



Utilization of a tetracycline-inducible system for high-level expression of recombinant proteins in *Francisella tularensis* LVS

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ARTICLE INFO

Keywords:

Francisella tularensis
Expression plasmid
ATc inducible
Regulated *bfr* promoter

ABSTRACT

Francisella tularensis is a Gram-negative intracellular pathogen causing tularemia. A number of its potential virulence factors have been identified, but their biology and functions are not precisely known. Understanding the biological and immunological functions of these proteins requires adequate genetic tools for homologous and heterologous expression of cloned genes, maintaining both original structure and post-translational modifications. Here, we report the construction of a new multipurpose shuttle plasmid – pEVbr – which can be used for high-level expression in *F. tularensis*. The pEVbr plasmid has been constructed by modifying the TetR-regulated expression vector pEDL17 (LoVullo, 2012) that includes (i) a strong *F. tularensis* *bfr* promoter, and (ii) two *tet* operator sequences cloned into the promoter. The cloned green fluorescent protein (GFP), used as a reporter, demonstrated almost undetectable basal expression level under uninduced conditions and a highly dynamic dose-dependent response to the inducer. The utility of the system was further confirmed by cloning the *gapA* and *FTT_1676* genes into the pEVbr vector and quantifying proteins expression in *F. tularensis* LVS, as well as by studying post-translational modification of the cloned genes. This study demonstrates that high levels of recombinant native-like *Francisella* proteins can be produced in *Francisella* cells. Hence, this system may be beneficial for the analysis of protein function and the development of new treatments and vaccines.

1. Introduction

Francisella tularensis is a highly infectious, Gram-negative intracellular pathogen causing the disease tularemia (Sjöstedt, 2007). A large number of virulence factors helping *F. tularensis* to infect hosts and avoid their immune defenses have been identified and characterized (Baron and Nano, 1998; Nano et al., 2004; Rowe and Huntley, 2015). Nevertheless, many of these proteins have unknown functions and no homology to known proteins from other bacteria. The lack of suitable genetic systems for controlled gene expression in *F. tularensis* has been a gap in this field of research. To date, just two controllable *Francisella* expression systems have been developed. One of these is a glucose-repressible system based on a *Francisella* promoter (FGRp) that is repressed by glucose and is active in macrophages (Horzempa et al., 2008). The other system was generated as an inducible and repressible gene expression system for *F. tularensis* based on the Tet repressor, TetR (LoVullo et al., 2012). This system allows inducible gene expression with the addition of the inducer anhydrotetracycline (ATc) and has been

shown to be functional in both broth and cell culture.

The aforementioned systems nevertheless exhibit limitations in the production of native proteins that preserve the original structure and modifications in amounts necessary for immunization or structural studies. High-level expression can be achieved in a system with a strong promoter and low level of basal expression due to tight regulation. We aimed to develop novel expression vectors enabling efficient protein overproduction in *F. tularensis*. Proteins that are identical to their native counterparts in great abundance are essential for structural and immunological studies, and this is especially true for membrane proteins, which play important roles in cellular functions (Pandey et al., 2016). Heterologous expression of membrane proteins is not always feasible because of toxicity to the host upon overexpression and often this is very difficult, if not impossible, due to differences in codon usage. Moreover, we are interested in maintaining a protein's post-translational modifications (PTMs), which are important for protein immunological and regulatory properties (Liu et al., 2016). Such PTMs, in particular, phosphorylation, glycosylation, acetylation, and prenylation have been

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<https://doi.org/10.1016/j.plasmid.2021.102564>

Received 26 June 2020; Received in revised form 14 January 2021; Accepted 27 January 2021

Available online 18 February 2021

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identified in *F. tularensis* (Balonova et al., 2010; Marakasova et al., 2020). Recombinant proteins produced in the commonly used host *E. coli* did not, in most cases, have these modifications and therefore their functions could be affected (Jia et al., 2016).

Our early attempts to modify well studied and commonly used sugar-responsive expression systems (such as xylose and rhamnose, which are functional in other Gram-negative bacteria) were unsuccessful in *F. tularensis*.

Previous work has demonstrated that the widespread *tet* regulatory system from *E. coli* could be successfully adapted for *F. tularensis* and is functional in bacteria grown both in culture medium and in eukaryotic cells (LoVullo et al., 2012). It has been used to study controlled virulence factors expression (Jia et al., 2016; Brodmann et al., 2018), as well as for gene silencing (LoVullo et al., 2012) and for mutant complementation (Moreau et al., 2018). We decided to adapt an existing TetR-regulated system to achieve a high level of protein expression in *Francisella*. The primary factor in constructing an expression system is the choice of a strong transcriptional promoter, because it is mainly linked to the expression levels of proteins (Sørensen and Mortensen, 2005; Bertram and Hillen, 2008). As has been shown in studies on the development of *tet* regulatory systems in other bacteria, such as *Mycobacterium* (Ehrt et al., 2005), *Bacillus subtilis* (Geissendörfer and Hillen, 1990), *Borrelia* (Whetstone et al., 2009), and *Helicobacter pylori* (Debowski et al., 2013), a strong host promoter is required for both fully fledged regulation and proper expression of a target gene. Such strong promoters should be tightly controlled in order to titrate protein production and in this manner prevent any harm and burden to cells which could be caused by gene expression greater than at its physiological level.

Relatively little is known about the nature of promoters in *F. tularensis*. Several strong promoters have previously been described and shown to be useful as genetic tools (Horzempa et al., 2008). The *rspL* promoter was used to generate systems for stably integrating genetic elements in single-copy into the *F. tularensis* genome (LoVullo et al., 2009). The expression of *iglE* in *trans* from *attTn7* under the control of the *Francisella* *rpsL* promoter showed that *IglE* is required for intramacrophage replication of strain Schu S4 in BMMs (bone marrow-derived macrophages) and of LVS in J774.A1 macrophage-like cells (Robertson et al., 2013). Another functional *Francisella* promoter is the *acp* promoter of the acid phosphatase gene, which effectively drives the expression of plasmid-carried *gfp* in *F. tularensis* LVS after a murine macrophage infection (Maier et al., 2004). The promoter *groESL* is the most-utilized and a well-studied promoter (Ericsson et al., 1997). It drives strong gene expression both *in vivo* and *in vitro* and has demonstrated effectiveness for the expression of exogenous genes such as GFP and antibiotic resistance genes as well as endogenous genes *in trans* (Maier et al., 2006; Gallagher et al., 2007; Kravchenko et al., 2007; Su et al., 2007). The TetR-based gene regulation systems for *F. tularensis* mentioned above also uses a modified *groESL* promoter to drive inducible or repressible gene expression (LoVullo et al., 2012).

Another *F. tularensis* promoter that has been characterized in detail is the strong *bfr* promoter, which drives expression of bacterioferritin in *Francisella* (Zaide et al., 2011). Previously, the *bfrA* promoter was used to overexpress various FPI (*Francisella* pathogenicity island) proteins and showed stronger promoter activity compared to *omp* and *groESL* promoters (Jia et al., 2016).

The *bfr* promoter was employed for complementation of the avirulent *pdpC* knockout strain *F. tularensis* Schu with wild-type *pdpC*, where it drove *PdpC* expression from a plasmid (Uda et al., 2014). The model antigen ovalbumin (OVA) in *F. tularensis*, when expressed under *bfr* promoter in *F. tularensis* allowed for the study of CD8⁺ T cell responses to the bacterium (Place et al., 2017). A dual reporter-labeled *F. tularensis* live vaccine strain for cellular and whole body imaging was successfully established using conjugated *gfp* and *lux* genes under the control of the bacterioferritin promoter (Kim et al., 2018).

Because the bacterioferritin promoter (*Pbfr*) has been identified as the most efficient *F. tularensis* promoter and demonstrated to be more

potent than the other *Francisella* promoters (Zaide et al., 2011), we combined one of the strongest *F. tularensis* promoters and a tetracycline-regulated system to generate a new expression vector. The function of the novel *bfr/tetO* promoter was demonstrated by cloning superfolder GFP (sfGFP) as a reporter and verifying its fluorescent signal after induction. Using this vector, two *Francisella* proteins with different cell localization and structure were expressed and efficiently purified from *F. tularensis* LVS lysates in soluble form using immobilized metal affinity chromatography (IMAC). In this work, we report the construction and analysis of an expression system that can be exploited to produce recombinant native-like *Francisella* proteins in sufficiently high levels.

2. Materials and methods

2.1. Bacterial strains and growing conditions

Strains and plasmids used in this study are detailed in Table 1. *Escherichia coli* strain HST08 (Stellar™; TaKaRa) was used for routine cloning procedures and was grown in selective Luria–Bertani broth or on solid plates (Sambrook and Russell, 2001). Hygromycin (200 µg/ml) was added to the medium where needed. Expression of recombinant proteins was effected in *F. tularensis* LVS. *F. tularensis* LVS was routinely cultivated on McLeod agar enriched with bovine hemoglobin (Becton Dickinson) and IsoVitalEx (Becton Dickinson). Electrocompetent *F. tularensis* cells were prepared and electroporated with plasmid DNA according to established protocols (LoVullo et al., 2006). Transformants were selected on McLeod agar enriched with bovine hemoglobin (Becton Dickinson) and IsoVitalEx (Becton Dickinson) and supplemented with hygromycin B (Hyg; Carl Roth, Karlsruhe, Germany). Chamberlain's selective liquid medium (CHI; Chamberlain, 1965) and brain-heart infusion (BHI) medium supplemented with 0.1% cysteine (Sigma–Aldrich) were inoculated with *F. tularensis* LVS colonies containing plasmids and incubated overnight at 37 °C and 200 rpm. Overnight cultures were pelleted and diluted in fresh medium at OD₆₀₀ = 0.2 and grown 24 h after induction. To induce expression, anhydrotetracycline hydrochloride (ATC; TaKaRa) was added at the concentrations stated when cultures reached OD₆₀₀ = 0.5–0.6. All cultures were grown aerobically at 37 °C and hygromycin B (Hyg; Roth) was added at 200 µg/ml

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Description	References/source
Strains		
<i>E. coli</i>	<i>F⁻, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, φ80d lacZΔ M15, Δ (lacZYA -argF) U169, Δ (mrr -hsdRMS -mcrBC), ΔmcrA, λ-</i>	TaKaRa
Stellar™		
<i>F. tularensis</i>		
LVS	<i>F. tularensis</i> subsp. <i>holarctica</i> live vaccine strain	American Type Culture Collection, ref. no. ATCC29684
pScherry-3	Ap ^R , source of Cherry tag	Delphi Genetics
pET28-1368	Km ^R , source of FTT_1368-HIS	Pavkova et al., 2017
pET28-1676	Km ^R , source of FTT_1676-HIS	Unpublished
pET28-sfGFP	Km ^R , source of sfGFP	Unpublished
pEDL17	Hyg ^R ; FTRp-mOrange2, <i>rpsLp-tetR</i>	LoVullo et al., 2012
pKK289	Km ^R , <i>E. coli</i> / <i>F. tularensis</i> shuttle vector	Bönquist et al., 2008
KmGFP	<i>PgroES</i> -GFP	
pKK-Pbfr-Cherry	Km ^R , <i>Pbfr</i> -Cherry	This work
pEPbr	Hyg ^R ; <i>Pbfr</i> -Cherry	This work
pEVbr-Cherry	Hyg ^R ; <i>Pbfr/tetO</i> -Cherry	This work
pEDL-1368	Hyg ^R ; FTRp-FTT_1368, <i>rpsLp-tetR</i>	This work
pEDL-1676	Hyg ^R ; FTRp-FTT_1676, <i>rpsLp-tetR</i>	This work
pEDL-sfGFP	Hyg ^R ; FTRp-sfGFP, <i>rpsLp-tetR</i>	This work
pEVbr-sfGFP	Hyg ^R ; <i>Pbfr/tetO</i> -sfGFP, <i>rpsLp-tetR</i>	This work
pEVbr-1368	Hyg ^R ; <i>Pbfr/tetO</i> -FTT_1368, <i>rpsLp-tetR</i>	This work
pEVbr-1676	Hyg ^R ; <i>Pbfr/tetO</i> -FTT_1676, <i>rpsLp-tetR</i>	This work

All plasmids generated and used in this study are derivatives of pKK289KmGFP (Bonquist et al., 2008) and pEDL17 (LoVullo et al., 2012), which replicate autonomously in both *E. coli* and *F. tularensis*. Plasmids for expression of exogenous sfGFP and *Francisella tularensis* Schu4 endogenous FTT_1368 and FTT_1676 proteins under the control of *Francisella* tetracycline-regulated promoter (*FTRp*) were constructed using the pEDL17 vector, a kind gift from Tom Kawula (Washington State University). DNA was amplified by PCR using vectors pET28-FTT_1368, pET-FTT_1676, and pET-sfGFP as templates. Genes *FTT_1368* and *FTT_1676* encoding a His-tag on the C-end were inserted into pEDL17 digested with *Mlu*I and *Acc*65I restriction enzymes by the In-Fusion cloning method (Supplementary Fig. 1A, 1B). The *sfGFP* gene was cloned into *Mlu*I-NheI restriction sites of pEDL17 in the same way (Supplementary Fig. 1C).

2.2.2. Creating plasmid pEVbr

To generate the expression vector pEVbr, we used the plasmid pEDL17 as the backbone. We replaced the promoter *FTRp* with the

[illegible]

tetracycline-regulated *bfr/tetO* promoter. First, the amplified promoter region of the *bfrA* gene from *F. tularensis* LVS and a PCR fragment of the Cherry tag from plasmid pScherry-3 were cloned into the *SpeI* and *EcoRI* restriction sites in pKK289KmGFP, resulting in pKKbfr-Cherry (Supplementary Fig. 2). The *tetO* operator sequence *tcctatcatgtgatagaga* (Table 2.) was then assembled from two complementary oligonucleotides, and subsequently inserted into the *SpeI* site in the *bfr* promoter region, and thus resulted in the pKK-Pbfr-Cherry plasmid. We used this vector as template for an amplification fragment containing both the *bfr/tetO* promoter and the Cherry tag. The generated PCR fragment was inserted into pEDL17 digested with *SbfI* and *KpnI*, thus resulting in the plasmid pEPbr. A second *tetO* sequence was placed immediately downstream from the predicted start of transcription within the *bfr* promoter as follows. First, the plasmid pEDL17 was linearized with restriction enzymes *NcoI* and *KpnI*. The plasmid pEPbr was used as template a PCR fragment with an *NcoI* flanking region and a sequence containing the start of transcription (TSS) and the new *tetO* sequence, as well as for a fragment including the first *tetO* sequence and *KpnI* flanking region. Finally, fragments were assembled into linearized pEDL17 using the In-Fusion HD Cloning Enzyme, thereby obtaining the pEVbr-Cherry plasmid. The Cherry reporter gene was then substituted with *sfGFP* (Supplementary Fig. 3) as follows. The modified *bfr* promoter with two *tetO* sequences and *sfGFP* was amplified by PCR using as template either pEVbr-Cherry or pET-sfGFP corresponding with primers containing 15 bp overlaps complementary to each other or to corresponding sequences on the pEDL17 vector and then inserted into *SbfI*- and *NheI*-digested pEDL17 using the In-Fusion HD Cloning Enzyme. The resulting plasmid was named pEVbr-sfGFP. Plasmids pEVbr-1368 and pEVbr-1676 carrying genes *FTT_1368* and *FTT_1676* were constructed in the same way, whereby the amplified gene and *bfr/tetO* promoter as described above were inserted into *SbfI*- and *NheI*- digested pEDL17. Plasmids pET28-FTT_1368 and pET28-FTT_1676 were used as the PCR templates.

2.3. GFP reporter assay

The fluorescence of cell cultures was measured with a FLUOstar OPTIMA microplate reader (BMG LabTech) using black microtiter plates (TPP Techno Plastic Products, Trasadingen, Switzerland). The excitation and emission wavelengths were set at 485 and 520 nm, respectively. Data were analyzed using the FLUOstar evaluation software (BMG Biotechnologies).

2.4. Protein purification

Colonies of *F. tularensis* LVS cells containing plasmids pEVbr-1368 and pEVbr-1676, respectively, were inoculated into BHI medium supplemented with 200 µg/ml hygromycin and incubated overnight at 37 °C and 200 rpm. Overnight cultures were pelleted and diluted in fresh medium at OD₆₀₀ = 0.2. Recombinant gene expression was induced by adding anhydrotetracycline (ATc) to a final concentration of 250 ng/ml to the cultures upon reaching OD₆₀₀ = 0.5–0.6. Bacteria were grown for 24 h after induction. Bacteria were harvested at 4 °C, pellets were lysed with xTractor Buffer (Clontech), and the recombinant His-tagged protein purification was performed using a HisTALON purification kit (Clontech) while following the manufacture's protocol. Briefly, 1.5 ml of TALON CellThru resin suspension was transferred into an Amicon® Pro device and equilibrated with Equilibration/Wash Buffer. Bacterial cells were lysed in xTractor buffer with gentle agitation at 8 °C for 30 min. Crude cell lysates were then loaded into columns and incubated at 8 °C for 20 min with mild shaking. Samples were next centrifuged for 10 min at 700 × g at 8 °C and the columns were washed twice by centrifugation with 10 ml of 1× Equilibration/Wash Buffer. Recombinant His-tagged proteins were eluted with Elution Buffer containing 150 mM imidazole. Concentrations of the proteins were determined using a BCA protein assay kit (Thermo Fisher Scientific), and purity of the proteins was confirmed by SDS-PAGE.

2.5. SDS-PAGE (1D gel electrophoresis) and immunodetection

For analysis of sfGFP and recombinant FTT_1368 and FTT_1676 protein production, *F. tularensis* was grown for 24 h and cells were harvested by centrifugation at 6500 rpm for 10 min at 4 °C. The cell pellets were washed twice with PBS, then resuspended in 3× SDS-PAGE sample loading buffer and incubated at 95 °C for 5 min. Proteins were separated by 12% SDS-PAGE electrophoresis and visualized by Coomassie blue staining. For immunoblot analysis, proteins were electrophoretically transferred from gels to PVDF membranes (Bio-Rad) using a Transblot-SD Semi-Dry Transfer Cell (Bio-Rad). Proteins FTT_1368 and FTT_1676 were detected using monoclonal anti-polyHistidine-HRP antibodies (Sigma-Aldrich). sfGFP protein was detected using recombinant rabbit monoclonal recombinant anti-GFP antibody (Abcam) as the primary antibody, and swine anti-rabbit IgG/HRP antibody (Dako) was used as the secondary antibody. Chemiluminescence was detected using a BM Chemiluminescence Blotting Substrate (POD) according to the manufacturer's instructions (Roche). Additionally, polyclonal rabbit anti-GapA antibody and FTT_1676 antibody (Apronex, Vestec, Czech Republic) were used for confirmation of protein production. Swine anti-rabbit IgG/HRP (Dako) was applied as the secondary antibody.

2.6. In-gel digestion of proteins and LC-MS analysis of protein glycosylation

The in-gel digestion and LC-MS analysis on an Ultimate 3000 RSLC nano interfaced to a Q-Exactive mass spectrometer (Thermo Fisher Scientific) was performed as previously described (Dankova et al., 2016) but with minor modifications.

The protein bands excised from a 1D-gel were destained, reduced with dithiothreitol, and the thiol groups alkylated with iodoacetamide. Digestion with trypsin (0.1 µg per protein band; Promega) was carried out in 50 mM ammonium bicarbonate at 37 °C for 17 h and then stopped by acidifying the samples with TFA. The samples were loaded onto a trap column, then separated using a 19 min linear gradient of B (4% to 36% B, 0.1% FA in 80% acetonitrile) at a flow rate of 0.3 µl/min on a PepMap RSLC C18, 2 µm, 100 Å, 0.075 × 150 mm analytical column. The full MS/Top10 data-dependent acquisition was used for identification of peptides and glycopeptides. Positive-ion full-scan MS spectra (*m/z* 350–1650) were acquired on a 1 × 10⁶ target ion population in the Orbitrap at resolution of 70,000 (at *m/z* 200). Precursor ions with charge state ≥2, minimum threshold intensity of 4 × 10⁴ counts, and not fragmented during previous 9 s were admitted for higher-energy collisional dissociation (HCD). Tandem mass spectra were acquired at resolution of 17,500 and with other parameters set to 1 × 10⁵ for AGC (automatic gain control) target value, 100 ms for maximum ion injection time, 27 for normalized collision energy, and *m/z* 120 for fixed first mass to allow for detection of diagnostic glycan-oxonium ions. For several glycopeptides that had been selected based on the glycan-oxonium ions pattern, the HCD spectra at stepped collision energy 14 and 24 were acquired to probe into the B- and Y-type glycosidic fragmentation. The spectra were acquired at resolution of 35,000, with AGC target value set to 2 × 10⁵ and 110 ms maximum ion injection time in this targeted analysis.

Peptides and proteins were identified by searching the raw files against the protein sequences database using the Mascot v2.4 search engine (Matrix Science) within Proteome Discoverer v2.4 (Thermo Fisher Scientific). The database contained protein sequences for the FTT_1676 and its C-term His6tag version, *F. tularensis* subsp. *holarctica* strain LVS (1678 sequences, Uniprot), and common contaminants (245 sequences, MaxQuant). The search parameters were as follows: trypsin was used to digest proteins, 2 missed cleavages were allowed, carbamidomethylation (C) was set as a fixed modification, oxidation (M) and deamidation (N, Q) were selected as variable modifications, and the mass tolerance of the precursor and fragment ions was set to 20 ppm and 0.02 Da, respectively. The peptide-modifying (N, S, T) glycan residues

"FtGlycan" (X P Hex (Balonova et al., 2012) HexNAc (Baron and Nano, 1998), $C_{43}H_{73}N_4O_{30}P$, monoisotopic mass 1156.40472) and "FtGlycan2" (Hex (Balonova et al., 2012) HexNAc (Baron and Nano, 1998), $C_{36}H_{59}N_3O_{25}$, 933.34377) with enabled neutral loss scoring were defined as additional variable modifications for glycopeptide identification. All tandem mass spectra were deisotoped and charge-deconvoluted using an IMP-MS2 Spectrum Processor node before searching. A false discovery rate of 0.01, estimated by processing of target-decoy results with the Qvalue algorithm, was used for accepting the identifications of the peptide groups and protein levels. The compositions of peptide-modifying glycans were concluded from manual interpretation of the glycopeptide HCD spectra, which were acquired using stepped collision energy (Supplementary Figs. 1–4). The mMass v5.5 software (Strohm et al., 2010) was used for manual interpretation and annotation of the glycopeptides spectra.

3. Results and discussion

3.1. Vector construction

We utilized one of the strongest known *F. tularensis* promoters and combined it with a tetracycline regulated system to generate a new expression vector. Plasmid pEVbr was designed based on the plasmid pEDL17 (LoVullo et al., 2012) by replacing the modified *groES* (*FTRp*) promoter in pEDL17 with the *bfr* promoter, which contained its own ribosome-binding site (RBS) and had one extra *tet* operator (Fig. 1). To achieve this objective, we inserted one *tetO* sequence directly at the TSS (transcription start) (Zaide et al., 2011) and one between the TSS and RBS. With this modification, we expected to achieve tight control over the *bfr* promoter while the tetracycline repressor (TetR) was constitutively expressed in the absence of inducer anhydrotetracyclin (ATc) (Bertram and Hillen, 2008; Debowski et al., 2013).

To assess the efficacy of the modified promoter and to examine its activation with ATc, we constructed the plasmid pEVbr-sfGFP using *sfGFP* as a reporter gene (Fig. 2A). sfGFP activity was quantified in *F. tularensis* LVS/pEVbr-sfGFP and cultivated in Chamberlain medium for 24 h after induction with 250 mM ATc. The strain exhibited bright fluorescence with a long-range UV light source (365 nm) after induction, while both controls, the non-induced strain LVS/pEVbr-sfGFP, as well as *F. tularensis* LVS without plasmid, emitted no fluorescence (Fig. 2B). The TetR system with the *bfr/tetO* promoter was further characterized by measuring GFP production after induction with increasing inducer concentrations. For the selection of expression condition, ATc concentrations of 50, 100, 250, 500, and 750 ng/ml were added when cultures reached $OD_{600} = 0.4$ – 0.5 . GFP production was determined after 24 h of culture with immunoblotting against GFP. Fig. 2C shows the linear dynamic range of ATc dependence in gene regulation, where the strongest effect was achieved with an ATc concentration of 750 ng/ml. Moreover, a comparison of the sfGFP fluorescence intensities demonstrated that

sfGFP activity was 5-fold higher when expressed under the control of *Pbfr/tetO* than when expressed under the control of *FTRp* (Supplementary Fig. 4).

Furthermore, we chose two *Francisella* genes to test the ability of the *bfr/tetO* promoter to overproduce and tightly control protein expression in *F. tularensis*. GapA, glyceraldehyde-3-phosphate dehydrogenase, (FTT_1368) was characterized as a 37 Da multifunctional protein. It has been demonstrated that GapA, despite being predicted to be cytosolic protein without a signal sequence, membrane-spanning motif, or functional domain other than a GAPDH NAD binding domain, could be localized on the outer membrane or even secreted into cell supernatant (Pavkova et al., 2017). The extracellular GapA (FTT_1368) then exhibits several size variants, thereby indicating its possible post-translational modification (Pavkova et al., 2017). The predicted membrane lipoprotein FTT_1676 has been identified as a virulence factor of *F. tularensis* subsp. *tularensis* Schu, playing an important role for both intracellular survival and proliferation in mice (Wehrly et al., 2009). FTT_1676 has been shown to induce a long-term antibody response in human cases (Janovská et al., 2007) and partially protected mice against *Francisella* infection after immunization with recombinant protein. It has been shown that the mature form of FTT_1676 is both acylated and glycosylated (Balonova et al., 2012).

Separate constructs containing the genes *FTT_1368* and *FTT_1676* with a C-terminal His-tag, under the control of *Pbfr/tetO*, were generated by the In-Fusion cloning method. The resultant vectors, pEVbr-1676 and pEVbr-1368, were then introduced into *F. tularensis* LVS. To evaluate promoter activity, we compared LVS strains expressing FTT_1368 and FTT_1676 from plasmids pEVbr-1368 and pEVbr-1676 carrying *bfr/tetO* promoter versus pEDL-1368 and pEDL-1676 containing *FTRp* respectively. Western blot analyses using monoclonal anti-polyHis antibodies showed that expression of FTT_1368 and FTT_1676 regulated with *bfr/tetO* promoter was 5- to 10-fold greater than did expression under the *FTRp* promoter (Fig. 3C).

To examine the impact of media composition on protein production from pEVbr in *F. tularensis* LVS, expression levels were analyzed using different media formulations. Cultures were grown in the complex media CHI and BHI, and protein-level expression was determined as described in the Materials and Methods. These media have previously displayed characteristics very favorable for growing *F. tularensis* LVS (Mc Gann et al., 2010). Production of the recombinant proteins was induced with ATc at equal cell densities and protein quantities were assessed by Western blot analysis. The final cell densities differed due to the different growth rates achieved in each of the media, and we analyzed equivalent numbers of cells. As predicted, the largest amounts of proteins were observed in those cells growing in BHI medium (Fig. 3C). Expression of FTT_1368 and FTT_1676 proteins from corresponding plasmids was inducer dependent (Fig. 3D, Supplementary Fig. 5). Additionally, we observed that the medium composition can also affect post-translation modifications (data not published). This

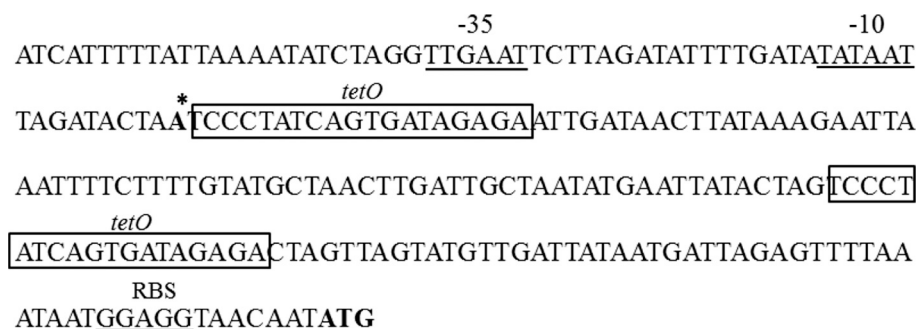


Fig. 1. Tetracycline-regulated promoter *Pbfr/tetO*. The –10 and –35 promoter sequences and ribosome-binding site (RBS) are underlined. The sequence (GGAGG) is similar to the sequence typical of the ribosome-binding sites in *E. coli*. The asterisk signifies the transcriptional start. The *tet* operator (*tetO*) sequences are indicated by boxes. ATG (shown in bold) represents the translational start.

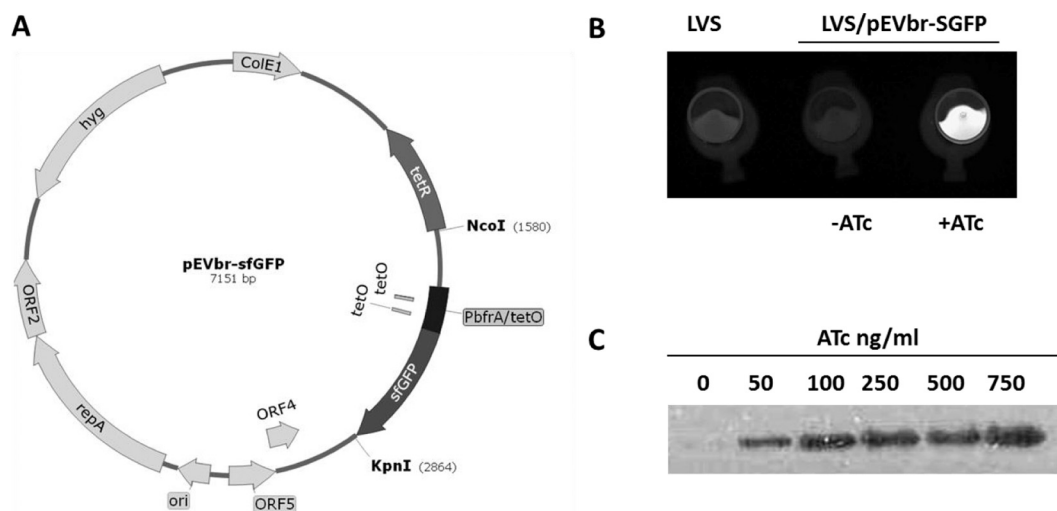


Fig. 2. TetR controlled of sfGFP expression in *F. tularensis* LVS. (A) Plasmid map pEVbr-sfGFP showing the sfGFP reporter gene under modified *Francisella bfr* promoter, and *tetR* gene constitutively expressed from the *Francisella rpsL* promoter. (B) The sfGFP expression in *F. tularensis* LVS in the present and absent of ATc. Bacterial cultures observed under UV light after 24 h growth in CHI medium supplemented 250 ng/ml of ATc. (C) Titration of sfGFP expression induced with different ATc concentrations. Cells were grown in BHI medium for 24 h in the presence of defined concentrations of ATc. Western blot analysis of sfGFP production was done using a primary rabbit monoclonal anti-GFP antibody.

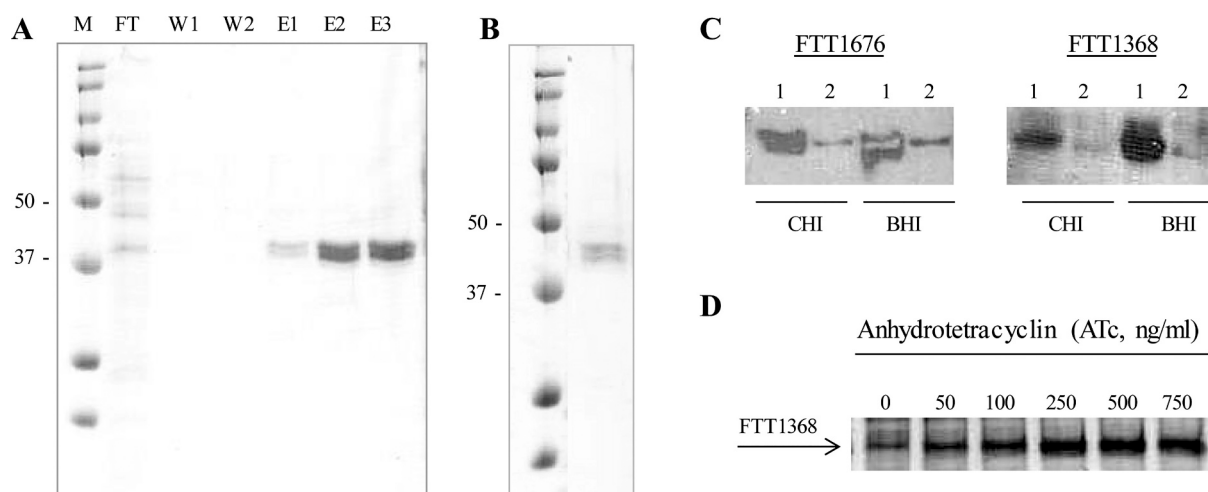


Fig. 3. SDS-PAGE and Western blot analysis of FTT₁₃₆₈ and FTT₁₆₇₆ expression. (A) Representative SDS-PAGE showing proteins in different fractions of IMAC purification of lysate LVS/pEVbr-1368. FT – flow-through, w1 and w2 – wash, and E1, E2, E3 – elution fractions. (B) SDS-page of purified FTT₁₆₇₆. Protein was produced in *F. tularensis* LVS and crude lysate was purified on TALON Cell Thru Resin. (C) Comparative expression analysis. FTT₁₃₆₈ and FTT₁₆₇₆ proteins production from vectors pEDL-1368, pEDL-1676, pEVbr-1368, and pEVbr-1676 were performed in different mediums in the same growing and induction conditions. Equivalent number of cells were analyzed by Western blot using Monoclonal Anti-poly Histidine-Peroxidase. Line 1 – promoter *Pbfr/tetO*, line 2 – promoter *FTRp*. (D) Titration of FTT₁₃₆₈ expression from vector pEVbr-1368 induced with varying amount of ATc. Strain LVS/pEVbr-1368 was grown in BHI until OD₆₀₀ = 0.6 and increasing amounts of ATc were added. Culture lysates were analyzed on 12% SDS-PAGE gel after 24 h growing.

corresponded to previous studies reporting *F. tularensis* growth differences in various media formulations (Morris et al., 2017). It has been shown that the protein profile expressed by *F. tularensis* in BHI medium was phenotypically indistinguishable from the profile of macrophage-passaged *Francisella* cells (Hazlett et al., 2008; Zarrella et al., 2011).

Likewise in pEVbr-sfGFP, expression of FTT₁₃₆₈ and FTT₁₆₇₆ proteins from corresponding plasmids was inducer dependent (Fig. 3D, Supplementary Fig. 5). SDS-PAGE analysis showed that induction was maximized at a concentration of 100–250 ng/ml ATc for the strains grown in CHI medium, and optimal concentration of ATc for growth in BHI medium was 250–500 ng/ml. The results from immunoblotting assays confirmed very low background expression levels in the uninduced cultures and efficient induction of protein expression with ATc.

To characterize proteins, we used IMAC with TALON CellThru resin

to purify the His-tagged proteins. Cultures were grown for 24 h to reach an optimal level of protein production, and cells were lysed in mild, non-denaturing conditions using xTractor Buffer. We loaded the resulting lysate directly onto a pre-equilibrated TALON CellThru column without centrifugation or filtration of the lysate (Fig. 3A). The identity of purified proteins was confirmed by immunoblotting using the monoclonal anti-polyHis antibodies or protein-specific polyclonal serum. The western blot analysis revealed that purified protein bands corresponded to the expected sizes for His-tagged fusion proteins. The concentrations of purified proteins were determined using a BCA assay. Concentrations up to 0.6 mg and 0.1 mg of protein per gram of cell biomass were obtained for FTT₁₃₆₈ and FTT₁₆₇₆ proteins, respectively, and high purity was further confirmed by 12% SDS-PAGE with Coomassie blue staining (Fig. 3A, B). We demonstrated that the expression level of FTT₁₃₆₈

produced in *F. tularensis* LVS was comparable with yields of soluble protein produced in *E. coli* (Pavkova et al., 2017). Our attempts to express the full-length FTT₁₆₇₆ as a recombinant protein using an *E. coli* expression system were unsuccessful.

3.2. Recombinant FTT₁₆₇₆ is modified with the same glycans as in native *Francisella* proteins

To determine glycosylation status of the recombinant FTT₁₆₇₆ protein, the purified protein was separated by SDS-PAGE and the protein digest was analyzed by LC-MS. The recombinant FTT₁₆₇₆ appeared on the gel as two narrowly separated bands (Fig. 3 B). It was identified as an abundant protein accounting for more than 93% of the total protein content and with protein sequence coverage exceeding 77% in both bands (Supplementary Table 1). The four unique peptide sequences of the protein FTT₁₆₇₆, ²⁷⁷ETTLASGSSISTIAK²⁹¹, ²⁷⁴EDKETTLASGSSISTIAK²⁹¹, ²⁶⁹ADEQREDKETTLASGSSISTIAK²⁹¹, and ²⁹²KPINQESSGSTIQTATK³⁰⁸, were identified as being modified by glycosylation. Each of the glycopeptides carried a single glycan moiety of two different glycans (Supplementary Table 1, Supplementary Figs. 6–11). The first identified peptide-modifying glycan was [X-Phos-HexNAc-HexNAc-Hex-Hex-HexNAc*], where X denotes an unknown moiety of a residue elemental composition C₇H₁₃O₂N, Phos signifies a phosphate residue HPO₃, and an asterisk labels the peptide-connecting monosaccharide. The second glycan had a monosaccharide sequence [HexNAc-HexNAc-Hex-Hex-HexNAc*] and the X-Phos moiety was therefore missing in this structure. The glycan [X-Phos-HexNAc-HexNAc-Hex-Hex-HexNAc] had previously been characterized in the homologous protein FTH₀₀₆₉ (Balonova et al., 2012) and another two *Francisella* proteins (Egge-Jacobsen et al., 2011; Thomas et al., 2011; Dankova et al., 2016). The second peptide-modifying glycan was not previously reported for the homologous protein FTH₀₀₆₉, but it was identified as one of the multiple glycans modifying PilA (Egge-Jacobsen et al., 2011). To conclude, we have demonstrated that the recombinant protein FTT₁₆₇₆ was glycosylated with two different glycans each of which have been previously identified on naturally occurring *Francisella* proteins.

In summary, we developed novel tetracycline-regulated expression vectors that enable efficient protein expression in *F. tularensis* LVS. We successfully expressed and purified two *Francisella* proteins in forms indistinguishable to their native forms using this modified *bfr/tetO* promoter. We optimized growth and induction conditions for strains transformed with plasmids and found that culturing in BHI medium with 250 ng/ml of ATc for induction was most optimal for recombinant expression.

These results indicate that the new expression system based on tetracycline-regulated bacterioferritin promoter is a functional and tightly controlled system. We showed an ability to produce and purify native *Francisella* proteins in quantity and quality suitable for functional assays, as well as for treatments and vaccine development.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plasmid.2021.102564>.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

We thank Thomas. H. Kawula (University of North Carolina at Chapel Hill) for providing us the inducible plasmids pEDL17, Irene MacAllister (US Army Engineer Research and Development Center) for helpful comments and suggestions, Lenka Luksikova and Jitka Zakova for their excellent technical assistance.

This work was supported by the Ministry of Defence of the Czech Republic - Long-term organization development plan Medical Aspects of Weapons of Mass Destruction of the Faculty of Military Health Sciences, University of Defence and Ministry of Education, Youth and Sports (SV/FVZ201801).

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