight. This overnight broth culture was then used to inoculate 10 ml of MH+ broth to an optical density at 600 nm (OD_{600}) of 0.05. For iron-restricted growth, the overnight culture was obtained by inoculating the bacteria from the aforementioned agar plate into 1 ml of MH− broth. This overnight broth culture was used to inoculate 10 ml of MH−/DF broth to an OD_{600} of 0.05. For iron add-back experiments, an overnight culture of the *F. tularensis* LVS in MH− medium was used to inoculate 20 ml of MH−/DF broth to an OD_{600} of 0.05. When the culture reached an OD_{600} of 0.4, the culture was divided into two cultures of 10 ml each. Fe-PP$_i$ was added to one culture, whereas an equivalent volume of sterile H_2O was added to the other culture. Both cultures were then grown as described above.

Preparation of whole-cell lysates. *F. tularensis* LVS or *F. novicida* U112 cells grown on MH+ plates were inoculated into 5 ml of MH+ or MH−/DF medium for iron-replete or iron-restricted growth, respectively. After overnight incubation, the cells were harvested by centrifugation at 5,800 × *g* for 10 min at 4°C. The cell pellet was resuspended in 3 ml of phosphate-buffered saline (PBS)/g (wet weight) of cells. A portion of 3×-concentrated sodium dodecyl sulfate (SDS) sample buffer (30) equivalent to half the volume of these suspended cells was then added, and the samples were heated at 100°C for 5 min. Western blot analysis was performed as previously described (10), with polyclonal antisera to various *F. tularensis* antigens; these antisera were diluted 1:3,000.

Preparation of cell envelopes of *F. tularensis* LVS. Cultures (100 ml) of the *F. tularensis* LVS were grown into stationary phase under iron-replete or iron-restricted conditions. After harvesting of the cells by centrifugation, the cells were washed twice in phosphate-buffered saline (PBS) and disrupted by sonication. Unbroken cells and cell debris were removed by centrifugation at 10,000 × *g* for 20 min at 4°C. The supernatant fluid was then subjected to centrifugation at 150,000 × *g* for 1 h at 4°C. The resultant cell envelope pellet was washed twice with PBS and resuspended in 0.5 ml PBS. The protein concentration of the cell

envelope preparation was determined by the Bradford method with the Protein Assay reagent (Bio-Rad, Hercules, CA), and approximately 20 μg of protein was loaded in each well for SDS-polyacrylamide gel electrophoresis (PAGE).

Protein identification. Proteins in cell envelope preparations were resolved by SDS-PAGE and stained with Coomassie blue. Selected protein bands were excised and identified by nano-high-performance liquid chromatography–tandem mass spectrometry performed by the Protein Chemistry Technology Center at the University of Texas Southwestern Medical Center.

Generation of polyclonal mouse antibodies against *F. tularensis* proteins. The predicted amino acid sequences of the *F. tularensis* Schu4 FigA and PdpB proteins were derived from the genome of this organism (19). A 21-amino-acid (aa) sequence (KDNNPQINHDDWQQFEYELDN) from FigA and a 24-aa sequence (KLQHFYGEITKLNKQNNTNDQNDK) from PdpB were synthesized with a cysteine residue added to the N terminus of each peptide. These peptides were covalently coupled to Imject maleimide-activated mariculture keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL) and used to immunize mice.

Nucleotide sequence analysis. PCR was used to amplify a 7-kb fragment from *F. novicida* that contained the *fur* gene and the *figABCD* gene cluster. Nucleotide sequence analysis of this DNA fragment showed that these five open reading frames (ORFs) from *F. novicida* (GenBank accession number DQ497185) had 97 to 100% identity with the same ORFs from both *F. tularensis* LVS and *F. tularensis* Schu4. The nucleotide sequences of these ORFs from the latter two strains are available from GenBank.

RNA isolation from *F. tularensis* LVS. *F. tularensis* LVS cells were grown under both iron-replete and iron-restricted conditions into the early logarithmic phase (OD_{600}, ~0.3). The cultures were harvested by centrifugation at 6,000 × *g* for 15 min at 4°C. Total RNA was isolated with the RNeasy Midi kit (QIAGEN, Valencia, CA) by following the manufacturer's protocol. During RNA extraction, treatment with the RNase-free DNase set (QIAGEN) was used to remove any DNA contamination. RNA concentration was determined by spectrophotometry (OD_{260}), and RNA integrity was verified by agarose gel electrophoresis.

DNA microarray development. Preliminary annotation of the incomplete *F. tularensis* Schu4 genome was accomplished with a combination of automated ORF prediction and homology searching. The collection of 48 contigs in the

August 2002 release of the *F. tularensis* Schu4 genome was obtained from http: //artedi.ebc.uu.se/Projects/Francisella/. The Glimmer 2.02 software was obtained from The Institute for Genomic Research and was used to predict most of the ORFs in the incomplete genome (9). Because Glimmer requires a complete, intact genome, the 48 contigs from the incomplete genome were concatenated into one contiguous sequence. The sequence 5′-TTAACTAACTAG-3′, which contains stop codons in all six reading frames, was inserted between contigs to prevent Glimmer from erroneously predicting false ORFs that spanned contig junctions. Glimmer was then used to predict putative ORFs (encoding proteins containing at least 50 aa) in this contiguous sequence. 2,051 putative ORFs were predicted and were translated with the standard genetic code. Seventy-mer oligonucleotides designed to anneal specifically to a region within the 5′ 70% of each of these 2,051 annotated ORFs were synthesized by QIAGEN and spotted in triplicate on Corning UltraGapII slides by Microarrays, Inc. (Nashville, TN). Subsequent to the preparation of these microarray slides, the definitive annotation of the *F. tularensis* Schu4 genome was published (19) and this more recent annotation was used in this study to identify relevant ORFs.

DNA microarray analysis. Genome-directed primers (3) were designed to anneal within the 3′ 30% of each ORF. These 47 genome-directed primers were synthesized by QIAGEN and used to prime the mRNA in total RNA preparations for cDNA synthesis. Equal quantities of total RNA from cells grown under iron-replete and iron-restricted conditions were used to generate cDNA with the CyScribe Post-Labeling kit (Amersham Biosciences, Piscataway, NJ). After reverse transcription, residual RNA was removed by alkaline treatment, followed by neutralization. The cDNAs were purified with a QIAquick gel extraction kit (QIAGEN). For each DNA microarray slide, one sample was labeled with Cy3-dCTP while another sample was labeled with Cy5-dCTP. Dye swap experiments were performed for each pair of samples to compensate for the different labeling efficiencies of Cy3 and Cy5. The labeling mixtures were cleaned by using YM-30 Microcon centrifugal filter devices (Millipore, Bedford, MA). Equal amounts of labeled cDNA from cells grown under both conditions were used to hybridize microarray slides in the hybridization buffer provided with the CyScribe Post-Labeling kit. Hybridization was carried out at 50°C for 16 h in the dark. After hybridization, the slides were washed in saline–sodium phosphate-EDTA buffer and scanned with GenePix scanner 4100A and GenePix Pro 5.0 software (Axon Instruments Inc., Union City, CA). Data from three independent RNA preparations hybridized on a total of seven slides were normalized and then analyzed with Acuity 4.0 software (Axon). Genes that were differentially regulated more than twofold were considered to be differentially transcribed. Selected results from these analyses are contained in Table 2.

Quantitative real-time reverse transcription-PCR (qRT-PCR). Twenty-one genes were selected for analysis of their transcription levels by qRT-PCR. For each of the genes tested, primers (Table 3) were designed with Primer Express software (Applied Biosystems, Foster City, CA). Total RNA was isolated from *F. tularensis* LVS cells grown under iron-replete and iron-restricted conditions as described above. To remove possible DNA contamination, an additional step of DNase treatment of the RNA samples was performed with the MessageClean kit (GeneHunter, Nashville, TN). Equal quantities of RNA from cells grown under both conditions were used in qRT-PCRs with SYBR Green PCR Master Mixture (Applied Biosystems) and MultiScribe reverse transcriptase (Applied Biosystems). Each reaction was carried out with a 25-μl volume containing 50% (vol/vol) SYBR master mix, the two oligonucleotide primers at a final concentration of 50 nM each, and 10 ng of RNA. PCR amplification was accomplished with a model 7500 real-time PCR system (Applied Biosystems). The relative levels of gene expression were calculated with 7500 System SDS software (Applied Biosystems) by the ΔΔCt method. The *F. tularensis* LVS *fopA* gene was chosen as an internal control because this gene apparently did not alter its level of expression between iron-replete and iron-restricted growth conditions as measured by DNA microarray and Western blot analyses (data not shown). Both no-reverse transcriptase reaction mixtures and no-template reaction mixtures were always included as negative controls.

RT-PCR. RT-PCR was performed with the SuperScript III kit (Invitrogen, Carlsbad, CA). Total RNA was isolated from *F. tularensis* LVS cells grown in iron-replete medium as described above. RNA (1 μg) was incubated with SuperScript III reverse transcriptase and appropriate oligonucleotide primers. PCR amplification with *Taq* DNA polymerase (New England Biolabs, Beverly, MA) was carried out at temperatures of 52°C for annealing and 72°C for extension.

Construction of *figA* mutants of *F. tularensis* LVS and *F. novicida* U112. All of the oligonucleotide primers used in this study were derived from the *F. tularensis* Schu4 genome (19). An ~1.0-kb fragment corresponding to the 5′ upstream region of the *F. tularensis* LVS *figA* gene was PCR amplified from chromosomal DNA with *Pfu* DNA polymerase (Stratagene, La Jolla, CA), primer 1 (5′-GCG**G**

GATCC**GCGCCAACAATCACTGATAAAAC-3′), and primer 2 (5′-**GGTACC** **GCATGCATGCAT**TTTAAAATCCCTACATGATAATG-3′). Another ~1.2-kb fragment corresponding to the 3′ downstream region of the *F. tularensis* LVS *figA* gene was amplified with primer 3 (5′-**ATGCATGCATGCGGTACC**GTAAAAG GTGAAATACACCAAAG-3′) and primer 4 (5′-GC**GGATCCC**AAAGGTAA CTCCAAGTATG-3′). Primers 2 and 3 shared an 18-nucleotide (nt) complementary sequence (in bold) at their 5′ ends, and a KpnI site (bold and underlined) was contained in these sequences. One-microliter portions of the two PCR amplicons were then mixed and used as the template in a bridging PCR (i.e., PCR sewing) (24, 29) with primers 1 and 4. This second PCR generated an ~2.2-kb product corresponding to the 5′ and 3′ flanking regions of the *figA* ORF with a KpnI site in the middle of this amplicon and BamHI sites (underlined in primers 1 and 4) at both ends. This fragment was digested with BamHI and ligated into BamHI-cut pACYC184 (New England Biolabs) to obtain pKD101.

A promoterless 850-bp kanamycin resistance cartridge (*np-kan*) (26) was amplified with *Pfu* DNA polymerase together with primer 5 (5′-ATA**GGTACC**G GGTGACTAACTA GGAGG-3′) and primer 6 (5′-ATA**GGTACC**GGGTCGC ATTATTCCCTCCA-3′) (KpnI sites underlined). The *np-kan* amplicon was digested with KpnI and ligated with KpnI-digested pKD101 to obtain pKD101-*np-kan*. This plasmid was used to transform *Escherichia coli* HB101 (35); plasmid purified from this recombinant strain by means of a QIAGEN Midi-prep kit was used to electroporate *F. tularensis* LVS. For electroporation, cell paste was scraped from two confluent MH+ agar plates and washed three times in 50 ml of 0.5 M sucrose by centrifugation at 5,800 × *g* for 10 min at 10°C. The washed cells were resuspended in 250 μl of 0.5 M sucrose and divided into individual 80-μl portions. A 7-μg portion of pKD101-*np-kan* (in a 10-μl volume) was added to an 80-μl portion of these cells, and the mixture was incubated at room temperature for 10 min. The mixture was then transferred into an electroporation cuvette (1-mm electrode gap) and electroporated at 2.0 kV, 10 μF, and 600 Ω. A 200-μl portion of MH+ medium was added to the cuvette, and the entire content of the cuvette was spread onto a chocolate II agar (CA II) plate (Becton Dickinson), which was incubated at 37°C in 95% air–5% CO_2 for 6 h. Next, growth on this CA II plate was scraped into 500 μl MH− medium, and the surface of the CA II plate was washed once with another 500-μl portion of this medium. The liquid was pooled and spread onto two MH− plates containing kanamycin (15 μg/ml) and incubated as described above for 36 to 48 h. Kanamycin-resistant colonies were patched onto MH−/DF agar, and *figA* mutants were identified by colony blot analysis with mouse antiserum to FigA. For construction of an *F. novicida* U112 *figA* mutant, the method used was the same as that described above, with two exceptions; the electroporated cells were incubated on the CA II agar plate for only 3 h, and the concentration of kanamycin for selection on MH− agar plates was increased to 30 μg/ml.

Colony blot analysis. *F. tularensis* LVS or *F. novicida* U112 colonies grown on MH− plates were patched onto MH−/DF plates. After incubation for 24 h at 37°C, the patches were blotted with a sterile nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was then placed for 5 min on Whatman filter paper that had been saturated with 20% (wt/vol) SDS, transferred to another filter paper saturated with denaturing solution (0.5 M NaOH, 1.5 M NaCl), incubated for 5 min, and finally placed on a filter paper saturated with neutralizing solution (1.3 M NaCl, 0.3 M Tris-HCl, pH 8.0) for 5 min. After drying at room temperature for 30 min, the membrane was gently agitated in PBS with 0.05% (vol/vol) Tween 20 (PBST) for 5 min with one change of PBST buffer. Acetic acid (0.1 N) was used to wash the membrane twice to remove residual agar and cell debris. Residual acid was removed by washing with PBST twice, and the membrane was blocked with 3% (wt/vol) skim milk in PBST for 1 h. Mouse antiserum to FigA was used as the primary antibody, and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G was used as the secondary antibody. Final color development was accomplished by using the 3,3′,5,5′-tetramethylbenzidine liquid substrate system (Sigma) according to the manufacturer's protocol.

Complementation analysis. The primers used to PCR amplify a 2.9-kb fragment of *F. tularensis* LVS chromosomal DNA containing the *figA* ORF and some flanking DNA were primer 7 (5′-CG**GGATCC**TTAATCAGTTTGAGTCAGC AGG-3′) and primer 8 (5′-CG**GGATCC**TAACTAAGTAATGTGGCTAAAA CACC-3′). Both primers contained a BamHI site (underlined) at their 5′ ends, and the PCR amplicon was ligated into the BamHI site in the vector pFNLTP6 (25) to obtain pKD105. A chloramphenicol resistance cassette (Δ*Ecat*) (15) was then ligated into the XhoI site of this plasmid to obtain pKD108. Digestion of this plasmid with BamHI yielded three fragments; these were the vector backbone, the *cat* cartridge, and the cloned *F. tularensis* LVS DNA insert. The gel-purified *cat* cartridge and vector backbone were religated to yield pFNLTP-CAT. The primers used to PCR amplify a 3.2-kb fragment containing the *F.*

TABLE 2. *F. tularensis* LVS genes whose expression was maximally altered by growth under iron-restricted conditions[a]

F. tularensis Schu4 ORF no.	Gene identity or function	Avg fold difference[b]
Genes up-regulated under iron-restricted growth conditions		
29c	Hypothetical protein (FigA)	19.70
27c	Diaminopimelate decarboxylase (FigC)	15.13
26c	Hypothetical protein (FigD)	14.78
28c	Hypothetical protein (FigB)	9.78
1565c	Glycosyl hydrolase, family 3, pseudogene	4.08
1702	Conserved hypothetical protein	3.87
1701	Hypothetical protein	3.62
1707	Conserved hypothetical protein	3.41
1542c	Outer membrane protein	3.29
1714c	Intracellular growth locus, subunit A (IglA)	3.26
383	Hypothetical protein	3.22
989	Hypothetical protein	3.19
1709	Conserved hypothetical protein	3.09
980	Aminotransferase, class II	3.08
1711c	Intracellular growth locus, subunit D (IglD)	3.06
1703	Conserved hypothetical protein	3.01
1700	Conserved hypothetical protein (PdpB)	2.99
1706	Conserved hypothetical protein	2.99
1717	Major facilitator superfamily transport protein	2.98
1712c	Intracellular growth locus, subunit C (IglC)	2.96
Genes down-regulated under iron-restricted growth conditions		
1214c	Haloacid dehalogenase-like hydrolase family protein	0.32
881c	Amino acid permease	0.39
265	ABC transporter, membrane protein	0.42
1269c	Chaperone protein, DnaK (heat shock protein family 70)	0.43
1696	Chaperone protein, GroEL	0.43
1624c	Hypothetical protein	0.44
1695	Chaperone protein GroES	0.44
938	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	0.46
331	30S ribosomal protein S3	0.47
172	Hypothetical membrane protein, fragment	0.48
327	50S ribosomal protein L23	0.49
413c	1,4-α-Glucan branching enzyme	0.49
328	50S ribosomal protein L2	0.49
140	50S ribosomal protein L11	0.50
326	50S ribosomal protein L4	0.50
1048c	Hypothetical protein	0.51
266	ABC transporter, ATP-binding protein	0.51
551	Conserved hypothetical protein, pseudogene	0.52
999c	ZIP metal transporter family protein, pseudogene	0.52
339	30S ribosomal protein S8	0.52

[a] For detailed DNA microarray data, see Table S1 in the supplemental material.

[b] The average fold difference indicates the gene expression level under iron-restricted conditions relative to that obtained under iron-replete conditions.

novicida U112 *figA* ORF and some flanking DNA were primer 9 (5′-CAGCTA GCTGTTGAGATACTTAATTTATTTG-3′) and primer 10 (5′-CTGCTAGCC AAAGGACCTATAGCGATAC-3′). Both primers contained an NheI site (underlined) at the 5′ end, and the PCR amplicon was cloned into this site in pFNLTP-CAT to construct pKD107. Both pKD107 and pKD108 were purified from *E. coli* DH5α and used to electroporate *F. novicida* U112 as described above. The desired transformants were selected with kanamycin (30 μg/ml) on MH+ plates. Plasmids pKD107 and pKD108 purified from *F. novicida* U112 were then used to electroporate the *F. novicida* U112 *figA*::*np-kan* mutant. Plasmid pKD107 purified from *E. coli* DH5α was used to electroporate the *F. tularensis* LVS *figA*::*np-kan* mutant. Transformants were selected with chloramphenicol (10 μg/ml) on MH+ plates.

Chrome Azurol S (CAS) assay. CAS/CDM− plates were prepared by incorporating CAS/Fe(III)/hexadecyltrimethylammonium (HDTMA) into Chamberlain's chemically defined medium (6) lacking FeSO₄ (CDM−). Briefly, 250 ml of sterile CAS-HDTMA solution was prepared as described by Payne (31). Four grams of the amino acid mixture (6) was mixed with 20 mg of spermine phosphate and dissolved in 150 ml of H₂O. This solution was extracted with 150 ml

of 3% (wt/vol) hydroquinoline in chloroform to remove contaminating iron. Traces of residual hydroquinoline were removed by another extraction with an equal volume of chloroform. Thiamine-HCl, L-calcium pantothenate, glucose, MgSO₄, KH₂PO₄, K₂HPO₄, and NaCl were added to this deferrated amino acid solution to the final concentrations described (6). The pH of this CDM− solution was adjusted to 6.2 to 6.4 before the volume was brought to 250 ml with H₂O. The CDM− solution was then filter sterilized and warmed to 55°C. A 250-ml portion of 2% (wt/vol) agar (in water) was sterilized and cooled to 55°C. This agar solution, the CDM− solution, and 50 ml of CAS-HDTMA were mixed gently and dispensed into petri dishes. *F. tularensis* LVS and *F. novicida* U112 strains were inoculated onto the CAS-CDM− plates and incubated as described above for either 2 to 5 days (for *F. tularensis* LVS) or overnight (for *F. novicida* U112). Siderophore (enterobactin)-producing *E. coli* strain W3110N and its enterobactin-deficient *entF* mutant were used as positive and negative controls, respectively, in the CAS assay.

Cross-feeding experiment. *F. novicida* U112 and its *figA* mutant U112 *figA*::*np-kan* were streaked in parallel in various combinations onto both a CAS/

TABLE 3. qRT-PCR analysis of gene expression[a]

Schu4 ORF no.	Putative function	Fold difference[b]		qRT-PCR primer pair (5′–3′)[d]
		Microarray	qRT-PCR	
26c	Hypothetical protein (FigD)	14.8	13.4	F: AAGCCTAATGGTAGCTGGTGAATC R: TTTTGGTGAGACTCCGTAAGTTTTT
29c	Hypothetical protein (FigA)	19.7	24.7	F: GGCTACTAAAGACAAAACACTAATGCC R: CAGACTCAGGCGATACTGTTCTTG
27c	Diaminopimelate decarboxylase (FigC)	15.1	13.0	F: GGTTTAGCAGGAATAAGCCCTACTC R: TTGTCGCGGCATATAGTTCTAAATC
28c	Hypothetical protein (FigB)	9.9	19.1	F: GAGTTTTTTAGCTCAGACCCGATAATC R: ATCGGCGCTGAAAATAGCA
651[c]	Proton-dependent oligopeptide family protein	2.4	0.6	F: CTCGCAAAAAAGACATTAGTATAAAATTAAAT R: AAGTTGCAAATTGTCCCCAAA
1712c	Intracellular growth locus, subunit c (IglC)	3.0	2.6	F: AAAAAGGAGAATGATTATGAGTGAGATG R: TGCAGTAGGATCAGTTCTCACATG
403	Peptide deformylase	2.8	3.2	F: CAGCTATAATATTTCAGCATGAATTTAATCA R: AGCAAATTTAGCTTGTAGTTCTTCGTT
1699	Conserved hypothetical protein (PdpA)	2.6	2.6	F: TGAGTTAATTTCAAACTCTGCCATATC R: GTTTGGGTATATGCCATTTCACAG
1780	Putative transposase	1.5	1.1	F: AAGAACCGGAGATTATAGTTCAAAGC R: AAATACTGTTCAATCAATGTTTTATCGG
651[c]	Proton-dependent oligopeptide family protein	1.3	0.7	F: CGTTGATAGTAAAACTATTGGTTTTGCA R: GGAAGAGCAACCACTTGATTTAATAGA
664c	Histidine decarboxylase	1.0	1.1	F: CATTTGATGGTGCTTTTCTACCG R: ATATTCCTGAAGGCATCGGATTAC
583	Outer membrane-associated protein	0.9	1.0	F: CAAATCTAGCAGGTCAAGCAACAG R: AACACTTGCTTGAACATTTCTAGATAGTTC
700	Conserved hypothetical protein	0.8	0.6	F: AAAATGGTGTTCGCCTTTTGG R: CCTTCGACATCATCTTTGTAGTCAAC
445	ABC transporter, ATP-binding, pseudogene	0.9	1.2	F: TTAGAAGAGTTTGTTGGAGGATATGATG R: ATTTTTCGACAACTCACTGTTATTTTTAGT
881c	Amino acid permease	0.4	0.6	F: CCAAGGTTCAGAAATTATCGGACTA R: GTGCTTAGCGATTCTTGGCAT
50	Translation initiation factor IF2	0.6	0.8	F: TTGGCGCGTATTCTGTTAAGAC R: GCTCGCATTGAAGTAAAAGCCT
140	50S ribosomal protein L11	0.5	0.8	F: ACGCCACCTGCTTCTTACTTAATT R: CGCACGAGTTATAGTTCCAACAA
350	DNA-directed RNA polymerase, alpha subunit	0.7	0.7	F: TTGACAAACCAAAGAGCATTTAGC R: TCTCAAGCTCGGTTGGTATCG
1695	Chaperone protein, GroES	0.4	0.5	F: GCTCAAGAGAAACCTAGCCAAGG R: ACATCCATAGGTAGCGTAGTGCC
326	50S ribosomal protein L4	0.5	0.7	F: AGTTTCTGGTGGCGGTGC R: GGTGAACGGATAGTACCCGCT
1696	Chaperone protein, GroEL	0.4	0.7	F: TTCAAAGACAGCGGATGTTGC R: GCGACAGCTTTTAGACCCTCTG

[a] qRT-PCR for each gene was performed with two individually prepared RNA samples, each in triplicate.
[b] The fold difference indicates the gene expression level under iron-restricted conditions relative to that obtained under iron-replete conditions.
[c] ORF 651 in the *F. tularensis* Schu4 genome was found to be fragmented into two separate ORFs in the *F. tularensis* LVS genome by the deletion of a single nucleotide.
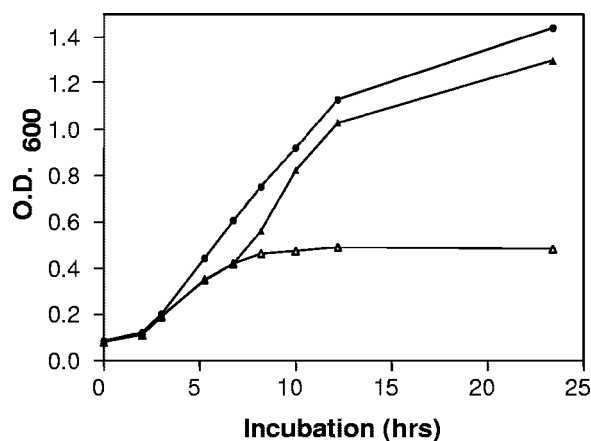[d] F, forward; R, reverse.

FIG. 1. Growth of *F. tularensis* LVS under iron-replete and iron-restricted conditions. *F. tularensis* LVS was grown overnight on an MH+ agar plate. For iron-replete growth (filled circles), bacteria from the plate were inoculated into 1 ml of MH+ broth and grown at 37°C overnight. This overnight culture was then used to inoculate 10 ml MH+ broth to an OD_{600} of 0.05. For iron-restricted growth, the overnight culture was obtained by inoculating the bacteria from the plate into 1 ml MH− broth. This overnight culture was used to inoculate 20 ml of MH−/DF broth. When the culture reached an OD_{600} of 0.4, the 20-ml MH−/DF culture was divided into two cultures of 10 ml each. Fe-PP$_i$ was added to a final concentration of 330 μM in one culture (filled triangles), whereas an equivalent volume of sterile H_2O was added to the other culture (open triangles).

CDM− plate and a CDM+ plate which was supplemented with FeSO$_4$ (2 μg/ml) (6). These plates were incubated at 37°C for 36 h.

Intracellular growth assay. Wild-type and mutant bacterial strains were used to infect mouse macrophage cell line J774A.1 (American Type Culture Collection, Manassas, VA) at a multiplicity of infection of ~10. Briefly, approximately 1×10^5 J774A.1 cells were seeded into the wells of a 12-well tissue culture plate containing Dulbecco's modified Eagle's medium (Cellgro-Mediatech, Inc., Herndon, VA) supplemented with 4 mM GlutaMax (Invitrogen), 10% (vol/vol) heat-inactivated FBS (HyClone, Logan, UT), and 1 mM sodium pyruvate 24 h before infection. Bacteria grown in MH− broth to an OD_{600} of 0.3 were washed with this growth medium three times and added to these monolayers (which were ~75% confluent) in triplicate. After 1 h incubation at 37°C in an atmosphere of 95% air–5% CO_2, the monolayers were washed three times with tissue culture medium, gentamicin was added to a final concentration of 100 μg/ml, and the plate was incubated for 30 min to kill extracellular bacteria. After gentamicin treatment, the monolayers were washed and the macrophages were incubated for 2 to 48 h. At various time points, the tissue culture medium was removed, the monolayers were washed once with tissue culture medium, and then 0.5% (wt/vol) octyl-β-D-glucopyranoside in PBS was added to lyse the macrophages. These lysates were plated onto MH+ agar to determine the number of bacteria recovered from the macrophages. The *F. novicida* U112 *iglC* mutant KKF24 (Δ*iglC*::*ermC*), which is deficient in the ability to grow intracellularly (20), was used as a negative control in these assays.

Accession number for DNA microarray data. The raw data from the DNA microarray experiments described here (Table 2) are located at the National Center for Biotechnology Information's GEO database under series accession number GSE3622.

RESULTS

Growth of *F. tularensis* LVS under iron-replete and iron-restricted conditions. To study iron-related gene regulation in *F. tularensis* LVS, it was necessary to determine growth conditions under which iron was limiting. The iron chelator DF was used to bind free ferric ions in the growth medium. In initial experiments, it was observed that *F. tularensis* LVS cells grown on MH+, when washed and inoculated into MH−/DF, con-
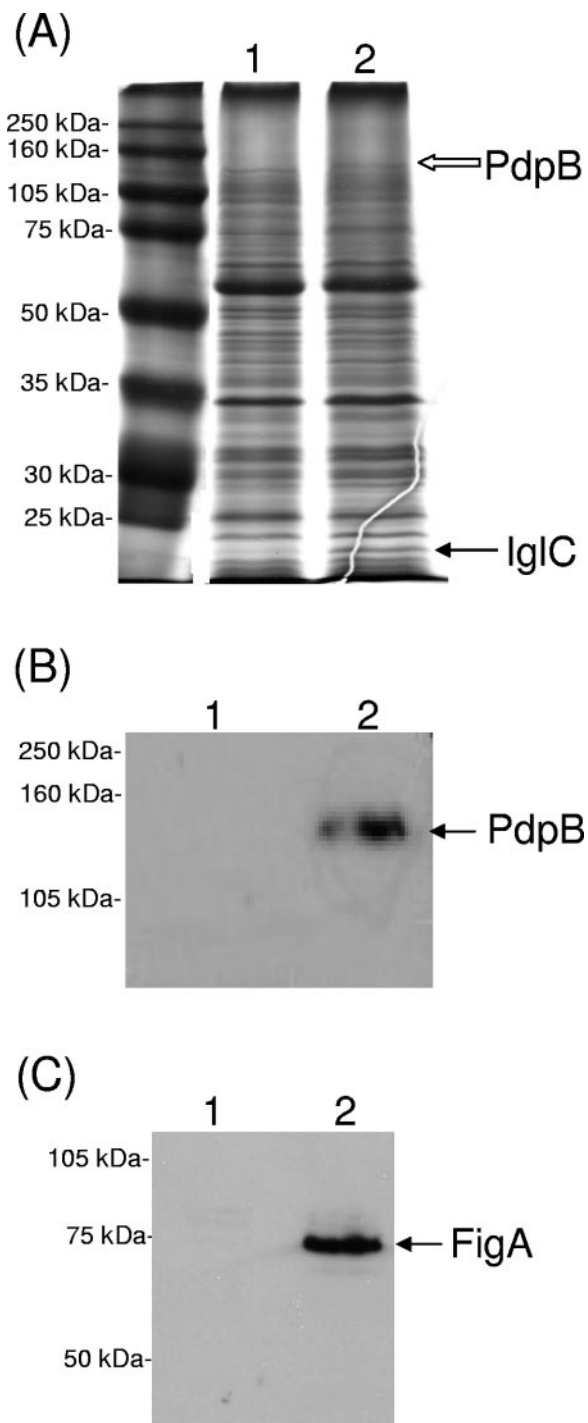

FIG. 2. Identification of *F. tularensis* LVS proteins with increased expression under iron-restricted conditions. *F. tularensis* LVS cells were grown to stationary phase under both iron-replete (lane 1) and iron-restricted (lane 2) conditions. Cell envelopes and whole-cell lysates were prepared as described in Materials and Methods. (A) Proteins in cell envelopes were resolved by SDS-PAGE and stained with Coomassie blue. Molecular mass standards are present in the far left lane. The black arrow indicates the IglC protein, and the white arrow indicates the PdpB protein. Proteins in cell envelopes (B) or in whole-cell lysates (C) were resolved by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes, and probed in a Western blot analysis with polyclonal mouse antiserum to PdpB (B) or FigA (C).
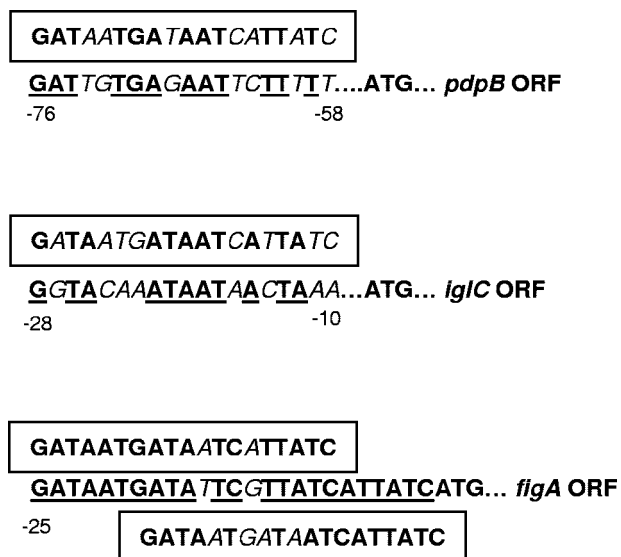
GAT*AATGA*TA*AT*C*ATT*ATC*

GAT*TGTGAG*AA**T***TC***TT***T*T....ATG... *pdpB* ORF
-76   -58

G*ATA*ATG*ATAATCA*TTA*TC*

GGTACAA**ATAAT**AA**CTA**AA...ATG... *iglC* ORF
-28   -10

GATAATGATA*ATC*ATTATC

GATAATGATA*TTCG*TTATCATTATCATG... *figA* ORF
-25   GATA*AT*G*AT*AATCATTATC

FIG. 3. Putative Fur boxes associated with the *F. tularensis* Schu4 *pdpB*, *iglC*, and *figA* ORFs. The ClustalW alignment was accomplished with the MacVector 6.5.3 software. The consensus Fur box is outlined. Identical bases in each pair are underlined and bold; nonidentity is indicated by italics.
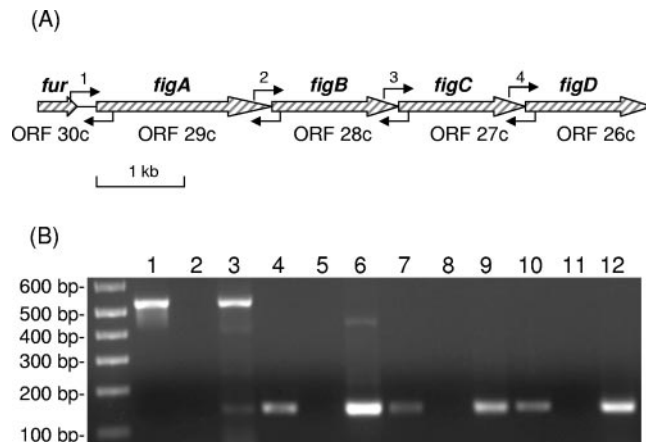
(A)

(B)

FIG. 4. *F. tularensis* Schu4 *figA* and adjacent ORFs. (A) Schematic drawing. (B) RT-PCR analysis of possible transcriptional linkage between *figA* and adjacent ORFs. RT-PCR was carried out as described in Materials and Methods, with oligonucleotide primer pairs spanning intergenic regions 1 (5′-GTGCTGAAATGATTGACTATAGTCTC-3′ and 5′-CT TGTCATTGTTTATATTGTAGAAGAATA-3′), 2 (5′-AGTGCGATA AAGATGACATGC-3′ and 5′-TAGCTGCTATGATTATAAGC-3′), 3 (5′-TATCTATTTGACCTTAAATC-3′ and 5′-TGAATAATTGTTTCA ATTTG-3′), and 4 (5′-TCAATACCAAGTTCTCAGAG-3′ and 5′-AC CTAAAAACCATGTTAAAAG-3′), respectively. Lanes 1, 4, 7, and 10, PCR products derived from *F. tularensis* LVS chromosomal DNA. The sizes of these PCR products are 547 bp (lane 1), 135 bp (lane 4), 120 bp (lane 7), and 121 bp (lane 10). Lanes 2, 5, 8, and 11, RT-PCR negative controls lacking reverse transcriptase. Lanes 3, 6, 9, and 12, products obtained when these same primers were used in an RT-PCR with *F. tularensis* LVS total RNA. Size markers are present on the left side of panel B.

tinued to grow normally (data not shown). However, if these cells were first grown overnight in MH− and then subcultured into MH−/DF, both the rate and extent of growth were severely limited (Fig. 1). Confirmation that this growth restriction was iron specific was obtained by adding iron to the MH−/DF medium and showing that both the rate and extent of growth were similar to those obtained in MH+ (Fig. 1). This latter experiment was necessary because it has been reported that DF can bind other metal ions in addition to iron (5).

Cell envelope protein profiles of *F. tularensis* LVS grown under iron-replete and iron-restricted conditions. To identify proteins whose expression was increased under iron-restricted conditions, SDS-PAGE was used to resolve proteins in cell envelopes prepared from bacteria grown under both iron-replete (Fig. 2A, lane 1) and iron-restricted (Fig. 2A, lane 2) conditions. Two proteins, with apparent sizes of approximately 23 kDa and 140 kDa, were more abundant in envelopes from cells grown under iron-restricted conditions (Fig. 2A, lane 2, black arrow and white arrow, respectively). Mass spectrometric analysis identified the 23-kDa protein as IglC and the 140-kDa protein as PdpB. Both the *iglC* and *pdpB* genes are located in the previously identified pathogenicity island of *F. tularensis* (27). Mouse antiserum raised against a PdpB peptide-KLH conjugate was used in a Western blot analysis to probe these same sets of cell envelopes, and it was found that these PdpB-reactive antibodies bound a 140-kDa protein in the iron-restricted sample (Fig. 2B, lane 2) and were unreactive with the iron-replete sample (Fig. 2B, lane 1).

Identification of putative Fur boxes in *F. tularensis*. Transcription of bacterial genes involved in iron acquisition is usually controlled by the ferric uptake regulator (Fur) protein, which binds ferrous ion and then itself binds to a DNA regulatory sequence (i.e., the Fur box), causing repression of transcription. The typical Fur box is located immediately upstream

from the iron-regulated ORF, and a 19-nt consensus Fur box has been defined (Fig. 3) (22). When the *iglC* and *pdpB* genes of *F. tularensis* Schu4 (19) were examined, sequences with similarity to the consensus Fur box were found (11/19 matches for *iglC* and 12/19 matches for *pdpB*) (Fig. 3). In addition, when the *F. tularensis* Schu4 genome was searched for consensus Fur box sequences, a number of ORFs were identified that had putative 5′ untranslated regions with possible Fur boxes (data not shown). The gene whose putative Fur box had the highest level of identity (17/19 matches) with this consensus sequence was *figA*. Interestingly, this same region contained a second putative Fur box with a slightly lower degree of identity with the consensus sequence (Fig. 3). The *figA* gene is located 253 nt downstream from the *fur* gene in the *F. tularensis* Schu4 genome (Fig. 4A) and encodes a predicted 74-kDa protein with 36% identity to the predicted FrgA protein (accession number NP_819459.1) from *Coxiella burnetii* and 34% identity to the LbtA (1) and FrgA (16) proteins from *L. pneumophila*. Expression of both of these *Legionella* proteins has been shown to be regulated by iron (1), and an *lbtA* mutant is deficient in expression of the *Legionella* siderophore legiobactin (1). Regulation of expression of the *F. tularensis* LVS FigA protein by iron availability was confirmed by the finding that mouse antibodies raised against a FigA peptide-KLH conjugate bound a 74-kDa protein in a whole-cell lysate of iron-restricted cells (Fig. 2C, lane 2) but did not react with an equivalent whole-cell lysate of iron-replete cells (Fig. 2C, lane 1).

DNA microarray analysis of *F. tularensis* LVS gene expression in response to iron availability. Having both established conditions for iron-restricted growth and identified several genes which appear to be iron regulated, DNA microarray analysis was used to provide a more comprehensive picture of the effect of iron limitation on gene expression by *F. tularensis* LVS. These DNA microarrays contained probes derived from the nucleotide sequence of the *F. tularensis* Schu4 genome (19). Approximately 80 of the *F. tularensis* LVS genes were found to be either up- or down-regulated at least twofold by iron limitation (see Table S1 in the supplemental material). Forty of the genes most affected by iron limitation, either positively or negatively, are listed in Table 2.

Among these genes, *figA* was found to be up-regulated nearly 20-fold by iron limitation. In the *F. tularensis* Schu4 genome, *figA* is located just downstream from the predicted *fur* gene and 5′ to a set of three consecutive ORFs (*figB*, *figC*, and *figD*; Fig. 4A) whose expression was also up-regulated as determined by DNA microarray analysis (Table 2). The predicted protein encoded by *figB* in the Schu4 genome is listed as a hypothetical protein and is 31% identical to a multidrug resistance efflux pump from *L. pneumophila* (accession number YP_095354.1). The predicted protein encoded by the Schu4 *figC* gene is described as a diaminopimelate decarboxylase and has 31% identity with a diaminopimelate decarboxylase from *Magnetospirillum magnetotacticum* (accession number ZP_00054090.1). The protein encoded by the Schu4 *figD* ORF is annotated as a hypothetical protein and is 38% identical to a drug resistance transporter from *L. pneumophila* (accession number YP_095353.1). RT-PCR was used to determine possible transcriptional linkage of these five ORFs. RNA was isolated from the *F. tularensis* LVS grown in MH+ medium and reverse transcribed with four pairs of oligonucleotide primers designed to span the four intergenic regions (Fig. 4A). Positive RT-PCRs were observed for all four sets of primers (Fig. 4B), indicating that these five ORFs are likely transcribed together to yield a polycistronic mRNA in *F. tularensis* LVS.

Validation of DNA microarray results. The qRT-PCR method was used to validate the DNA microarray data in Table 2. Transcript levels were determined for 21 genes, including 9 whose expression, as measured by DNA microarray analysis, appeared to be up-regulated, 8 with apparent down-regulation, and 4 whose expression appeared relatively unaffected by growth under iron-limiting conditions. For most of the genes tested, the values obtained by qRT-PCR (Table 3) correlated well ($R^2 = 0.94$) with those obtained from DNA microarray analysis (Table 2).

Generation of *figA* mutants of the *F. tularensis* LVS and *F. novicida* U112. Expression of the *figA* gene was shown to be substantially increased under iron-restricted growth conditions by both DNA microarray analysis and qRT-PCR. To determine the relevance of the FigA protein to iron acquisition, *figA* deletion mutants were constructed in both *F. tularensis* LVS and *F. novicida* U112. PCR was used to amplify the *F. tularensis* LVS chromosomal DNA region immediately upstream from the *figA* gene and, separately, a small portion of the 3′ end of the *figA* gene and some flanking DNA (Fig. 5A). A promoterless *kan* cartridge (26) was ligated between these two fragments to create pKD101-*np-kan* as described in Materials and Methods. This plasmid was used to electroporate both *F.*
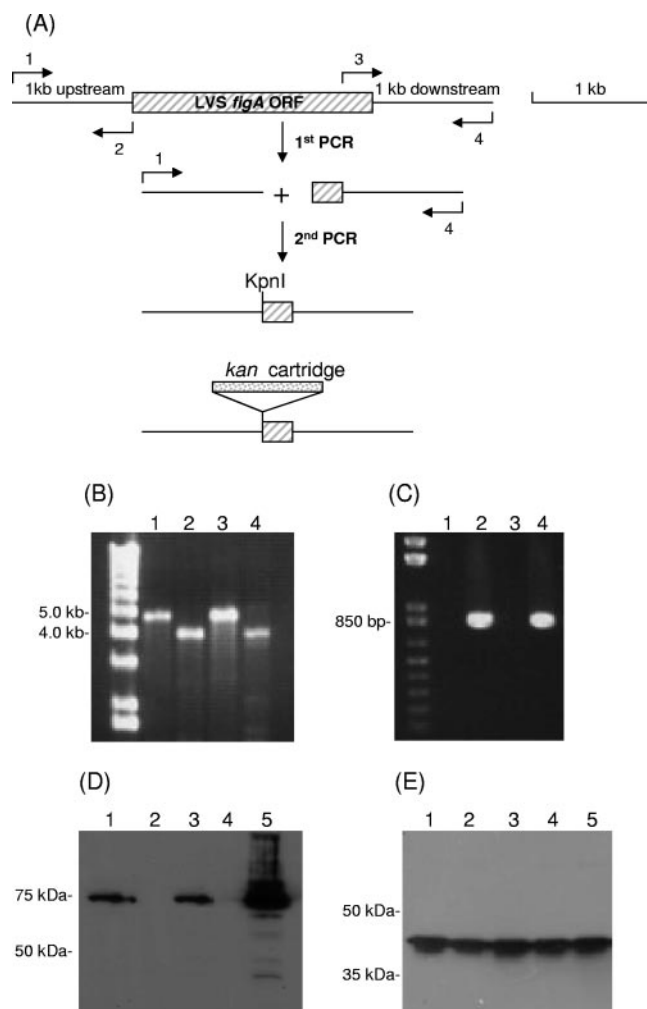

FIG. 5. Construction and characterization of *figA* mutants. (A) Schematic of PCRs used to amplify and sew flanking regions of the *F. tularensis* LVS *figA* gene. The promoterless *kan* cartridge was ligated into this cloned PCR product at the KpnI site. (B) PCR products obtained from the chromosomal DNAs of wild-type and *figA* mutant strains with oligonucleotide primers (described in Results) binding 1.5 kb from the 5′ and 3′ ends of the *figA* gene. Lane 1, wild-type *F. tularensis* LVS; lane 2, *F. tularensis* LVS *figA*::*np-kan*; lane 3, wild-type *F. novicida* U112; lane 4, *F. novicida* U112 *figA*::*np-kan*. The positions of 4.0-kb and 5.0-kb size markers are indicated on the left. (C) PCR products obtained from chromosomal DNAs of wild-type and mutant strains with primers binding within the *kan* cartridge; lanes are the same as in panel B. The position of an 850-bp size marker is shown on the left. (D) Western blot analysis of whole-cell lysates of these wild-type and mutant strains grown in MH−/DF broth. Lanes 1 to 4 contain whole-cell lysates of the same strains as in panel B; lane 5 contains a whole-cell lysate of *F. novicida* U112 *figA*::*np-kan* (pKD107). The primary antibody was mouse FigA antiserum. (E) Western blot analysis of these same lysates with rat polyclonal *F. tularensis* LVS FopA antiserum (i.e., a loading control).

tularensis LVS and *F. novicida* U112, and kanamycin-resistant transformants were tested by colony blot analysis with the mouse antiserum to FigA. Chromosomal DNA was purified from putative *figA* mutants and tested in PCR with oligonucleotide primers 5′-GCAAAGAGTCCCAGCAACAATAA G-3′ and 5′-GAATAATAGTGTTTAGAGATTGAG-3′

(which bind outside of the chromosomal DNA insert in pKD101) (Fig. 5B). A second PCR was performed with primers for the *kan* cartridge to confirm its presence in these two mutants, designated *F. tularensis* LVS *figA*::*np-kan* and *F. novicida* U112 *figA*::*np-kan* (Fig. 5C). Finally, Western blot analysis was used to confirm that these two mutants did not express FigA (Fig. 5D).

Assessment of potential siderophore production by *Francisella* species. The predicted FigA proteins of *F. tularensis* strains Schu4 and LVS have 34% identity with the *L. pneumophila* LbtA protein, which has been shown to be necessary for siderophore production by this pathogen (1). Although siderophore production has not been reported to date for *F. tularensis*, it was appropriate to test whether these *figA* mutants differed from their parent strains in the production of possible siderophore activity. To accomplish this, the CAS assay was used with CDM− agar plates. The CAS assay is a universal method to detect siderophores independent of their structure (39).

The CAS/CDM− agar plates were tested first with a well-studied *E. coli* strain and its mutant deficient in enterobactin synthesis. *E. coli* W3110N produces the siderophore enterobactin, which results in removal of Fe^{3+} from the CAS/Fe(III)/HDTMA complex and release of the free (orange) dye. Orange halos were present around each colony of *E. coli* W3110N grown on CAS/CDM− agar (data not shown). In contrast, the *entF* mutant of W3110N did not show any siderophore activity (data not shown). This CAS/CDM− medium was shown to be iron limiting for growth of *F. tularensis* LVS (data not shown). When wild-type *F. tularensis* LVS was grown on this medium, a modest orange zone appeared around the bacterial growth after 2 to 5 days (Fig. 6A, sector 1). The *F. tularensis* LVS *figA*::*np-kan* mutant formed an orange zone that appeared to be only slightly smaller (Fig. 6A, sector 2). In contrast, a much larger orange zone formed around *F. novicida* U112 (Fig. 6B, sector 1) after 24 h of growth and its *figA* mutant produced little or no orange zone (Fig. 6B, sector 2).

Complementation of the *F. novicida* U112 *figA* mutant. The significant difference in CAS test results obtained with the wild-type *F. novicida* U112 strain and its *figA* mutant facilitated subsequent complementation analysis. This was necessary to eliminate the possibility that an unlinked secondary mutation was responsible for the phenotype of the *F. novicida figA* mutant in the CAS assay. To accomplish this, the wild-type *F. novicida figA* gene was cloned into the pFNLTP-CAT vector to construct pKD107 in *E. coli* DH5α as described in Materials and Methods. Several attempts involving electroporation of *E. coli*-derived pKD107 into *F. novicida* U112 *figA*::*np-kan*, followed by chloramphenicol selection, were unsuccessful, so this *E. coli*-derived plasmid was used to electroporate the *F. novicida* U112 wild-type strain, followed by selection with kanamycin for plasmid-containing transformants. (Kanamycin cannot be used to select this plasmid in this kanamycin-resistant mutant.) When pKD107 propagated in *F. novicida* U112 was used to electroporate the *F. novicida* U112 *figA*::*np-kan* mutant, chloramphenicol-resistant transformants containing this plasmid were obtained. This same mutant was transformed with the vector pFNLTP-CAT (propagated first in *F. novicida* U112). When introduced into *F. novicida* U112 *figA*::*np-kan*, the wild-type *F. novicida figA* gene in *trans* restored both ex-
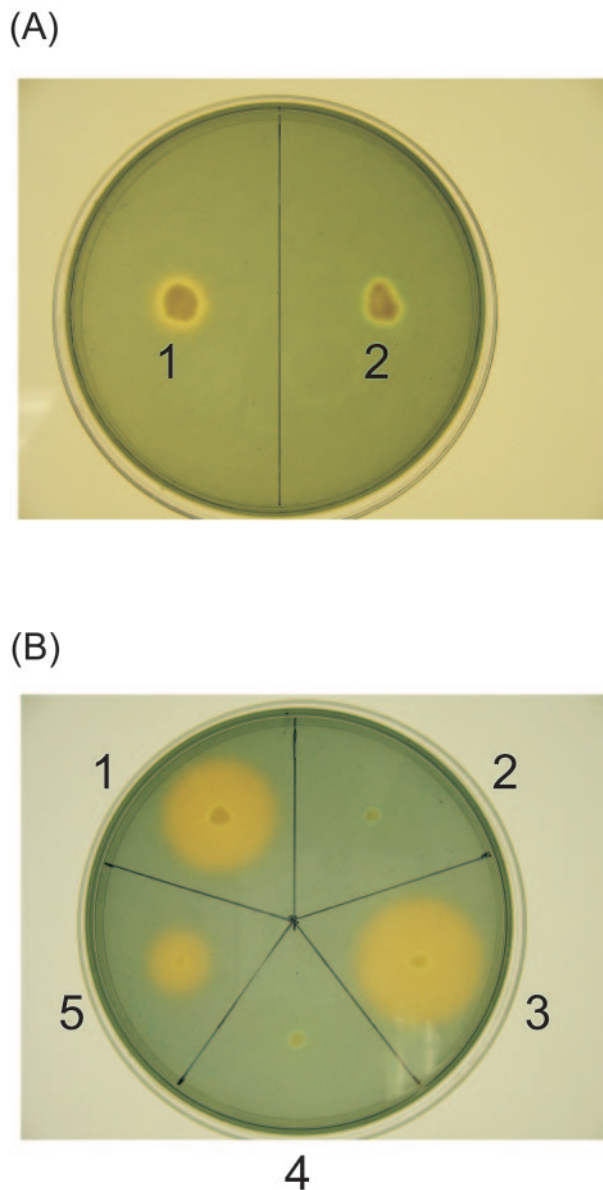
FIG. 6. CAS assay. Wild-type *F. tularensis* LVS and *F. novicida* U112 and mutant and complemented mutant strains of both were grown on CAS/CDM− agar plates. (A) Wild-type *F. tularensis* LVS (sector 1) and *F. tularensis* LVS *figA*::*np-kan* (sector 2) grown for 5 days. (B) Wild-type *F. novicida* U112 (sector 1), *F. novicida* U112 *figA*::*np-kan* (sector 2), *F. novicida* U112 *figA*::*np-kan*(pKD107) (sector 3), *F. novicida* U112 *figA*::*np-kan*(pFNLTP-CAT) (sector 4), and *F. novicida* U112 *figA*::*np-kan*(pKD108) (sector 5) grown for 24 h.

pression of the FigA protein (Fig. 5D) and production of a large orange zone in the CAS assay (Fig. 6B, sector 3). Similarly, the presence of the wild-type *F. tularensis* LVS *figA* gene (in pKD108) in this *F. novicida figA* mutant also resulted in the production of a readily detectable orange zone in the CAS assay (Fig. 6B, sector 5). The presence of only the pFNLTP-CAT vector in this same mutant did not affect the phenotype of this strain in the CAS assay (Fig. 6B, sector 4).

Effect of the *figA* mutation on extracellular growth. To address whether inactivation of the *figA* gene in *F. tularensis* LVS
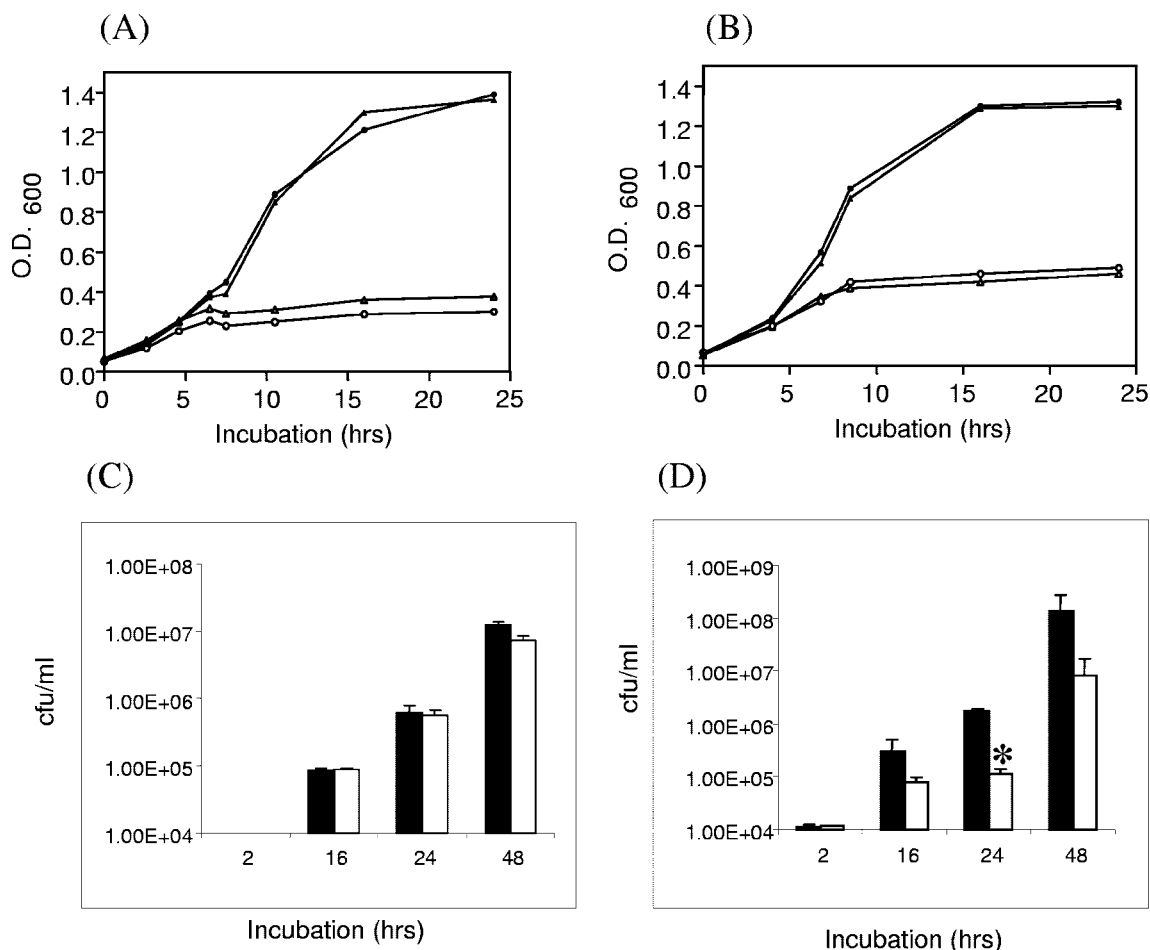
FIG. 7. Extracellular and intracellular growth of wild-type and mutant strains of *F. tularensis* LVS and *F. novicida* U112. For extracellular growth (A and B), bacteria were grown in both iron-replete (filled symbols) and iron-restricted (open symbols) media. The wild-type *F. tularensis* LVS strain (panel A, circles), its *figA* mutant (A, triangles), the wild-type *F. novicida* strain (B, circles), and its *figA* mutant (B, triangles) were grown as described in Materials and Methods. To assess intracellular growth (C and D), these same strains were grown in the macrophage cell line J774A.1 as described in Materials and Methods. (C) Wild-type *F. tularensis* LVS (filled columns) and its *figA* mutant (open columns). (D) Wild-type *F. novicida* (filled columns) and its *figA* mutant (open columns). These data are the means from two independent experiments. In panel D, the difference between the *F. novicida* wild-type and mutant strains was significant only at 24 h postinfection (asterisk; $P = 0.0096$, Student's t test).

and *F. novicida* U112 had any effect on extracellular growth, the *figA* mutants were compared with their respective parent strains for the ability to grow in broth medium under iron-replete and iron-restricted conditions. Chelation of free iron in MH−/DF medium was shown to inhibit growth of the *F. novicida* U112 wild-type strain (data not shown). However, unlike *F. tularensis* LVS, which had to be prestarved for iron to obtain iron-restricted growth, *F. novicida* U112 was more sensitive to iron limitation and did not require prestarvation in MH− medium prior to inoculation into MH−/DF. As shown in Fig. 7A and B, respectively, the *F. tularensis* LVS and *F. novicida* U112 wild-type strains and their respective *figA* mutants grew at similar rates and to the same extent in iron-replete medium. Similarly, both wild-type strains and their *figA* mutants exhibited the same degree of growth inhibition in MH−/DF medium.

When the *F. novicida* wild-type strain and its *figA* mutant, which exhibited the greatest difference in the CAS assay (Fig. 6), were inoculated separately onto CAS/CDM− agar, only the

wild-type strain exhibited abundant growth (data not shown). To determine whether the siderophore-like activity secreted by the *F. novicida* wild-type strain could enhance the ability of its *figA* mutant to grow under these iron-restricted conditions, a cross-feeding experiment was performed. When *F. novicida* U112 *figA::np-kan* (Fig. 8A, streaks 3 and 4) was streaked in parallel on a CAS/CDM− plate, this mutant grew very poorly compared to this same mutant (Fig. 8B, streaks 3 and 4) streaked in parallel on CDM+ medium. In contrast, when this *figA* mutant (Fig. 8A, streak 2) was streaked alongside the *F. novicida* wild-type strain (Fig. 8A, streak 1), this mutant apparently grew as well as the wild-type strain. These results indicated that the siderophore-like activity evidenced by *F. novicida* U112 in the CAS assay was able to enhance the growth of its *figA* mutant under iron-restricted conditions.

Effect of the *figA* mutation on intracellular growth. The intracellular growth abilities of these wild-type and mutant strains were compared in mouse macrophage cell line J774A.1. Over the 48-h experimental period postinfection, there was no
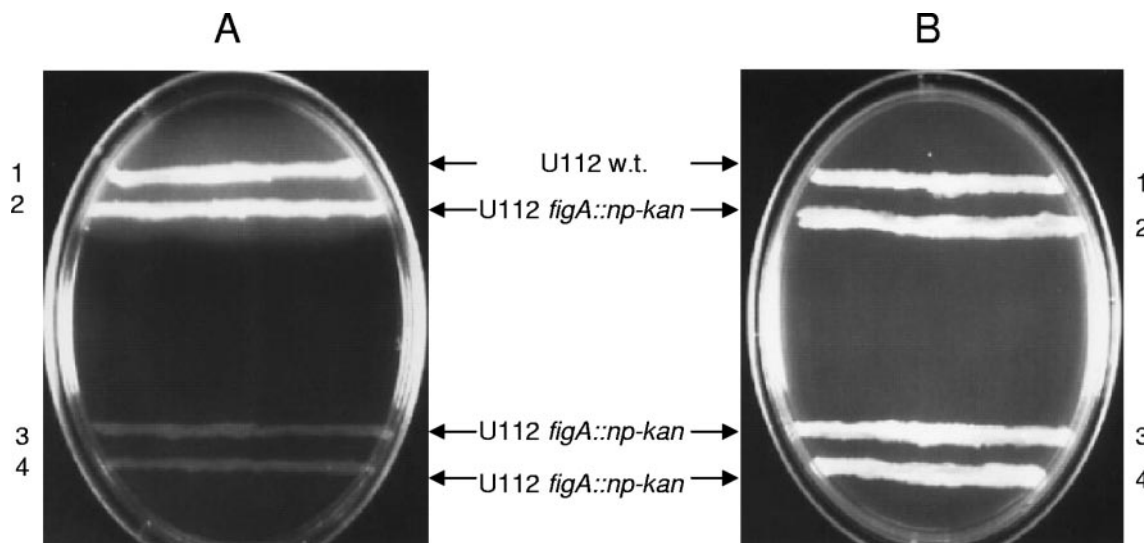
FIG. 8. Cross-feeding of the *F. novicida figA* mutant by the *F. novicida* U112 wild-type (w.t.) strain. The *F. novicida* U112 wild-type strain (streak 1) and its *figA* mutant (streaks 2, 3, and 4) were inoculated onto both CAS/CDM− (A) and CDM+ (B) agar plates and grown for 36 h.

significant difference between wild-type *F. tularensis* LVS and its *figA* mutant (Fig. 7C). In contrast, the *F. novicida* U112 *figA* mutant appeared to grow less well in these macrophages although the difference was statistically significant only at the 24-h time point (Fig. 7D).

DISCUSSION

In marked contrast to the abundance of information about iron acquisition by both enteric and nonenteric gram-negative pathogens, the data available concerning iron uptake by *F. tularensis* are rudimentary at best. Moreover, much of the published data on this subject date back more than 4 decades. Studies by early workers indicated that in vitro growth of small inocula of the virulent SCHU-S4 strain of *F. tularensis* was stimulated by ferric chloride, ferric sulfate (41), and two ferric hydroxamates, ferrichrome and ferrioxamine B (14). In addition, *F. tularensis* has been shown to produce a growth-stimulating compound whose function could be at least partially replaced by iron salts or siderophores (14). This latter finding raised the possibility that *F. tularensis* could produce some type of siderophore. Other facultative intracellular pathogens, including *Y. pestis* (32) and *Mycobacterium* species (18), have been shown to produce siderophores. Moreover, *L. pneumophila*, long thought to be unable to produce a siderophore, was recently shown to release an iron chelator into culture supernatant fluid under certain growth conditions (23) and gene products involved in the expression of this siderophore have been identified (1).

In the present study, a combination of genomic screening, DNA microarray technology, and mutant analysis was used to identify *F. tularensis* genes encoding products whose expression was affected by iron limitation. Prior to this study, there was a single published report in which a rifampin-resistant mutant of the *F. tularensis* LVS was grown under conditions of limited iron availability (2). These authors subjected this organism to a number of stresses, including increased temperature, low pH,

and iron limitation, independently. SDS-PAGE analysis of cell envelopes obtained from these bacteria grown under iron-replete and iron-restricted conditions showed apparent increases in the expression of a few proteins, but these proteins were not identified (2).

To obtain conditions under which iron was the limiting factor for growth, *F. tularensis* LVS had to be starved for iron by growth in MH− broth overnight prior to inoculation into MH−/DF medium. Interestingly, prestarvation of *F. novicida* was not necessary to achieve iron-limited growth, a result which suggests that these two *Francisella* species may have different iron storage capabilities. Under iron-limiting conditions, a few *F. tularensis* LVS proteins detectable in cell envelope preparations were apparently up-regulated and at least two of these (IglC and PdpB) were identified by mass spectrometry. Examination of the nucleotide sequence of the *iglC* and *pdpB* genes in the *F. tularensis* Schu4 genome (19) revealed the presence of putative Fur boxes immediately upstream of both of these ORFs (Fig. 3). Both *iglC* and *pdpB* are located in possible operons (*iglABCD* and *pdpAB*, respectively) in the Schu4 genome, and examination of the nucleotide sequences immediately upstream from both *iglA* and *pdpA* also showed the presence of putative Fur boxes (data not shown). When the consensus Fur box sequence was used to search the entire Schu4 genome, the putative Fur box that had the highest degree of identity (17/19) with the consensus sequence was located in front of the *figA* gene and overlapped with a second predicted Fur box. In *L. pneumophila*, the iron-regulated gene *lbtA*, which encodes a protein that may be functionally similar to FigA, also has two overlapping Fur boxes (1).

Subsequent DNA microarray analysis confirmed that expression of the *figA* gene, as well as that of about 80 other *F. tularensis* LVS ORFs, was up- or down-regulated at least twofold by growth under iron-restricted conditions (Table S1 in the supplemental material). These iron-regulated ORFs included both the *iglC* and *pdpB* genes previously shown by protein analysis to be up-regulated under these growth condi-

tions (Fig. 2). Genes that were repressed under iron starvation were mainly those involved in protein synthesis (Table 2). These DNA microarray data were verified for several ORFs by the use of qRT-PCR (Table 3). The genes located in the recently identified *F. tularensis* pathogenicity island involved in intracellular growth (27), including *pdpA*, *pdpB*, *pdpC*, *iglA*, *iglB*, *iglC*, and *iglD*, were all up-regulated at least twofold under iron-restricted conditions (Table S1 in the supplemental material). It is possible that, under this iron-restricted growth condition in vitro, the bacteria may sense an environment similar to that encountered within macrophages. The relatively low concentrations of iron encountered in the host cell may function as a signal to the bacterium to increase the production of virulence or survival factors that would be beneficial in this location. It must be noted that MglA has been shown to be a transcriptional activator for these genes (21), and additional studies are necessary to determine the relative importance of iron limitation in affecting expression of these genes.

Among the genes that were up-regulated by iron restriction, the tightly linked *figABCD* genes had the greatest increases in expression as assessed by both DNA microarray and qRT-PCR analyses. The *figA* gene was found to be located ~250 nt downstream from the predicted *fur* gene in the Schu4 genome. The *figABCD* ORFs, together with the predicted *fur* gene, likely constitute an operon on the basis of RT-PCR analysis (Fig. 4). Interestingly, expression of the *fur* gene was apparently not affected substantially by iron limitation (Table S1 in the supplemental material). Exactly how *fur* and these other four genes are differentially regulated by iron restriction remains to be determined.

F. tularensis LVS and *F. novicida* U112 *figA* mutants were constructed with a kanamycin resistance cartridge (26) designed to prevent or minimize polar effects on downstream ORFs. Under iron-restricted conditions in broth, these two *figA* mutants grew at a rate and to a final density that were similar, if not identical, to those obtained with the respective wild-type parent strains (Fig. 7). The intracellular growth ability of the *F. tularensis* LVS *figA* mutant was the same as that of its parent wild-type strain, whereas the *figA* mutant of *F. novicida* seemed to be slightly deficient in the ability to grow in macrophages (Fig. 7). Further investigation of these mutants did reveal a striking difference between the *F. novicida* U112 *figA* mutant and its wild-type parent strain in an assay originally designed to detect siderophore production by enteric bacteria (31). Interestingly, an *L. pneumophila* mutant unable to express the LbtA protein, which resembles FigA, was also deficient in siderophore production as assessed in a CAS-based assay (1). A difference between the *F. tularensis* LVS wild-type strain and its *figA* mutant (Fig. 6A) was detected in the CAS assay but was very modest compared to that observed with the *F. novicida* wild-type–mutant pair. This latter pair of strains was also used in a cross-feeding experiment (Fig. 8) which demonstrated that the siderophore-like activity released by *F. novicida* in the CAS assay could enhance the growth of the *F. novicida figA* mutant under these iron-restricted conditions on CAS/CDM− agar.

The role of the FigA protein in the CAS plate phenotype was confirmed by the use of complementation analysis (Fig. 6B). When the cloned *F. novicida figA* gene in pKD107 was expressed in the *F. tularensis* LVS *figA* mutant, this comple-

mentation in *trans* did not increase the reactivity of this mutant in the CAS assay even though the FigA protein could be detected by Western blot analysis (data not shown). However, when we cloned and expressed the wild-type *F. tularensis* LVS *figA* gene in the *F. novicida figA* mutant, this recombinant did express a readily detectable reaction zone on this same medium (Fig. 6B). Taken together, these results raise the possibility that the very weak reactivity of the wild-type *F. tularensis* LVS in the CAS assay is the result of a secondary mutation elsewhere in the genome that affects the synthesis or release of the siderophore-like activity and is not caused by a nonfunctional *F. tularensis* LVS FigA protein. It is also noteworthy that expression of the cloned *F. novicida figA* gene in *F. novicida* was subject to regulation by iron availability (data not shown).

This study provides the first identification of iron-regulated genes in *F. tularensis* LVS and evidence for the production of a siderophore-like molecule by *F. novicida* U112. Whether this siderophore-like molecule, the FigA protein, or both are necessary for virulence expression by *F. tularensis* remains to be determined.

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