Characterization and Application of a Glucose-Repressible Promoter in Francisella tularensis

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Francisella tularensis, the causative agent of tularemia, is a category A biodefense agent. The examination of gene function in this organism is limited due to the lack of available controllable promoters. Here, we identify a promoter element of F. tularensis LVS that is repressed by glucose (termed the Francisella glucose-repressible promoter, or FGRp), allowing the management of downstream gene expression. In bacteria cultured in medium lacking glucose, this promoter induced the expression of a red fluorescent protein allele, tdTomato. FGRp activity was used to produce antisense RNA of iglC, an important virulence factor, which severely reduced IgC levels in vitro and in vivo and restored IgC levels, indicating the usefulness of this promoter for controlling both exogenous and chromosomal gene expression. Moreover, FGRp was shown to be active during the infection of human macrophages by using the fluorescence reporter. In this environment, the FGRp-mediated expression of antisense iglC by F. tularensis LVS resulted in reduced bacterial fitness, demonstrating the applicability of this promoter. An analysis of the genomic sequence indicated that this promoter region controls a gene, FTL_0580, encoding a hypothetical protein. A deletion analysis determined the critical sites essential for FGRp activity to be located within a 44-bp region. This is the first report of a conditional promoter and the use of antisense constructs in F. tularensis, valuable genetic tools for studying gene function both in vitro and in vivo.

Francisella tularensis is classified by the Centers for Disease Control and Prevention as a category A biodefense agent. It is therefore imperative that researchers have sufficient genetic tools to study this organism. Historically, difficulties in the genetic manipulation of F. tularensis were due partially to poor recognition of exogenous promoter elements by Francisella transcriptional machinery (41). A recent study by Charity et al. showed that the transcriptional machinery of this organism is quite unique (7). Unlike all other bacteria, F. tularensis possesses two distinct α subunits of RNA polymerase, and these subunits are divergent even within the domains involved in promoter contact (7). This feature may explain, in part, the incompatibility of foreign promoters. These complications necessitated the design of genetic tools in which the expression of selectable and counterselectable markers was driven by Francisella promoters (43). This knowledge has been applied toward developments in the repertoire of F. tularensis molecular tools that includes shuttle vectors (25, 26), transposons and mutant libraries (4, 12, 15, 19, 27), and strategies for constructing chromosomal deletions (1, 14, 24).

Some promoter sequences have been characterized and adapted for use as genetic tools for F. tularensis. One of the most frequently exploited and well-studied F. tularensis promoters is that of groEL (11). The groEL promoter has been especially useful, as it drives strong gene expression both in vivo and in vitro (12, 21, 25–27, 30, 38). This promoter was first characterized by sequencing the groE operon of F. tularensis LVS, which led to the discovery of a DNA sequence similar to the motif of σ70-dependent promoters of Escherichia coli (11). Primer extension was used to identify the transcriptional start site, which is located 6 bases downstream of the putative −10 region. Additionally, a distinctive inverted repeat in the 5′ untranslated region, which is characteristic of groE operons from other organisms lacking σ32-dependent promoters, has been identified (11). Another functional Francisella promoter used for molecular manipulation is that of the respiratory-burst-inhibiting acid phosphatase gene, acpA (34). Putative −10 and −35 regions were identified by a sequence analysis of cloned acpA. This promoter element was used previously to drive the expression of plasmid-carried gfp in F. tularensis LVS during a murine macrophage infection (26), demonstrating its effectiveness for the expression of exogenous genes. In F. novicida, a useful promoter was discovered after the chromosomal incorporation of a kanamycin resistance cartridge into the open reading frame of a hypothetical protein gene (FTN_1451) (12). Gallagher et al. subsequently generated a derivative of this translational fusion using this promoter element to mediate the expression of antibiotic resistance for the selection of transposon mutants (12). Apart from the aforementioned studies, little research has been done to elucidate F. tularensis promoters. To address this problem, new strategies, including the construction of promoter traps, have been developed to allow the assessment of F. tularensis promoter activity under a variety of conditions (22, 32, 33).

Controllable promoters are fundamental molecular tools for the differential expression of recombinant proteins in model
systems. Two of the most widely used systems for recombinant gene expression in microorganisms are the classic lactose- and arabinose-inducible promoters (16, 39). These systems are useful and practical, as the addition of a relatively inexpensive carbohydrate into growth medium manifests the induction of gene expression in microorganisms are the classic lactose- and arabinose-inducible promoters (16, 39). These systems are useful and practical, as the addition of a relatively inexpensive carbohydrate into growth medium manifests the induction of.

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

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<tr>
<th>Strain, plasmid, or primer</th>
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MATERIALS AND METHODS

**Francisella strains and growth conditions.** Bacterial strains, plasmids, and primers used in this study are listed in Table 1. *F. tularensis* LVS strains, raised on chocolate plate, were used to inoculate cysteine heart broth (2.5% [wt/vol] heart infusion broth, 0.5% [wt/vol] proteose peptone, and 0.1% [wt/vol] i-cysteine hydrochloride monohydrate) supplemented with a carbon source (i.e., o-glucose [Glc], o-sucrose [Suc], o-galactose, glycerol, glycine, or citric acid) at a concentration of 1% (wt/vol). Broth cultures were grown to stationary phase at 37°C for 20 to 24 h, with shaking at 250 rpm, to an optical density at 600 nm (OD600) of ≥0.5. For genetic manipulations, *E. coli* strains were grown at 37°C aerobically on Luria-Bertani agar plates or in Luria-Bertani broth (DBco) while being shaken at 250 rpm. When required, the concentrations of antibiotics for *E. coli* were 150 µg/ml for ampicillin and 35 µg/ml for kanamycin and 10 µg of kanamycin/ml was used for *F. tularensis* LVS.

**Promoter trap library construction.** A plasmid comprising a promoterless fluorescent reporter, f8tdTomato, was first constructed by subcloning the tdTomato-containing BamHI/EcoRI fragment from pRSSET1tdTomato into pFNLT8 that had been digested by these same enzymes. To generate a promoter trap library, *F. tularensis* genomic DNA was partially digested with Sau3AI and genomic fragments, approximately 0.5 to 1.6 kb, were isolated from agarose gels. These fragments were cloned into pF8tdTomato that had been digested with BamHI and treated with alkaline phosphatase (40). Additionally, a different red fluorescent protein allele, mcherry (37), was used similarly for the construction of an alternative promoter trap library. The successful cloning of *F. tularensis* LVS fragments was confirmed by restriction analysis (data not shown). In addition, fluorescence microscopy analysis of *E. coli* harboring this promoter trap library
revealed the presence of fluorescent colonies on solid medium (see Fig. 1A), indicating the acquisition of additional DNA. We observed that approximately 11% of the E. coli colonies harboring this promoter trap library were fluorescent (data not shown). DNA sequencing of genomic fragments was conducted by the University of Pittsburgh core facility.

Promoter deletion mutagenesis. By using primers listed in Table 1, amplicons (containing truncated promoters-damodamo fusions) were generated by PCR to delete 5‘ portions (1,363, 1,595, 1,535, 1,491, 1,476, 1,466, or 1,228 bp) of the cloned F. tularensis LVS genomic fragment in pTC3D. In these PCRs, forward primers (t1_BamHI, t2_BamHI, t3_BamHI, t4_BamHI, SIGforward, and SIG-delforward) were all paired with the reverse primer Rev_EcoRI and pTC3D was used as a template. The PCR products were cloned into pGEM-T by the protocol of the manufacturer (Promega). Subsequently, the EcoRI/BamHI fragments containing the promoter-Δtmdo fusion were subcloned into pFNLT8 digested with these same enzymes, producing pTC3D1, pTC3D2, pTC3D3, pTC3D4, pSIG, and pSIGdel (Table 1). Restriction analysis and DNA sequencing were used to confirm plasmid construction.

Construction of pGRP and pGRPGlc-rev. With pTC3D as a template and primers GRP1_Sall and GRP2_NdeI, PCR was used to amplify the cloned F. tularensis LVS genomic fragment. The amplicon was cloned into pGEM-T by the protocol of the manufacturer (Promega). Subsequently, the NdeI/SalI fragment containing the F. tularensis LVS genomic DNA was subcloned into pFNLT8 that had been digested with these same enzymes, producing pGR. To generate pGRGlc-rev, iglC was amplified by PCR using IgC_Ndel and IgC_NotI as primers and LVS genomic DNA as a template. The amplicon was cloned into pGEM-T by the protocol of the manufacturer (Promega). The gglC-containing NdeI/Pell fragment of this vector and the NdeI/Ndel fragment of the pGEM-T containing the fluorescent-repressible promoter were ligated together. This step produced a construct in which the glucose-repressible promoter was fused with the antisense form of gglC. This fusion was subcloned into an F. tularensis shuttle vector by ligating the Sall/Not fragment containing the glucose-repressible promoter-antisense gglC fusion with pFNLT8 that had been digested with these same enzymes. This procedure produced pGRGlc-rev.

Electroporation for F. tularensis LVS. Plasmids were mobilized into F. tularensis by electroporation as previously reported (4). Briefly, F. tularensis LVS Trypticase soy broth cultures (approximately 50 ml) supplemented with 0.1% cysteine were grown to an OD600 of at least 0.4, washed three times with 500 mM sucrose, and resuspended in 4 ml of 500 mM sucrose. For electroporation, 1 µl (approximately 1 µg) of plasmid DNA was mixed with 50 µl of electrocompetent cells and the mixture was pulsed in a 0.2-cm-gap cuvette (Bio-Rad) at 2.5 kV, 150 µF, and 25 µF. Immediately after, cells were resuspended in 1 ml of Trypticase soy broth supplemented with 0.1% cysteine and grown at 37°C with shaking at 250 rpm for 1 to 4 h before selection on cysteine heart agar or chocolate II agar plates supplemented with kanamycin.

Fluorescence detection and quantification. A fluorometric analysis of F. tula-
rensis LVS broth cultures was performed using a Molecular Dynamics M2 plate reader to evaluate the expression of Δltdo (at a 553-nm excitation wavelength and a 585-nm emission wavelength) (37). Fluor emission with three independent cultures in 96-well opaque, clear-bottom plates were measured in triplicate, and growth differences were normalized using OD600 values.

Flow cytometry. Actively growing F. tularensis LVS broth cultures, diluted to an OD600 of 0.3, were resuspended in phosphate-buffered saline (PBS) containing 200 µg of gentamicin/ml and incubated at 25°C for 15 min prior to flow cytometry analysis. The preservation of bacterial cell integrity and the mainte-
nance of fluorescence following gentamicin treatment were assessed by fluores-

cence microscopy (data not shown). Fluorescence measurements for individual bacteria were collected, as previously described (41), with a FACSCalibur cyto-
tometer equipped with an argon laser (excitation wavelength, 488 nm) using the FL2 photomultiplier tube (band-pass filter, 585 nm). The analysis and quantitation of fluorescence were performed with CellQuest software (Beckton Dickin-
son).

Macrophage infection and fluorescence microscopy. Human macrophages were differentiated from monocytes by in vitro culture as previously described (5). Briefly, monocytes were purified from human buffy coats from blood dona-
tions (Central Blood Bank, Pittsburgh, PA) using Ficoll gradients (Amersham Biosciences) to isolate peripheral blood mononuclear cells, Optiprep gradients (Axis-Shield) to enrich for monocytes, and passing on plastic to further purify monocytes (final purity, >95% based on microscopy). Cells were cultured in 60-mm-diameter culture dishes for 7 days at 37°C with 5% CO2 in 7 ml of Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 20% fetal calf serum (Invitrogen), 10% human serum (Gemini Biosciences), 25 mM HEPES ( Gibco), and 1% GlutaMAX (Invitrogen). On day 7, macrophages were removed from the culture dish by using a lidocaine-EDTA solution (5 mM EDTA and 4 mg of lidocaine/ml in PBS, pH 7.2). Cells were washed and resuspended in Dulbecco’s modified Eagle’s medium supplemented with 1% human serum, 25 mM HEPES, and 1% GlutaMAX and then plated onto Lab-
Tec II eight-well chamber slides at a density of 5.0 × 104 to 1.0 × 105 cells per well. Actively growing bacterial cultures were adjusted to an OD600 of 0.3 (ap-
proximately 8.0 × 107 CFU/ml) and diluted to achieve a multiplicity of infection (MOI) of 500, which typically yields an infection rate of >98% (5, 23). Actual MOIs were measured by plating serial dilutions onto chocolate II agar plates. Macrophages were exposed to this MOI for 2 h at 37°C with 5% CO2 and then incubated with Hanks balanced salt solution (Gibco) containing gentamicin (20 µg/ml) for 20 min to kill extracellular bacteria. The cells were washed three times with warm Hanks balanced salt solution and then incubated at 37°C with 5% CO2 for another 22 h with fresh culture medium. The macrophages were viewed under a Zeiss Axiosvert 200 microscope at 24 h postinfection. All images were captured at a magnification of ×200 with equal fluorescence exposure times. All use of human-derived cells was approved by the University of Pittsburgh Insti-
tutional Review Board.

For the competition studies (10), human primary macrophages or murine RAW 267.4 macrophages were infected as described above. Macrophages were cultured with an ~1:1 mixture (determined by the OD600 of LVS/pGRP and LVS or LVS/pGRPGlc-rev and LVS, yielding a total MOI of 500. Twenty-four hours following infection, macrophages were lysed with 0.02% sodium dodeyl sulphate and lysates were serially diluted and subsequently plated onto chocolate II agar plates with or without kanamycin for CFU analysis. The number of vector-harboring bacteria (those on plates with kanamycin) were subtracted from the number of total bacteria (those on plates without kanamycin) to determine the amount of viable wild-type LVS bacteria. Competition ratios (ratios of vector-harboring bacteria to the respective wild-type bacteria) were normalized to the ratio for the control infections (pGRP-harboring bacteria to LVS bacteria) to account for small differences in the inocula, as has been done previously (10).

Knockdown experiment. F. tularensis LVS/pGRPGlc-rev or F. tularensis LVS/ pGRP was streaked onto cysteine heart agar plates consisting of cysteine heart broth supplemented with 2% agar, 5% rabbit blood, 1% Glc or Suc, and 10 µg of kanamycin/ml. Following incubation, bacteria were harvested in PBS and the cell concentration was normalized by the OD600. Cell suspensions were sonicated (three 5-s bursts) and mixed with 5% β-mercaptoethanol and NuPage LDS sample buffer (Invitrogen) to a 1× concentration. This material was boiled for 10 min, separated on a 12% acrylamide–Tris–glycine gel, and electroblotted onto nitrocellulose paper. The nitrocellulose was blocked using 0.5% bovine serum albumin, 0.5% milk, and 0.025% sodium azide in PBS, pH 7.4. The membrane was probed with rabbit polyclonal antibody to heat shock protein 70 (GencTex) and mouse anti-IgC monoclonal antibody (Immuno-Precise Antibodies Ltd.). Bands were detected using peroxidase-conjugated anti-rabbit and anti-mouse antibodies (Sigma) and Amersham ECL kit Western blotting detection reagents (GE Healthcare). Bands were quantitated using Quantity One 4.4.0 software (Bio-Rad).

RESULTS

Isolation of F. tularensis LVS promoter elements. To identify F. tularensis promoter elements, we cloned random genomic fragments upstream of a promotorless red fluorescent protein allele. The resulting plasmids were first introduced into E. coli, and transformants were analyzed for in vitro fluorescence by stereomicroscopy (Fig. 1A). We hypothesized that some of the cloned F. tularensis LVS DNA fragments containing promoter elements would be active in E. coli. In fact, colonies with a range of fluorescence levels, from bright fluorescence to undetectable levels, were observed (Fig. 1A). Insert DNA from six plasmids that contained functional promoter elements was sequenced, revealing the chromosomal locations of the cloned fragments (Fig. 1B). Together, these data validated the con-
struction of this promoter trap library.

One of the cloned promoter elements containing an active promoter in E. coli consisted of genetic material from F. tula-
rensis LVS that was upstream of a putative sugar transporter gene (Fig. 1B, panel 6) (pTC3D). This cloned fragment was ∼1,700 bp in length, containing all of FTL_0579 and a portion

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of FTL_0580, which encode a putative nicotinic acid phosphoribosyltransferase and a conserved hypothetical protein, respectively (35). Because this region originated upstream of a putative sugar transporter gene, FTL_0581 (Fig. 1B, line 6), we hypothesized that the presence or absence of a specific carbohydrate influenced its regulation in *Francisella*, raising the possibility that its activity could be manipulated.

**Promoter characterization.** To assess the regulation of the promoter region cloned into pTC3D, we introduced this construct into *F. tularensis* LVS by electroporation. *F. tularensis* LVS/pTC3D, *F. tularensis* LVS/pF8tdTomato (carrying promoterless tdTomato), or *F. tularensis* LVS alone was grown in cysteine heart broth supplemented with an additional carbon source (1% [wt/vol]) (Fig. 2A). This medium was used because, unlike the chemically defined medium for *Francisella* (6), cysteine heart broth will support growth when supplemental Glc is removed or replaced with other carbon sources (data not shown). We selected a range of carbon sources reflecting common carbohydrate medium supplements, in addition to alternative metabolites (glycine and citric acid) (Fig. 2A). Cultures were analyzed for fluorescence by using a plate reader where relative intensity was normalized to the OD_{600}. There were high levels of fluorescence produced by LVS/pTC3D in the presence of all supplemental sugars except Glc (Fig. 2A). In addition, strong fluorescence was observed when the medium was supplemented with only citric acid or glycine (Fig. 2A). This result suggested that expression from the pTC3D promoter element was inhibited by the presence of Glc or that all other supplemental carbon sources used induced expression (Fig. 2A). To clarify this point, we assessed the production of...
fluorescence from cultures grown in cysteine heart broth without carbohydrate supplementation. Here, LVS/pTC3D produced high levels of fluorescence similar to those shown in Fig. 2A for cultures supplemented with carbon sources other than Glc (data not shown). Importantly, the control vector (pF8tdTomato), comprising tdTomato with no upstream promoter element, produced no fluorescence, like Francisella LVS alone (Fig. 2A). These data confirmed that the chromosomal fragment cloned upstream of tdTomato in pTC3D (Fig. 1B) was responsible for the fluorescence produced as indicated in Fig. 2A.

To validate the fluorescence data seen in Fig. 2A, we analyzed F. tularensis/pTC3D cultures supplemented with Glc or Suc by flow cytometry (Fig. 2B). Suc was selected for this experiment as this carbohydrate is common and inexpensive and it supported strong fluorescence in the previous experiment (Fig. 2A). Flow cytometry indicated that individual bacteria were more fluorescent when cultured with Suc rather than Glc (Fig. 2B). Data here are consistent with the normalized fluorescence readings and observations presented in Fig. 2A.

We wanted to verify the observed Glc repression of the promoter cloned into pTC3D. Therefore, bacteria were cultured with various concentrations of Glc and Suc (Fig. 3). As Francisella medium typically contains 1% Glc (wt/vol), we used 1% (wt/vol) as the total sugar concentration for these experiments. High levels of fluorescence were observed when bacteria were cultured in 0.1% Glc–0.9% Suc (Fig. 3). However, fluorescence reached background levels when the Glc concentration was increased to 0.25% (with 0.75% Suc) (Fig. 3). Together with the data indicating that strong fluorescence was observed in the absence of a supplemental carbohydrate in the medium, these data support the conclusion that Glc represses the expression of the promoter upstream of tdTomato in pTC3D (Fig. 3). Moreover, these data indicate that Glc repression occurs at Glc levels greater than or equal to 0.25% (wt/vol) (Fig. 3). The trapped Francisella glucose-repressible promoter of pTC3D is referred to as FGRp throughout the remainder of this paper.

Minimal levels of fluorescence in F. tularensis LVS/pTC3D cultured in Glc were detected (Fig. 2 and 3), due likely to promoter leakiness. We wanted to determine if this expression could be diminished by adding more Glc. Similar levels of fluorescence from F. tularensis LVS/pTC3D cultures grown in cysteine heart broth supplemented with 0.25 and 1% Glc (Fig. 2 and 3) and 5 and 10% Glc (data not shown) were observed, however. These results indicate that maximal repression is achieved at Glc levels greater than 0.25%.

To determine if the phenomenon of the repression and induction of gene expression controlled by FGRp was reversible, we inoculated Suc-supplemented broth with bacteria raised in the presence of Glc. Conversely, F. tularensis LVS/pTC3D grown in Glc-containing medium was used to inoculate broth supplemented with Suc (Fig. 4). Fluorescence and OD readings were obtained at the postinoculation time points indicated in the figure. The fluorescence intensity of F. tularensis LVS/pTC3D following the shift from Suc to Glc decreased steadily until 3.5 h postinoculation (Fig. 4). After this time point, fluorescence intensity plateaued until the experiment was terminated (Fig. 4). The remaining fluorescence seen may have been the result of a combination of promoter leakiness and remnant tdTomato protein, since this protein has been shown to be extremely stable (37). F. tularensis LVS/pTC3D that had been shifted from Glc-containing medium to broth supplemented with Suc increased in fluorescence after 3.5 h (Fig. 4). This was the same time point at which repression was complete when F. tularensis LVS/pTC3D was shifted from Suc to Glc-containing medium (Fig. 4). Fluorescence was not detected in bacteria harboring vectors with promoterless tdTomato in either medium (Fig. 4). These data indicate that the activation of repressed FGRp was possible following the removal of Glc from the medium and that the repression of
formerly activated FGRp was possible following culturing in Glc (Fig. 4).

Together, the Glc-Suc expression data shown in Fig. 2, 3, and 4 indicate that FGRp possesses a regulatory mechanism that can be manipulated by researchers. With these data, we investigated potential uses of this promoter element in Francisella research applications.

Application of FGRp. *F. tularensis* replicates within macrophages, and thus, many researchers choose to study this pathogen within this intracellular environment. Therefore, we tested FGRp activity inside macrophages. Human monocyte-derived macrophages were infected with strain LVS/pTC3D or LVS/pF8tdTomato (control vector) grown in medium supplemented with Glc. Macrophages were analyzed by fluorescence microscopy 24 h following infection (Fig. 5A). Strong fluorescence was observed in macrophages infected with *F. tularensis*/pTC3D, whereas no fluorescence from bacteria harboring the promoterless control vector (*F. tularensis*/pF8tdTomato bacteria) was visible. (B) *F. tularensis* LVS/pTC3D was cultured in medium supplemented with 1% Suc or 1% Glc and observed by fluorescence microscopy in parallel with the macrophage infections. Data shown are representative of three experiments.

![FIG. 5. FGRp expression is induced in macrophages. (A) Cells were analyzed by bright-field (BF) and fluorescence (Fluor.) microscopy at 24 h postinfection. All images were captured at a magnification of ×200 with equal fluorescence exposure times. Strong fluorescence was produced by *F. tularensis*/pTC3D, whereas no fluorescence from bacteria harboring the promoterless control vector (*F. tularensis*/pF8tdTomato bacteria) was visible. (B) *F. tularensis* LVS/pTC3D was cultured in medium supplemented with 1% Suc or 1% Glc and observed by fluorescence microscopy in parallel with the macrophage infections. Data shown are representative of three experiments.](http://aem.asm.org/)

We wanted to ensure that the fluorescence observed within macrophages was due to the induction of *tdTomato* and not simply background expression from promoter leakiness, as observed in bacteria cultured in Glc (Fig. 2, 3, and 4). To test this, we simultaneously cultured strains LVS/pTC3D and LVS/pF8tdTomato in cysteine heart broth containing 1% Glc or 1% Suc for 24 h during the macrophage infection. These broth cultures were analyzed by fluorescence microscopy along with the infected macrophages (Fig. 5B). The only broth cultures that contained visibly detectable fluorescent bacteria were those containing Suc (Fig. 5B), consistent with our previous data (Fig. 2 to 4).

A controllable promoter can be used to generate antisense RNA (8). In other bacteria, including *E. coli* (31), *Helicobacter pylori* (8), *Staphylococcus aureus* (20), and *Mycobacterium smegmatis* (29), the induction of antisense RNA has been used previously to knock down specific gene expression. We therefore hypothesized that the production of antisense RNA by FGRp should reduce the amount of the corresponding protein product in the absence of Glc in *F. tularensis* LVS. However, when Glc is present, wild-type protein levels should be observed. We therefore generated pGRPiIgC-rev, in which the reverse complement of *iglC* (intracellular growth locus C, a gene necessary for growth in macrophages [2, 14, 15, 23, 28]) was cloned behind FGRp in an *F. tularensis* shuttle vector. When bacteria harboring this vector were cultured in medium containing Glc, IgIC levels were comparable to those in bacteria harboring the control vector (pGRP) as determined by
mixture of either LVS/pGRPiC-rev and LVS or LVS/pGRP and LVS as a control. Twenty-four hours following infection, there was a >3-fold reduction of viable LVS/pGRPiC-rev bacteria compared to the level of wild-type *F. tularensis* LVS bacteria in both human primary and murine RAW macrophages (Fig. 6B). This result confirmed that FGRp was active during macrophage infection and demonstrated a functional application of this promoter.

**Identification of a minimal fragment of FGRp.** As FGRp in pTC3D is located within a ~1,700-bp region, it is likely that much of this DNA is unimportant for the Glc repression and gene regulation observed. To identify the essential components of this promoter, we deleted segments of FGRp, generating promoter-*tdTomato* fusions in which the promoter-harboring segment was reduced to roughly 50, 100, 200, or 400 bp (Fig. 7A and B). These fusions, designated pTC3Dt1, pTC3Dt2, pTC3Dt3, and pTC3Dt4, respectively (Fig. 7A and B), were introduced into *F. tularensis* LVS by electroporation. *F. tularensis* LVS strains harboring these vectors were grown in medium supplemented with either Glc or Suc. Fluorescence was not detected from *F. tularensis* LVS harboring pTC3Dt1 or pTC3Dt2 or from controls (LVS or LVS/pF8tdTomato) in medium containing either carbohydrate (Fig. 7C). A modest amount of fluorescence was detected from *F. tularensis* LVS/pTC3Dt3 when this strain was cultured in Suc, and the fluorescence subsided during growth in Glc (Fig. 7C). This result suggested that pTC3Dt3 may be lacking a DNA sequence essential for full transcription. However, pTC3D and pTC3Dt4 produced comparable fluorescence levels in Suc while showing similar repression levels in Glc. This finding suggested that pTC3Dt4 contained all of the FGRp components necessary for full functionality (Fig. 7C). Moreover, the functional nucleotides of FGRp on the LVS chromosome are directly upstream of and adjacent to FTL_0580 (Fig. 7). This arrangement indicates that this promoter controls the expression of FTL_0580 (Fig. 7), which encodes a hypothetical protein (Fig. 1B).

An analysis of the FGRp sequence near and upstream of the truncation of pTC3Dt3 with the BPROM program (Softberry) revealed the presence of putative ~10 and ~35 sites (Fig. 7B). Therefore, we deleted portions of FGRp in pTC3D to test their importance. pSIG, which contains the FGRp sequence through the putative ~10 and ~35 sites, produced fluorescence levels similar to those produced by pTC3D (Fig. 7D), suggesting that pSIG contained the complete functional promoter. The deletion of the putative ~10 and ~35 sites (pSIGdel) produced only background levels of fluorescence, indicating that this segment of DNA is critical for the transcriptional activation of FGRp. This result indicated the DNA sequence that is necessary for optimal transcription to be within the 44 base pairs between the deletions of pSIG and pSIGdel (Fig. 7B). Interestingly, the low-level fluorescence produced by LVS/pSIGdel and LVS/pTC3Dt3 (Fig. 7C and D) was detected only when these bacteria were cultivated in Suc, suggesting that these constructs may also contain Glc repressor and alternative ~10 and ~35 sites.

**DISCUSSION**

Previous work characterizing the *groEL*, *acpA*, and FTN_1451 *Francisella* promoters has been crucial for the construction of
shuttle plasmids, transposon systems, and suicide constructs (11, 12, 25, 26, 34), all essential tools for bacterial geneticists. Although these tools are indispensable, the potential application of these promoters is limited since the regulation of the promoters is undefined, prohibiting researchers from controlling the conditional expression of downstream genes. In this work, we have described a *Francisella* promoter, FGRp, whose expression is repressed by Glc (Fig. 2, 3, and 7). The application of these promoters is limited since the regulation of the promoters is undefined, prohibiting researchers from controlling the conditional expression of downstream genes. In this work, we have described a *Francisella* promoter, FGRp, whose expression is repressed by Glc (Fig. 2, 3, and 7). The application of these promoters is limited since the regulation of the promoters is undefined, prohibiting researchers from controlling the conditional expression of downstream genes. 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In this work, we have described a *Francisella* promoter, FGRp, whose expression is repressed by Glc (Fig. 2, 3, and 7). The application of these promoters is limited since the regulation of the
cation of this carbohydrate-regulated promoter will allow researchers to control the expression of exogenous genes. In cultures supplemented with Suc, rather than Glc, we utilized this promoter to drive the expression of a red fluorescent protein allele (tdTomato), as well as antisense RNA of iglC, an important *F. tularensis* virulence factor (2, 13–15, 23, 36). Upon cultivation in Glc, FGRp-controlled gene expression was reduced to background levels in *tdTomato* reporter assays (Fig. 2, 3, 4, and 7) and antisense iglC constructs did not reduce target protein levels (Fig. 6A). Along with defining the regulation of FGRp by the presence or absence of Glc, these data show that antisense RNA is capable of reducing target protein expression levels in *F. tularensis* LVS. The strategy of generating isogenic knockdown mutants rather than knockout mutants may be more desirable when there is more than one chromosomal copy of a gene, such as iglC and others carried on the *Francisella* pathogenicity island. The FGRp-mediated expression of antisense iglC during a macrophage expression assay reduced the fitness of *F. tularensis* LVS (Fig. 6B). However, the attenuation of the iglC knockdown strain was less severe than that of previously described chromosomal mutants (14, 15, 23). This is probably because minute levels of IgIC still remained after the production of antisense iglC (Fig. 6A).

FGRp may also be useful to generate strains that are deficient in the expression of genes essential to central metabolism, DNA replication and repair, and transcription-translation which may be of interest in drug discovery. The controllable promoter presented here would allow such genes to be identified quickly by the production of an antisense knockout library (17).

As many other conditional expression systems, FGRp does have shortcomings. For example, the alteration of Glc levels may disrupt critical metabolic and central cellular processes. Also, stable proteins may persist well after the addition of Glc in the antisense system (Fig. 4). Finally, we showed this promoter’s controllable repression and expression in cell lines heart broth base medium. The possibility remains that under other conditions, additional signals may contribute to the regulation of FGRp. Nevertheless, in the field of *Francisella* genomics, information on functional promoters is limited. Thus, the FGRp system is a useful molecular tool under the culture conditions described here.

A deletion analysis indicated that the sites essential for FGRp activity are within a 44-bp region and that this sequence regulates the expression of a gene (FTL_0580) encoding a hypothetical protein (Fig. 7). Interestingly, the gene directly downstream (150 bp) of this open reading frame (FTL_0581) is in the same orientation and encodes a putative sugar transporter (Fig. 1B, panel 6). Based on the regulation of FTL_0580 and the putative function of FTL_0581, it is possible that these two genes are in an operon. The putative protein encoded by FTL_0580, DUF465 (PF04325), belongs to the Pfam family of proteins, which includes homologous conserved hypothetical proteins, as well as the heavy chains of eukaryotic myosin and kinesin (18). Although its definitive function remains unknown, the activity of the FTL_0580 promoter under low-Glc conditions provides a starting point for future studies of glucose-regulated gene expression. It will be of further importance to determine if the Glc-dependent regulation seen here was due to the central regulator of carbohydrate metabolism, referred to as carbon catabolite repression (42), or an independent process (9).

The Glc concentration is an important signal for virulence factor expression in many bacterial systems (9). For example, a number of virulence genes of *Listeria monocytogenes*, such as *hly* and *plcA*, that are controlled by the transcription activator PrfA are repressed by Glc (9). In addition, the expression of several virulence determinants of *Streptococcus pyogenes* rely upon MgA (9). The promoter of *mga* is downstream of an operator site that is the target for the carbon catabolite repressor complex. It remains to be determined if low Glc levels cue *F. tularensis* to alter the expression of virulence factors following entry into host cells. The results presented here indicated that FGRp was active in macrophages, suggesting that FTL_0580 would also be expressed in this environment, due likely to the limitation of Glc. Future studies should focus on determining the contribution of FTL_0580 to infection, in addition to the global response of *F. tularensis* to low Glc.

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REFERENCES