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Identification and Characterization of Novel and Potent Transcription Promoters of *Francisella tularensis*▽†

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Two alternative promoter trap libraries, based on the green fluorescence protein (*gfp*) reporter and on the chloramphenicol acetyltransferase (*cat*) cassette, were constructed for isolation of potent *Francisella tularensis* promoters. Of the 26,000 *F. tularensis* strain LVS *gfp* library clones, only 3 exhibited visible fluorescence following UV illumination and all appeared to carry the bacterioferritin promoter (*Pbfr*). Out of a total of 2,000 chloramphenicol-resistant LVS clones isolated from the *cat* promoter library, we arbitrarily selected 40 for further analysis. Over 80% of these clones carry unique *F. tularensis* DNA sequences which appear to drive a wide range of protein expression, as determined by specific chloramphenicol acetyltransferase (CAT) Western dot blot and enzymatic assays. The DNA sequence information for the 33 unique and novel *F. tularensis* promoters reported here, along with the results of *in silico* and primer extension analyses, suggest that *F. tularensis* possesses classical *Escherichia coli* σ^{70}-related promoter motifs. These motifs include the −10 (TATAAT) and −35 [TTGA(C/T)A] domains and an AT-rich region upstream from −35, reminiscent of but distinct from the *E. coli* upstream region that is termed the UP element. The most efficient promoter identified (*Pbfr*) appears to be about 10 times more potent than the *F. tularensis groEL* promoter and is probably among the strongest promoters in *F. tularensis*. The battery of promoters identified in this work will be useful, among other things, for genetic manipulation in the background of *F. tularensis* intended to gain better understanding of the mechanisms involved in pathogenesis and virulence, as well as for vaccine development studies.

The bacterium *Francisella tularensis* is a Gram-negative, facultative intracellular human pathogen which was recognized as the etiological agent of tularemia at the beginning of the 20th century (39). As of today, the disease is relatively rare in the Western world and is efficiently treated by prompt antibiotic administration (40). Yet, owing to the low bacterial dose necessary for the onset of inhalatory infection and the potential airborne route of dissemination, *F. tularensis* was recently classified by the Centers for Disease Control and Prevention as a category A biothreat select agent. This has led to a surge of studies of this human pathogen in an attempt to better understand the pathogenesis of the bacteria and to design novel approaches for diagnostics, prophylaxis, and treatment strategies. Such studies strongly depend on the availability of genetic tools that enable the examination of individual bacterial proteins in a variety of experimental approaches (e.g., directed disruption of genes and/or controlled expression of heterologous proteins), and the paucity of these tools severely limited *F. tularensis* research for many years (10). We therefore decided to search for, isolate, and characterize different *F. tularensis* promoters to increase the number of genetic tools that will allow the modulation of gene expression in the background of *F. tularensis*.

To date, a small number of functional *Francisella* promoters have been adapted for such purposes, among which is the *groEL* promoter (9) that has been widely used for gene expression both *in vivo* and *in vitro* (16, 24, 27, 31, 35, 36). Other promoters include the *acpA* promoter (34), which was used to drive the expression of green fluorescent protein (GFP) in *F. tularensis* strain LVS during a murine macrophage infection (27), and the FTN_1451 promoter (11), which was used to express the kanamycin resistance gene in the process of adapting the Targeton system for use in *F. tularensis* (35, 36). Promoter trap studies were previously conducted in *F. tularensis* LVS, resulting in the identification of several promoters that were active *in vivo*, but to the best of our knowledge, their identity was not disclosed (18, 33). In addition, an LVS promoter trap library constructed and screened in *Escherichia coli* resulted in the characterization of the FTL_0580 glucose-repressible promoter (15).

The relatively limited repertoire of *F. tularensis* promoters available for genetic and recombinant DNA manipulations (such as allelic exchange and complementation experiments) may stem from the fact that *F. tularensis* RNA polymerase possesses two distinct and unique α subunits (6, 20). Indeed, some studies suggested that the expression of heterologous genes is more efficient in *F. tularensis* when their transcription is driven by endogenous rather than heterologous promoters. For example, the transformation efficiency of the *F. tularensis* Schu S4 strain with a plasmid carrying the *aphA* kanamycin resistance gene was significantly lower when the *aphA* gene was transcribed from its native promoter than when *aphA* was transcribed from the *F. tularensis groEL* promoter (24). In another study, it was observed that a transposon mutant *F. tularensis* subsp. *novicida* library exhibits a significant insert orientation bias in favor of the direction of the gene residing upstream from the insertion site. Such orientation probably enabled the expression of the antibiotic resistance gene from

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain or plasmid	Description or sequence[a]	Source or reference
Strains		
F. tularensis LVS	*F. tularensis* subsp. *holarctica* live vaccine strain	ATCC 29684
E. coli DH5α	*endA1 recA1*	Clontech
Plasmids		
pRIT5	Ap^r in *E. coli*, Cm^r in Gram-positive organisms, pC194 origin of replication	Pharmacia (29)
pTE	pRIT5 vector in which the protein A gene was deleted; *E. coli-F. tularensis* shuttle vector	This study
pWH1012	Souce of the gfp^+ gene	37
pTRAP	Promoterless gfp^+ gene from pWH1012; inserted as an EcoRI-PstI fragment in pTE	This work
pASC-1	*E. coli-Bacillus* expression vector, carrying the *B. anthracis pagA* gene	8
pKK214	Tet^r in *E. coli* and *F. tularensis*; p15A origin of replication in *E. coli*; *ori*FT in *F. tularensis*; promoterless *cat* gene	18
pKK202	Tet^r in *E. coli* and *F. tularensis*; p15A origin of replication in *E. coli*; *ori*FT in *F. tularensis*	30
pTRAP (*groEL-gfp*)	pTRAP containing the *groEL* promoter upstream from the *gfp* gene	This work
pKK (*bfr-cat*)	pKK214 containing the *bfr* promoter upstream from the *cat* gene	This work
Primers		
GFP-F	gtcgatcatcgaattctctagagatcttacaataaggagtacgtatATGGCTAGCAAAGGAGAAG	
GFP-R	tgaccatagcctgcagtggtacccgggTTATTTGTAGAGCTCATCCATGC	
GFP-seq	TTTGTGCCCATTAACATCACC	
CAT-seqF	CCAAAACGATCTCAAGAAGATC	
CAT-seqR	GATGCCATTGGGATATATCAACGG	
PE P2A[b]	GACGAATGTTCATAACAATCTTACTCC	
PE P2B	GCACGACGAACTAATACTCTATCTTG	
PE P3A	CCACAGATACCTAAAATATGAATATG	
PE P3B	CTAATACTGCTAAAGAACCCATAAAAG	
PE P39A	GCTCAACTATTATATGGTTAACTCTAG	
PE P39B	CTTCTGGCTTAAGATCTTCTTC	
PE *Pbfr*A	CTTGTTTATTTTCTAATTTAAGTTCC	
PE *Pbfr*B	CGAGTTCTAAGATTTTATTTAATTG	
PE P18A	GTTTGATTTTTTTCATTGTATTGC	
PE P18B	CTAACTAGGGTAAGTGTAGCTAATG	
CAT-F	ATGGACAACTTCTTCGCCCC	
CAT-R	CAAACGGCATGATGAACCTG	
Tet-F	CTAACAATGCGCTCATCGTCA	
Tet-R	CCGGCAGTACCGGCATAAC	
Det-P2F	TGGTAATGCTCAAGAGAAACCTAG	
Det-P2R	CTTGTTTTGCAGCCATTTTTAATTTC	
Det-P39F	CTACAGATATGACTGATAAACTAACTG	
Det-P39R	TTCTTCTTTAACGCCTAATTGCTC	
Det-P29F	CTAGATAAGATTGAAAATTAAACTC	
Det-P29R	CTGTTAACATAAATTTACTCC	
Det-P*bfr*F	GCATAATATCATTTTTATTAAAATATC	
Det-P*bfr*R	TGTTTATTTTCTAATTTAAGTTC	
groEL-F	GCCAAAAAACagatctCCTATTGTATGGATTAGTCGAGC	
groEL-R	CGAATGTTCtacgtaATCTTACTCCTTTG	
bfr-F	caatactgcatctagaGATCCATACCCATGATGGTTAC	
bfr-R	cataagaattcctgcagGATCAATAATTTCTTGTTTATTTTC	

[a] The region of homology to the coding sequence is in uppercase letters. The restriction sites, used for cloning of the corresponding PCR fragments, are underlined.
[b] PE primers were used for primer extension analyses of the various promoters as indicated by their numbers.

promoters of the genes residing upstream from the insertion sites, overcoming the poor expression of the kanamycin resistance marker from its native promoter (11).

In the present study, we describe the use of two alternative promoter trap screening procedures in order to identify *Francisella* promoters. The first procedure, which is a nonselective method, relies on the expression of the GFP gene as a reporter gene, while the other is dependent on the selection of chloramphenicol-resistant (Cm^r) colonies due to expression of the *cat* gene. The screening and selection procedures resulted in the identification of numerous novel promoters, representing different intergenic chromosomal loci, which exhibit a wide dynamic range of heterologous gene expression in the background of *F. tularensis*. Inspection of the sequences of 33 promoters, as well as primer extension analyses of some of them, provides new insight into the architecture of *F. tularensis* promoters which could serve as new tools for genetic manipulation of *F. tularensis* genes.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* DH5α was grown in Luria-Bertani (LB) medium containing 100 μg/ml of ampicillin or 10 μg/ml tetracycline. *F. tularensis* LVS wild-type and recombinant strains were grown in TSBC broth (0.1% L-cysteine, 3% tryptic soy broth) or CHA agar (1% hemoglobin, 5.1% Bacto heart infusion) supplemented with 2 μg/ml chloramphenicol (Cm) or 10

μg/ml tetracycline when they contained the pTRAP or the pKK214 vector, respectively. In experiments in which the possible enrichment of highly potent promoters was evaluated, the bacteria were cultured in medium supplemented with up to 80 μg/ml Cm. Cultures were grown to exponential or stationary phase at 37°C under vigorous agitation (200 rpm) for 12 to 16 h to an optical density at 600 nm (OD_{600}) of 0.4 or >1.0. *F. tularensis* agar plates were typically incubated for 72 h at 37°C.

Electroporation of *F. tularensis*. Plasmids were introduced into *F. tularensis* by electroporation as previously reported (2). Briefly, *F. tularensis* LVS TSBC cultures (150 ml) were grown to an OD_{600} of 0.2 to 0.4, washed twice with wash solution (0.5 M sucrose, 15% glycerol) and resuspended in 250 μl of the same solution. Plasmid DNA was mixed with 200 μl of electrocompetent cells, and the mixture was pulsed in a 0.2-cm-gap cuvette (Bio-Rad) at 2.5 kV, 600 Ω, and 25 μF. Immediately after being pulsed, the cells were resuspended in 2 ml of TSBC and incubated for 4 h (37°C, 150 rpm) prior to selection on CHA plates.

Construction of a *gfp* promoter trap library and related clones. An *E. coli-F. tularensis* shuttle vector, pTE, was constructed by SalI digestion and self-ligation of the pASC-1 plasmid. A promoterless version of the gfp^+ reporter gene was amplified by PCR from plasmid pWH1012 using primers GFP-F and GFP-R. The PCR product was ligated into a linearized pTE vector as an EcoRI-PstI fragment, and the resulting plasmid was designated pTRAP. Chromosomal DNA from *F. tularensis* subsp. *holarctica* LVS was isolated by the method of Marmur (28). The purified DNA was partially digested with Sau3AI and size fractionated by agarose gel electrophoresis. DNA fragments ranging from 0.3 to 2.0 kb were purified and ligated into the BglII site of pTRAP. The ligation mixture was introduced into *E. coli* DH5α cells, and transformants were plated on LB agar containing 100 μg/ml ampicillin, resulting in about 9,000 colonies. Visualization of fluorescent colonies expressing the gfp^+ gene was carried out at a wavelength of 365 nm using a UV illuminator. Estimation of the number of insert-containing colonies was carried out by PCR analysis using primers GFP-F and GFP-seq. The extent of genome coverage of the library was calculated using the equation $N = \ln(1 - P)/\ln(1 - a/b)$, where N is the number of library clones needed for full genome coverage at the desired probability (P), a represents the average insert size (bp), and b is the complete genome size. One microgram of plasmid DNA prepared from a pool of the *E. coli* clones was electroporated into competent LVS cells and plated on large (10- by 10-cm) CHA agar plates containing 2 μg/ml Cm. Screening of the resulting colonies was performed using UV light. Fluorescent colonies were isolated and stored for further analysis.

The pTRAP (*groEL-gfp*) vector was constructed by PCR amplification of the *groEL* promoter region using primers *groEL*-F and *groEL*-R and subsequent ligation into the BglII-SnaBI linearized pTRAP vector. Similarly, a PCR fragment containing the *bfr* promoter region was amplified using the *bfr*-F and *bfr*-R primers and ligated to an XbaI-PstI linearized pKK214 vector to obtain the pKK (*bfr-cat*) plasmid.

Analysis of GFP fluorescence. Quantification of fluorescence in cultures of the pTRAP(*groEL-gfp*) and pTRAP(*bfr-gfp*) clones was performed with a Spectrafluor Plus (Tecan) fluorimetric microplate reader, using a 485-nm filter for excitation and a 510-nm filter for emission. Culture samples were diluted to an OD_{600} of 1.0 with TSBC broth prior to analysis. Flow cytometry data were obtained from exponential-phase bacteria that were washed twice in phosphate-buffered saline (PBS) and resuspended in PBS supplemented with 1% bovine serum albumin (BSA) at a concentration of 10^7 CFU/ml. Flow cytometry analysis was performed with a FACSCalibur (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Bacterial labeling for fluorescence-activated cell sorter (FACS) analysis was performed with the total Alexa 647-conjugated IgG collected from hyperimmune antiserum prepared by immunization with formalin-killed LVS cells as previously described (1). Labeling was carried out at a concentration of 1 μg/ml for 30 min in 4°C. Data were analyzed using the FlowJo software.

Construction of *cat*-based promoter trap library. The *F. tularensis* subsp. *holarctica* LVS genomic DNA preparation was partially digested with AluI, and DNA fragments ranging from 50 to 1,500 bp were purified and ligated into the SmaI-linearized pKK214 plasmid. The ligation products were used to transform *E. coli* DH5α cells as a means for plasmid amplification. The resulting recombinant colonies were plated on LB medium containing 10 μg/ml tetracycline. One hundred nanograms of plasmid DNA prepared from a pool of the *E. coli* colonies was electroporated into LVS cells and incubated in 2 ml of TSBC broth containing 5 μg/ml Cm at 37°C under agitation (150 rpm) for 3 h, diluted 1:20 into fresh medium, and incubated under the same conditions for 2 additional hours. The same procedure was then repeated by a 1:20 dilution of the propagated bacteria in fresh medium supplemented with 10 μg/ml Cm. One hundred-microliter fractions of the 2-ml bacterial culture originating from the last liquid medium propagation were then plated on CHA plates containing 10 μg/ml Cm, and the plates were incubated at 37°C for 3 days. Forty of 2,000 Cm^r colonies obtained were arbitrarily isolated for further characterization.

PCR analysis for specific promoters in the promoter trap libraries. In order to identify specific *cat*-isolated promoters in the original *gfp* library, a plasmid DNA preparation (50 ng) made from the pool of *gfp* clones served as a template for PCR analysis using primers Det-P2F and Det-P2R, Det-P39F and Det-P39R, and Det-P29F and Det-P29R (Table 1) for identification of the *PgroEL*, P39, and P29 promoters, respectively. Similarly, the presence of the *bfr* promoter in the original *cat* library was verified by PCR analysis of the plasmid pool using primers Det-P*bfr*F and Det-P*bfr*R (Table 1).

Qualitative evaluation of expression levels of CAT by immunoblot analysis. For determination of intracellular chloramphenicol acetyltransferase (CAT) levels, individual LVS Cm^r library clones were cultured in a TSBC broth supplemented with 10 μg/ml Cm to an OD_{600} of 0.5. Cell pellets originating from 1-ml cultures were harvested by centrifugation and resuspended in SDS sample buffer (50 mM Tris-Cl, pH 6.8, 100 mM dithiothreitol [DTT], 2% SDS, 0.1% bromophenol blue, 10% glycerol) to an OD_{600} of 10.0 and boiled for 10 min. Western blot analysis was performed according to standard procedures. For dot blot analysis, 0.5- to 1-μl amounts of samples were spotted onto a dry Hybond ECL nitrocellulose membrane (Amersham Biosciences) and handled as described for the Western blots. Immunoblotting was performed using commercial anti-CAT antibodies (Sigma) and developed with horseradish peroxidase (HRP)-labeled donkey anti-rabbit immunoglobulin G (Sigma) according to standard procedures.

Quantification of CAT activity by an enzymatic analysis. A mid-log-phase bacterial cell culture of each of the 40 recombinant Cm^r LVS library clones grown in TSBC broth at 37°C under aeration was centrifuged, and cell pellets were adjusted to a calculated turbidity of 0.2 OD_{600}/ml. Pellets were subjected to a single freeze-thaw cycle, resuspended in 500 μl of cold (4°C) disruption buffer (50 mM Tris, pH 7.8, 30 μM DTT), vigorously vortexed for 30 s, and centrifuged, and the protein concentration in the soluble fraction was determined using the Bradford assay (3). Equal amounts of proteins from the supernatant fractions were used for the spectrophotometric assay of CAT activity according to the method of Shaw (38). Briefly, total protein (1 μg) from each sample was diluted 1:10 into a 100-μl CAT reaction buffer {0.4 mg/ml DTNB [5,5′-dithiobis(2-nitrobenzoic acid)], 0.1 mM acetyl-coenzyme A, and 0.1 M Tris buffer, pH 8.0}. CAT activity was determined at 25°C by monitoring the changes in absorbance at 405 nm under conditions of excess substrate.

Characterization of chromosomal inserts in selected Cm^r and GFP-expressing clones. Cm^r LVS colonies were analyzed by PCR using primers CAT-seqF and CAT-seqR for Cm library clones or GFP-F and GFP-seq for *gfp* library clones (Table 1). The PCR amplicons were electrophoretically analyzed to determine insert sizes. Plasmid DNA isolated from LVS was prepared using Qiagen miniprep spin columns. The insert fragments were sequenced by the dideoxy termination method with primers CAT-seqF and CAT-seqR (for Cm library clones) or GFP-F and GFP-seq (for *gfp* library clones) and analyzed with an automated sequencing system (Applied Biosystems). The insert sequences were analyzed by GenBank database searches using the National Center for Biotechnology Information BLAST web server (http://www.ncbi.nlm.nih.gov/BLAST).

Total RNA isolation, primer extension, and quantitative real-time PCR (qRT-PCR) analyses. Total RNA from *F. tularensis* strain LVS grown to mid-log phase in TSBC broth was extracted using a RiboPure-bacteria kit (Ambion). Residual genomic DNA was removed by DNase I treatment according to the manufacturer's instructions. The RNA was quantified spectrophotometrically, and its integrity was examined by agarose gel electrophoresis. Primer extension (PE) reactions were carried out according to the method of Lloyd and colleagues (22). Briefly, for each reaction, the respective FAM (6-carboxyfluorescein)-labeled primer (Table 1) (10 nM) was added to 15 to 20 μg of total RNA, and first-strand cDNA synthesis was performed using the avian myeloblastosis virus (AMV) reverse transcriptase enzyme (Promega). FAM-labeled cDNAs were purified using a Performa DTR gel filtration cartridge (Edge Bio) according to the manufacturer's instructions and then air-dried with a heated Speed-Vac centrifuge and resuspended in 25 μl of UltraPure formamide (Invitrogen) with 1.0 μl MapMarker 1000 (BioVentures, Inc.). The mixture was heated to 90°C for 2 min, chilled on ice for 5 min, and then used for electrophoresis, using an ABI310 genetic analyzer (Applied Biosystems). The DNA fragments were sized using GeneScan Analysis software version 3.1 (Applied Biosystems).

For qRT-PCR, cDNA was generated with Omniscript reverse transcriptase (Qiagen), 10 μM random primers (BioLabs), and 250 ng of RNA prepared from each of the tested clones. A 1:1,000 dilution of each of the cDNA preparations was amplified in two separate 50-μl reaction mixtures using 500 nM *cat*-specific primers (CAT-F and CAT-R) or tetracycline (*tet*)-specific primers (Tet-F and Tet-R), 5 mM magnesium, 0.25 mM deoxynucleoside triphosphate, PCR buffer,

100 nM Super ROX (BioSearch Technologies), AmpliTaq Gold DNA polymerase (Applied Biosystems), and EVA green (Biotium, Inc.). The experiments were repeated at least twice for each clone tested and analyzed using a 7500 ABI real-time PCR system (Applied Biosystems). The pKK214 plasmid DNA (10 pg/ml to 10,000 pg/ml) served as a reference for cDNA quantification. The relative potency of each clone tested was determined by normalizing the amount of *cat* PCR products generated to the amount of common *tet* PCR products.

Antibiotic sensitivity tests (Etests). Isolated individual LVS colonies, originating from a CHA agar plate, were suspended in TSBC broth to an OD_{660} of 0.15. A 100-μl aliquot of each suspension was plated to a lawn on a CHA agar plate and left to dry for 10 min, and an Etest strip (AB bioMérieux, Solna, Sweden) was placed on the plate. The plates were incubated at 37°C for 48 to 76 h, and MICs were determined following the clear emergence of the bacterial monolayer according to the manufacturer's instructions.

Use of chloramphenicol for direct selection of highly potent promoters from the *cat*-based promoter trap system. The 2,000 Cm^r colonies isolated from the *cat*-based promoter trap system were pooled and grown in the presence of 5 μg/ml Cm and then diluted into increasing concentrations of Cm (20, 40, and 80 μg/ml). Total plasmid DNA was isolated to determine variations in plasmid abundance from the bacteria cultured at the lowest (5 μg/ml) and the highest (80 μg/ml) antibiotic concentrations. The abundance of plasmids was analyzed by qRT-PCR using primers specific for six representative promoters of different strengths (P2, P3, P18, P36, P39, and *Pbfr*). All plasmids, regardless of the promoter potency, could be identified in cultures with both the low and the high concentration of Cm.

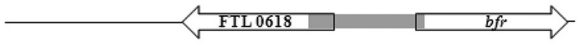
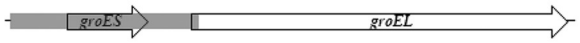
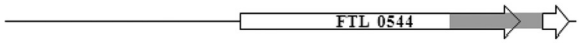
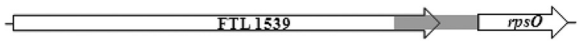
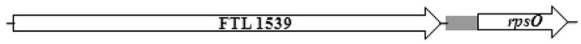
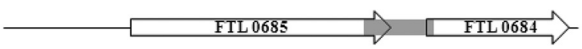
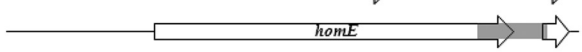
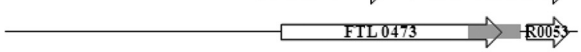
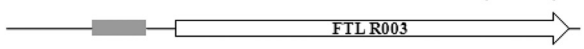
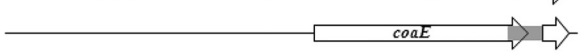
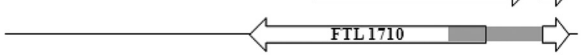
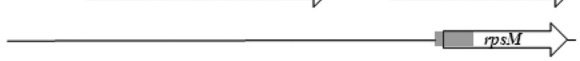
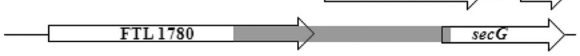
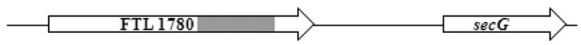
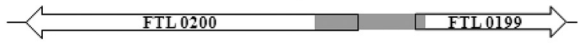
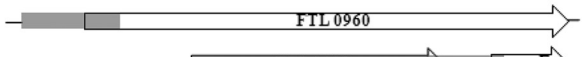
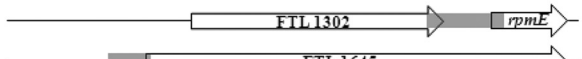
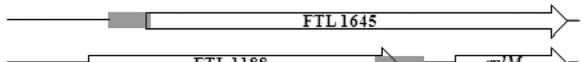
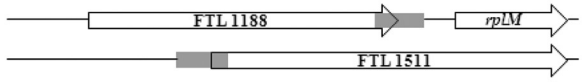
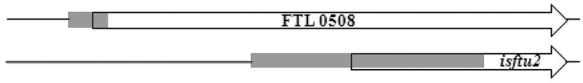
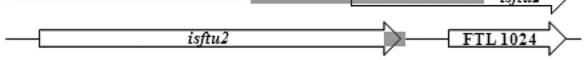
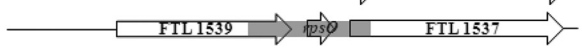
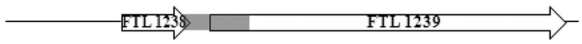
Computational analysis of promoter elements. The sequences of all the unique promoter clone DNA inserts were subjected to regulatory element analysis using the BPROM bacterial promoter prediction program (Softberry). Multiple alignment of predicted promoter elements was carried out using the T-coffee algorithm (32) and visualized using the BOXSHADE viewer (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html).

RESULTS

Screening an *F. tularensis* LVS promoter trap library using *gfp* as a reporter gene. The initial attempt to identify *F. tularensis* promoters was based on screening of a *gfp*-based promoter trap system. For this purpose, a novel pTRAP *E. coli-Francisella* shuttle vector was constructed based on a derivative of the pRIT5 vector (29) (Pharmacia, Uppsala), which contains the promiscuous pC194 origin of replication (14) (see Materials and Methods). The GFP vector includes a promoterless copy of the gfp^+ gene (37) located downstream from a synthetic ribosome binding site (RBS) which is separated from the gfp^+ start codon by seven nucleotides that comprise a SnaBI restriction site. Sau3A-digested *F. tularensis* LVS chromosomal DNA fragments were inserted into the pTRAP plasmid vector. This ligation mixture was used to transform *E. coli* cells, resulting in about 9,000 colonies, many of which exhibited visible fluorescence. DNA restriction analysis performed on a sample of 50 colonies revealed that about 50% of the transformants contained chromosomal inserts, with an average size of 410 ± 50 bp (average ± standard deviation). Based on the *F. tularensis* genome size (1.9 Mbp), the calculated genome coverage achieved by the inserts in the library is about 50% (see Materials and Methods). Plasmid DNA isolated from the pool of *E. coli* transformants was used to transform *F. tularensis* LVS. Of the 2.6×10^4 *F. tularensis* transformant colonies that were inspected by UV illumination, only three colonies exhibited visible fluorescence (in marked contrast to the many fluorescence-positive clones in the *E. coli* pool). DNA restriction and sequence analysis revealed that all three clones carry the same chromosomal fragment, which contains the bacterioferritin promoter (*Pbfr*) region upstream from the *gfp* reporter. The map of the cloned fragment present in all three of these clones is provided in the first line of the chromosomal locus tabulation in Fig. 1. As will be shown below, *Pbfr* is the most potent among all the promoters analyzed in this study.

Use of a *cat*-based promoter trap library for the selection of *F. tularensis* LVS promoters. In view of the limited results obtained with the *gfp* promoter trap system, we decided to employ a different screening approach that relies on *cat* as a reporter gene. The *cat*-based promoter library was constructed from partially AluI-digested *F. tularensis* LVS chromosomal DNA fragments that were cloned upstream from a promoterless *cat* gene in the *E. coli-F. tularensis* shuttle vector pKK214 (18). Since attempts to construct the library directly in the LVS background resulted in an insufficient number of colonies on chloramphenicol (Cm) selection plates, we first amplified the library in *E. coli.* Plasmids isolated from the pool of *E. coli* transformants were electroporated into LVS cells and resulted in a total of 2,000 Cm^r LVS clones. Of these 2,000 Cm^r clones, we arbitrarily selected 40 colonies for further characterization. Restriction analysis revealed that all of the clones contained LVS DNA fragments of various sizes (50 to 1,500 bp; average size, 350 ± 50 bp).

Identification of the chromosomal loci of the LVS promoters analyzed. The cloned chromosomal DNA fragments from all of the 40 clones isolated from the *cat* library, as well as those of the 3 clones which exhibited fluorescence in the *gfp* library, were sequenced, and the genomic location of each was determined based on the sequence of the *F. tularensis* subsp. *holarctica* LVS genome in the NCBI databank (Fig. 1). As mentioned above, all three clones originating from the *gfp* library appeared to be identical and all were derived from the *bfr* promoter region; hence, they are represented as a single clone in the first line of Fig. 1. Of the clones isolated from the *cat* library, three, P1, P10, and P38, contained identical fragments, as did clones P8 and P34. Some chromosomal regions were represented in more than one clone, yet they clearly represent independent cloning events, since their respective inserts were not identical (P4 and P5; P12 and P26; P14 and P29; and P6, P35, and P40) (Fig. 1; also see Table S1 in the supplemental material). Most of the clones include inserts derived from intergenic fragments, consistent with their expected location in regulatory regions. Furthermore, the orientation of these inserts with respect to the *cat* reporter gene is in line with the orientation expected for transcription in the context of the genome. The vast majority of the trapped DNA regulatory fragments contained a putative ribosome binding site (RBS). In the case of the five inserts that did not contain an apparent RBS (P9, P7, P16, P22, and P36), it appears that the expression of the reporter gene exploited the existing RBS in the expression vector. Six clones, P7, P22, P27, P32, P33, and P36, were found to include DNA inserts that resulted from the ligation of two separate AluI genomic fragments, yet in each case, only one of the two ligated fragments was found to represent a promoter-like 5′ untranslated region of an open reading frame (ORF). We note that, apart from P2, which is located upstream of the previously described *groEL* operon (9), all of the sequenced clones represent novel *F. tularensis* promoters. In summary, of 43 analyzed clones originating from both the *gfp* and the *cat* libraries, 38 clones contained different genomic fragments representing 33 unique promoters.

Potency group	Clone No.	Functional annotation	Genomic context of the trapped fragment
I	*bfr*	Putative bacterioferritin	
	39	Acyl carrier protein	
	2	Chaperonin protein	
II	12	tRNA-Met3	
	26	tRNA-Met3	
	14	30S ribosomal protein S15	
	29	30S ribosomal protein S15	
	15	tRNA-Lys1	
	13	Hypothetical protein	
	27	Hypothetical protein	
	33	tRNA-Met2	
	5	Conserved hypothetical lipoprotein	
	4	Conserved hypothetical lipoprotein	
	9	50S ribosomal RNA	
	7	16S ribosomal RNA	
	28	tRNA-Arg3	
	11	tRNA-Met1	
	16	50S ribosomal protein L10	
III	1,10,38	30S ribosomal protein S13	
	32	50S ribosomal protein L34	
	6	Preprotein translocase SecG	
	35	Preprotein translocase SecG	
	40	Preprotein translocase SecG	
	18	Hypothetical protein	
	17	Serine transporter	
	8,34	NADH dehydrogenase I, A subunit	
	19	Pyridine nucleotide transhydrogenase	
	25	50S ribosomal protein L31	
	24	Major facilitator transporter	
	36	50S ribosomal protein	
	37	Glycerophosphoryl diester phosphodiesterase family protein	
	3	UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-Meso-diaminopimelate ligase	
	20	Transposase FTL 0042	
	22	30S ribosomal protein S6	
	23	Transposase FTL 0270	
	31	Transposase FTL 0079	
	21	polyribonucleotide nucleotidyltransferase	
	30	Signal recognition particle protein, Ffh	

***In silico* compilation and experimental analysis of *F. tularensis* promoters.** Computational analysis of the newly identified promoters was carried out in a search for regulatory elements, using the BPROM tool (Softberry). Consensus sequences were derived from multiple alignment of the 33 unique sequences (Fig. 2A). Accordingly, a putative −10 region matching the consensus hexamer of the σ^{70} family promoters (TATAAT) was identified 4 to 8 nucleotides upstream from the predicted transcription start point (TSP), and a putative −35 region highly similar to the classical TTGACA consensus sequence was found to be located 14 to 21 bp upstream from the −10 region (Fig. 2A). Inspection of the LVS promoter sequences located upstream from the −35 consensus (positions −38 to −59) revealed a typical AT-rich region that resembled the upstream region, termed the UP element, that is necessary for efficient transcription from some promoters in both Gram-positive and Gram-negative bacteria (12).

In order to confirm that the predictions based on this *in silico* analysis reflect the actual TSPs, we further selected five promoters for primer extension analysis. Total RNA prepared from *F. tularensis* LVS bacteria grown to mid-logarithmic phase served as a template for a modified primer extension analysis (see Materials and Methods) using FAM-labeled primers as previously described (22). Two independent extension reactions, using two different primers complementary to the 5′ region of the relevant open reading frame, were carried out for each promoter inspected. As depicted in Fig. 2B, a single TSP was determined for each of the five clones except for P18, in which two TSPs were identified. All of the TSPs that were experimentally determined were located within a range of 1 to 5 nucleotides of the TSP predicted by the *in silico* analysis. The TSP of P2, which is located upstream of the *groEL* operon, was mapped to reside 91 bp upstream from the *groES* start codon, which is in excellent agreement with previously published results (9). The TSPs of the open reading frames regulated by the *Pbfr*, P39, and P3 promoters are located 119, 45, and 25 bp upstream from the respective ATG start codons. The first transcriptional initiation site of promoter 18 (TSP1) is located 19 bp upstream from the translational start codon of its putatively regulated gene, FTL_0199. The second transcription start point (TSP2) is located 385 bp upstream from the FTL_0199 translational start codon and is a part of the FTL_0200 ORF. However, TSP2 is not a part of the cloned *cat* library fragment, and therefore, *cat* transcription in clone 18 is driven by the proximal rather than the distal promoter.

It was of interest to determine to what extent the sequences of the promoters originating from the *F. tularensis* subsp. *holarctica* LVS strain are similar to or diverge from those of the virulent *F. tularensis* subsp. *tularensis* Schu S4 strain. It appears that of the 33 LVS promoter sequences, 26 are completely identical to their corresponding loci in the Schu S4 strain, while 7 promoters exhibited minor differences consisting of single-nucleotide replacement/deletion/insertion, all within the region spanning between the −10 and −35 elements. This region is known to be promiscuous with respect to both sequence composition and length.

Relative potencies of the analyzed *cat*-derived promoters. In order to assess the relative potencies of the promoters derived from the *cat* library, we conducted a dot blot Western analysis of cell extracts from each clone using specific anti-CAT antibodies (Fig. 3A). This analysis revealed that all the clones tested interact specifically with the anti-CAT antibodies, while control LVS cells carrying the promoterless *cat* cassette showed no Western blot signal. Furthermore, the tested clones exhibited various signal intensities, suggesting a wide dynamic range of CAT expression levels.

In order to better quantify the potencies of the different cloned promoters, an enzymatic assay was performed using equal amounts of cell extracts derived from each clone (see Materials and Methods). The CAT activity levels determined by the assay were consistent with the results derived from the dot blot analysis in most cases. Based on the enzymatic assay, the promoters could be arbitrarily classified into three potency groups (Fig. 3B). The relative potencies of selected promoters were further manifested by SDS-PAGE and Western blot (for example, see Fig. 3C). Both the Western and the enzymatic analyses established that 2 out of the 40 clones analyzed (5%) entail promoters of high potency, 15 clones (37%) represent promoters of moderate potency, and 23 clones (58%) include relatively weak promoters (it should be noted that in E-tests, even the weakest LVS promoter [e.g., promoter P30] provided at least a 5-fold-higher MIC value than that of pKK202 [30] in the LVS background). In view of the presence of clones containing plasmids with different promoter strengths, we tested the possibility of direct selection of highly potent promoters using increasing Cm concentrations. However, no enrichment of clones containing the more potent promoters was attained by this selection procedure (see Materials and Methods).

To directly assess the relative transcription initiation potencies of the promoters, the number of *cat* mRNA molecules generated under the control of various promoters was determined by qRT-PCR analysis. The pKK214 *tet* gene, which confers tetracycline resistance, was used as an internal control in the RT-PCR assays. The relative levels of *cat* amplicons in promoter clones P39, P2, and P3 were 100%, 67%, and 7%, respectively. These results confirm the potencies of the promoters inferred from the CAT assay and the Western analysis and demonstrate that these assays correctly reflect the promoter potency at the transcriptional level.

FIG. 1. Chromosomal gene arrangement and genomic context of the strain LVS promoters analyzed. The maps of the 38 clones compiled in this figure represent 33 unique *F. tularensis* promoters that were isolated from the *gfp* and the *cat* promoter trap libraries. Gray boxes represent the regions of the LVS cloned fragments. The chromosomal loci of the various promoters are depicted in the orientation that is compatible with expression of the reporter gene (*cat* or *gfp*). The genes adjacent to the cloned fragments are indicated as open arrows, and their locus tags (FTL numbers) according to the *F. tularensis* subsp. *holarctica* complete genome sequence in the NCBI database are indicated. Whenever available, the genes are designated by their name symbols. Promoters are arranged according to their relative potencies (Fig. 3B and text). Note that clones 1, 10, and 38 represent the same DNA fragments and thus appear together in their respective entries, as is the case for clones 8 and 34. For more details on the cloned fragments, please refer to the text and Table S1 in the supplemental material.

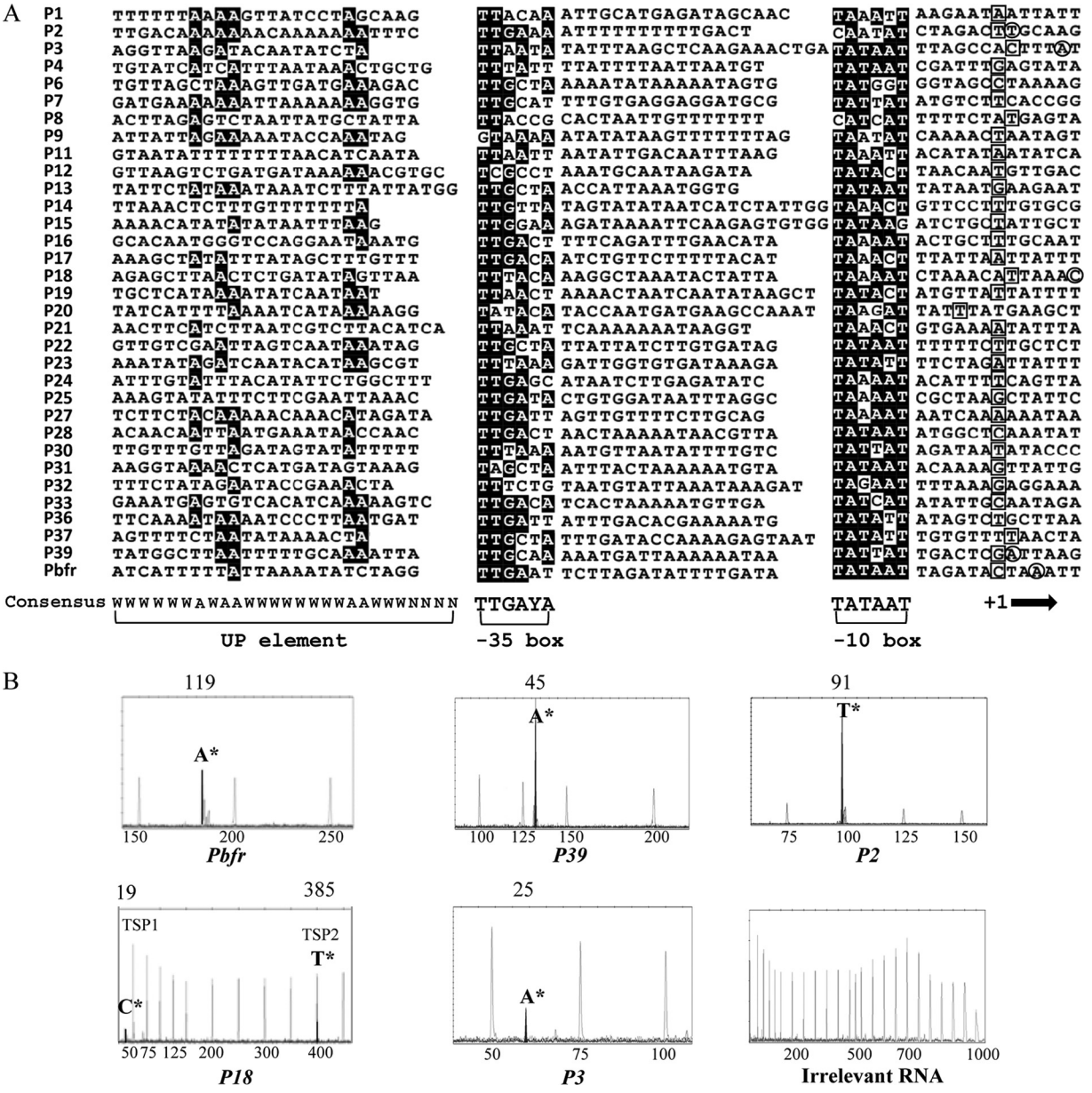


FIG. 2. Sequence analysis of the cloned *F. tularensis* promoters. (A) Multiple alignment of all unique promoter sequences determined in this study. The promoters are arranged in ascending order. The TSP, −10, and −35 boxes were predicted using the BPROM tool and aligned using the T-coffee multiple-alignment tool. Nucleotides conserved among more than 50% of the sequences are indicated by black boxes. The predicted TSP nucleotide is boxed, while the 5 experimentally determined TSP nucleotides are circled. The consensus sequences of the −10, the −35, and the UP element are indicated at the bottom. The arrow on the bottom right indicates the TSP (nucleotide position +1). (B) Analysis of primer extension (PE) products specific for the indicated promoters. PE fragments were sized using GeneScan Analysis software (Applied Biosystems) (for detailed experimental conditions, see Materials and Methods). MapMaker 1000 internal lane standards are included in each electropherogram. The first 5′ nucleotide of the transcript is marked by an asterisk above the translation start diagnostic peak. The number above each panel refers to the length of the 5′ untranslated region of each promoter (i.e., the distance between the transcription starting point and the respective translation starting point). Note the absence of cDNA signal in the irrelevant-RNA negative-control panel.

Potency of the *bfr* promoter and limitations of the GFP screening method. While the Cm-based selection allowed the isolation of numerous promoters, the *gfp*-based promoter trap system essentially generated only 3 clones, all representing the *bfr* promoter (*Pbfr*). In order to determine the reason for this extremely low prevalence of fluorescent LVS clones, we probed the original plasmid pool of the *gfp* library for the presence of 3 potent promoters (P39, P2, and P29) which were identified in the *cat* library (see Materials and Methods). The PCR analysis revealed that the DNA fragments of each of these promoters is present in the *gfp* library. This result strongly suggests that the GFP expression level driven by these 3 promoters was probably insufficient to allow visible fluorescence in the LVS background (unlike that driven by *Pbfr*), in

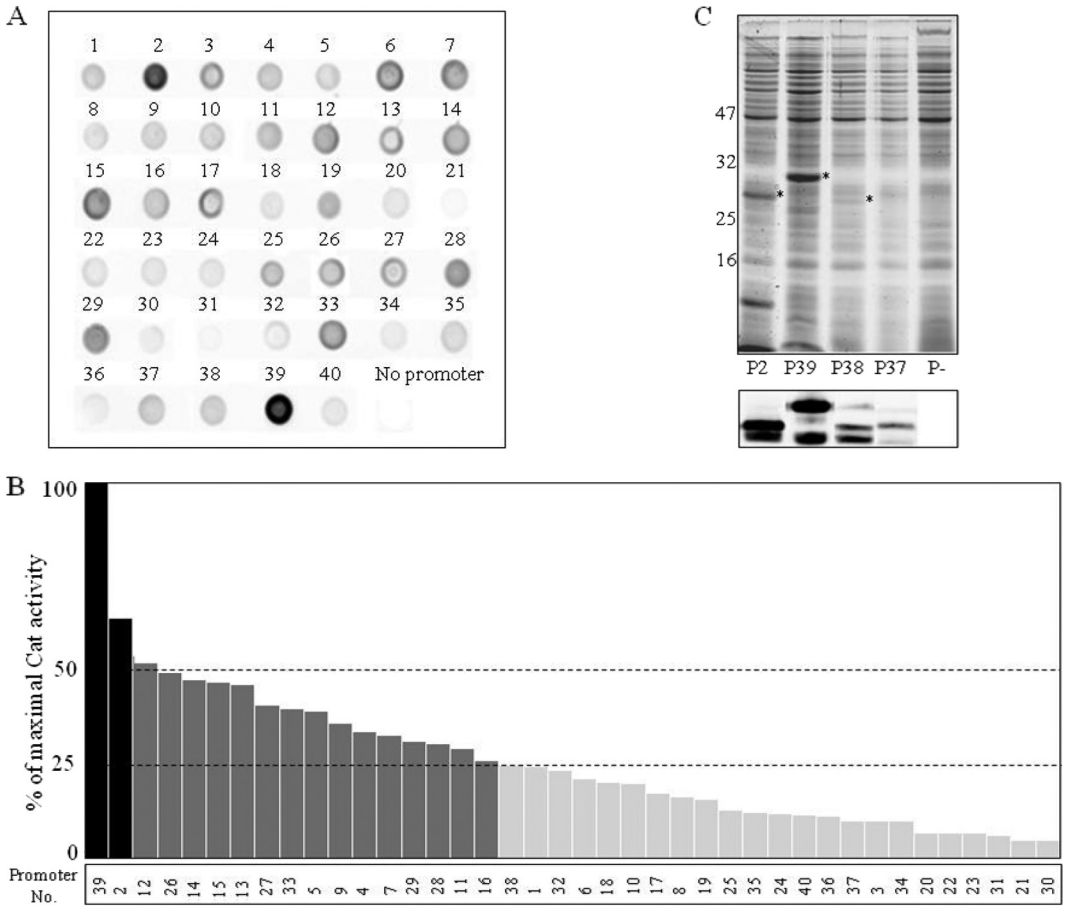


FIG. 3. Expression level of chloramphenicol acetyltransferase (CAT) from selected clones isolated from the *cat* library. (A) Western dot blot analysis for determination of CAT levels in cytosolic extracts, using anti-CAT antibodies. (B) Relative CAT activity in cytosolic cell extracts determined by CAT enzymatic assays. Promoters are ordered according to descending levels of potency. Black, dark-gray, and light-gray bars denote strong, moderate, and weak promoters, respectively. (C) SDS-PAGE (Coomassie, top) and immunoblot (anti-CAT, bottom) analyses from four selected promoter clones. CAT protein is indicated by asterisks (the additional high-molecular-weight CAT form generated in the P39 clone probably stems from an alternative upstream translation initiation codon within the P39 insert). The control lane designated (P−) represents cells carrying the promoterless *cat* plasmid pKK214.

spite of their relative strength. To further confirm this notion, we decided to compare the GFP expression levels in the cells carrying the *bfr* promoter to those generated by the well-characterized *F. tularensis* promoter *groEL* (identified as P2 by the Cm screen and classified as one of the two most potent promoters). Accordingly, the *groEL* (P2) promoter was cloned upstream from the promoterless gfp^+ (in the pTRAP vector), and the level of GFP production in the LVS transformants was compared to that of the *bfr*-GFP cells. Indeed, the levels of GFP expressed by the LVS cells as analyzed by SDS-PAGE, as well as by inspection of the fluorescence emitted by colonies grown on solid medium, were significantly higher when the transcription was driven by the *bfr* promoter (Fig. 4A and B). Furthermore, quantitative fluorimetry, as well as FACS analysis of cell extracts, allowed us to estimate that the fluorescence level driven by *Pbfr* was nearly 10-fold higher than that driven by *PgroEL* (Fig. 4C and D).

While the *bfr* promoter was not identified among the 40 randomly selected *cat* colonies, its presence within the *cat* library plasmid pool was verified by PCR analysis (see Materials and Methods). To further directly evaluate the potency of the *bfr* promoter relative to the potencies of other promoters in the *cat* context, we cloned the *bfr* promoter fragment (isolated from the *gfp* library) upstream from the promoterless *cat* gene in the pKK214 vector and compared the CAT expression level to that driven by promoter 39 (the strongest promoter identified in the *cat* library). This assay showed that the activity of the CAT enzyme driven by the *bfr* promoter was higher than that driven by P39 (80 OD_{405}/min/OD_{600} and 60 OD_{405}/min/OD_{600}, respectively [OD_{405} is the measurement for CAT enzyme activity and OD_{600} is the measurement for culture density]). Based on all these observations, we conclude that the *bfr* promoter is the most potent among all promoters identified in the present work and is probably one of the most potent promoters in *F. tularensis*.

DISCUSSION

The previously identified *groEL*, *acpA*, and FTN_1451 *Francisella* promoters provided essential genetic tools for studying and manipulating genes of *F. tularensis*. Nevertheless, the number of *Francisella* promoters available to date is rather limited,

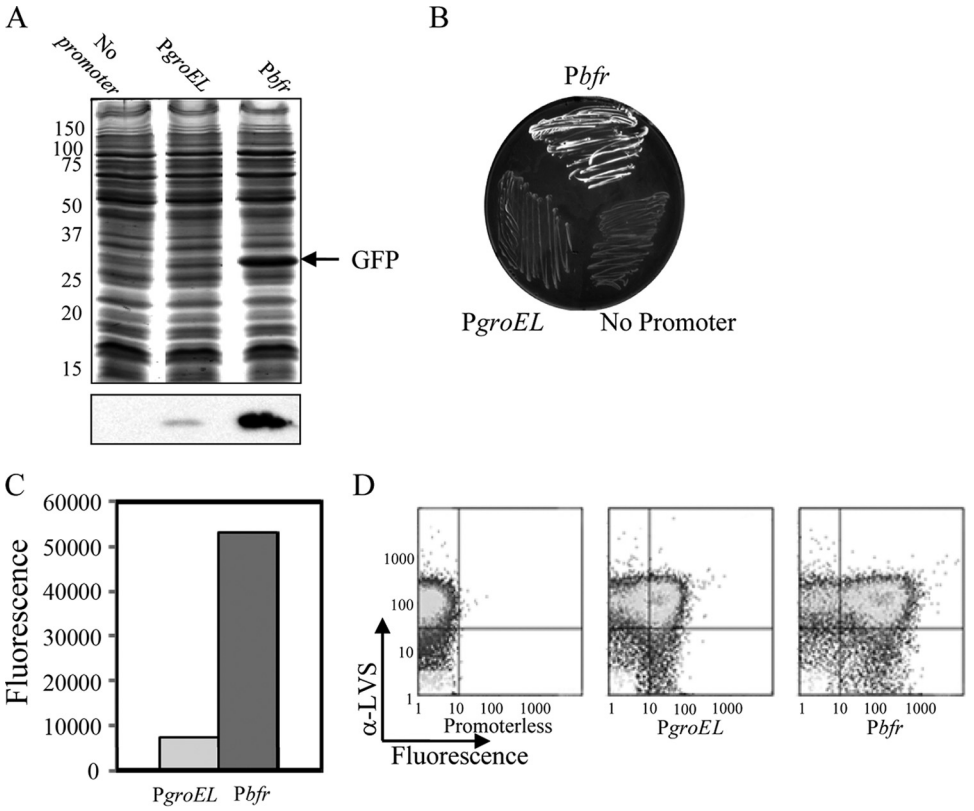


FIG. 4. Comparison of relative potencies of the *bfr* and *groEL* promoters determined by GFP expression. (A) SDS-PAGE (top) and Western blot (anti-GFP, bottom) analyses of *F. tularensis* LVS cell extracts expressing GFP from indicated promoters cloned in the pTRAP vector. Bacterial cultures were collected 12 h postinoculation. (B) Direct inspection of fluorescence emitted by cells expressing GFP from the indicated promoters. (C) Quantification by direct fluorimetry of GFP fluorescence levels emitted by cells expressing GFP from either the *bfr* or *groEL* promoter. Fluorescence quantification was carried out with cultures of identical densities. (D) FACS analysis for quantification of GFP fluorescence levels emitted by equal numbers of cells expressing GFP from either the *bfr* or *groEL* promoter.

and those identified are still poorly characterized. In the current study, two alternative *in vitro* large-scale screening procedures were employed in an attempt to identify new potent *Francisella* promoters. The first approach relied on *gfp* as a reporter gene. This procedure allowed the identification of a highly potent promoter (the *bfr* promoter). In fact, this promoter was the only promoter that could drive the expression of *gfp* in an amount that enabled the visualization of the fluorescent clone on agar plates using UV light. This phenomenon is clearly not due to underrepresentation of insert-containing clones, since we estimated that 50% out of the initial 9,000 library clones contained LVS sequences. The fact that many LVS promoters were able to generate visible fluorescence in the background of *E. coli* but failed to do so in the LVS background may be due to one or a combination of the following reasons. (i) The visible GFP fluorescence elicited in LVS is somehow quenched, and thus, only very potent promoters can allow the detection of GFP-expressing colonies. Indeed, while GFP expression from the *groEL* promoter was confirmed by Western blot analysis, the fluorescence level of the *groEL-gfp* clone was hardly detectable in the LVS background (Fig. 4). Actually, detection of the fluorescence emitted by a *gfp* clone required that *gfp* expression be driven by a promoter 10 times stronger than the *groEL* promoter, such as the *bfr* promoter (Fig. 4A and B). We note that in another study, the expression of GFP from the *F. tularensis rpsL* promoter was noted to be poorly visible in strain LVS, while it was clearly visible in the *E. coli* background, as judged by confocal microscopy imaging (23). (ii) A certain proportion of the cloned chromosomal segments that promoted GFP fluorescence in *E. coli* may not represent authentic *Francisella* promoters. It is well established that the *E. coli* σ^{70}-dependent promoters are capable of driving significant levels of transcription solely upon recognition of an AT-rich −10 consensus promoter element (TATAAT) together with a TGn −10 motif (4, 17). Since the *F. tularensis* genome is AT rich, it is likely that some *F. tularensis* chromosomal DNA regions may be recognized as promoters by the *E. coli* transcription machinery just by virtue of the high frequency of AT-rich regions. Similar observations were reported for the pKK214 vector, which contains a promoterless *cat* gene and still confers chloramphenicol resistance when introduced into *E. coli* (18). (iii) It is possible that a higher copy number of the pTRAP vector in *E. coli* could have contributed to the high prevalence of fluorescent *E. coli* clones.

The second screening approach that was implemented in this study relied on direct selection of Cm^r colonies through expression from *cat* as a reporter gene. Many studies of bacterial pathogens, including *F. tularensis*, have used *cat*-based promoter trap systems in order to select for bacterial genes

that are specifically induced during infection (7, 18, 25, 26). Here we used, for the first time in *F. tularensis*, an *in vitro* approach that took advantage of the strong Cm-mediated selective pressure in order to select *Francisella* promoters in a genomic library. Out of 2,000 Cm^r LVS colonies, we arbitrarily selected 40 clones for further characterization. All of these clones appeared to contain inserts of various sizes upstream of the *cat* reporter gene, as attested by both restriction and sequence analyses. Sequence analysis revealed that 37 of the 40 *cat* library clones contain different genome fragments and 32 represent unique promoter sequences. The low level of redundancy of the cloned promoters in the population suggests that the 2,000 *cat* library clones provide good coverage of most if not all *F. tularensis* promoters.

The two screening approaches (*gfp* and *cat* cassette) yielded 33 different and unique promoters with various levels of potency. The 3 most potent promoters were found to belong to a putative bacterioferritn (*Pbfr*, FTL_0617), an acyl carrier protein (*PacpP*, FTL_1138), and a heat shock protein which belongs to the *groESL* operon (*PgroEL*, FTL_1715). The high potency exhibited by *Pbfr* and *PgroEL* is in line with previous reports documenting that Bfr and the 2 proteins residing on the *groESL* operon (i.e., GroES and GroEL) are among the 12 major proteins secreted by LVS cells (21). The side-by-side quantitative comparison of GFP expression driven by the *bfr* and the *groEL* promoter suggests that the *bfr* promoter is about 10-fold more potent than the *groEL* promoter. Interestingly, 16 of the 33 unique clones contain promoters that regulate genes belonging to components of the translational machinery (6 clones contain promoters that regulate the transcription of tRNAs, 2 clones include rRNA promoters, and the rest of the clones harbor various ribosomal protein promoters). This high prevalence of promoters related to the translational machinery is surprising since only 5.5% of the entire *F. tularensis* genome encodes such functions (2% encodes tRNAs [38 genes], 0.5% encodes rRNA [10 genes] and less than 2.7% encodes ribosomal proteins). We note in this context that the promoters of genes encoding rRNA, tRNA, and ribosomal proteins are among the most active in the bacterial cell (19).

Very little is known about the regulation of gene expression in *Francisella* or the architecture of the promoters. Apart from the *groEL* operon, in which the transcription start point was determined (9), no other *Francisella* promoters have been characterized at the molecular level. Multiple sequence alignment of the 33 newly identified promoters revealed a σ^{70}-relevant −10 motif with a 6-nucleotide conserved sequence identical to the TATAAT consensus sequence from *E. coli* (13). This *F. tularensis* −10 motif is found at the expected location upstream of the predicted transcription start point (Fig. 2). Within the −35 region, we could determine a TTGA (C/T)A consensus sequence which is in good agreement with the *E. coli* TTGACA consensus sequence. The spacer DNA segment between the two conserved regions ranged from 17 to 21 bases in most cases, and the 2 most potent promoters (*Pbfr* and P39) exhibited a 17-bp spacer, in line with the architecture of strong promoters previously reported in *E. coli* (13). The results of primer extension analyses carried out for five selected promoter clones are in good agreement with the predicted location of the transcription initiation site and, thus, provide additional experimental support for the *in silico*-predicted consensus promoter elements of *Francisella tularensis*. In addition to the −10 and −35 regions, a 22-nucleotide upstream region (termed the UP element) exhibiting an AT-rich sequence mediating recognition by the α-subunit of RNA polymerase is often needed for efficient transcription (12). We observe that such an AT-rich region is present in all the promoters analyzed. And yet, no consensus sequence which resembles the *E. coli* UP element consensus sequence could be found in this region. Collectively, these results show that the two well-established promoter elements (the −10 and −35 regions) described in other microorganisms as σ^{70}-binding sites can also be identified in *F. tularensis*. However, the inability to distinguish the canonical *cis*-acting UP element consensus among *F. tularensis* promoters may imply that recognition of this DNA element by the *F. tularensis* α-subunit is different from that described for *E. coli.*

The battery of promoters identified in the present work may serve in future studies to achieve controlled levels of expression of selected proteins in the genetic background of *F. tularensis* strains, as well as for various studies aimed at understanding the mechanisms of pathogenesis and virulence. Furthermore, *F. tularensis* strain LVS has been used as a vaccine for many years; however, its efficacy against the virulent Schu S4 strain has been questioned (5). Our study could provide the means for selected expression of specific LVS-/Schu S4-derived proteins in the background of LVS and, thus, may allow the development of a more efficacious *F. tularensis* vaccine.

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