Genetic engineering of *Francisella tularensis* LVS for use as a novel live vaccine platform against *Pseudomonas aeruginosa* infections

Cory M Robinson, Brianna N Kobe, Deanna M Schmitt, Brian Phair, Tricia Gilson, Joo-Yong Jung, Lawton Roberts, Jialin Liao, Chelsea Camerlengo, Brandon Chang, Mackenzie Davis, Leah Figurski, Devin Sindeldecker, and Joseph Horzempa

1Biomedical Sciences Department; West Virginia School of Osteopathic Medicine; Lewisburg, WV USA; 2Department of Natural Sciences and Mathematics; West Liberty University; West Liberty, WV USA; 3Biology Department; Briar Cliff University; Sioux City, IA USA; 4Department of Pathology; Microbiology, & Immunology; University of South Carolina School of Medicine; Columbia, SC USA

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*Francisella tularensis* LVS (Live Vaccine Strain) is an attenuated bacterium that has been used as a live vaccine. Patients immunized with this organism show a very long-term memory response (over 30 years post vaccination) evidenced by the presence of indicators of robust cell-mediated immunity. Because *F. tularensis* LVS is such a potent vaccine, we hypothesized that this organism would be an effective vaccine platform. First, we sought to determine if we could genetically modify this strain to produce protective antigens of a heterologous pathogen. Currently, there is not a licensed vaccine against the important opportunistic bacterial pathogen, *Pseudomonas aeruginosa*. Because many *P. aeruginosa* strains are also drug resistant, the need for effective vaccines is magnified. Here, *F. tularensis* LVS was genetically modified to express surface proteins PilA<sub>Pa</sub>, OprF<sub>Pa</sub>, and FliC<sub>Pa</sub> of *P. aeruginosa*. Immunization of mice with LVS expressing the recombinant FliC<sub>Pa</sub> led to a significant production of antibodies specific for *P. aeruginosa*. However, mice that had been immunized with LVS expressing PilA<sub>Pa</sub> or OprF<sub>Pa</sub> did not produce high levels of antibodies specific for *P. aeruginosa*. Therefore, the recombinant LVS strain engineered to produce FliC<sub>Pa</sub> may be able to provide immune protection against a *P. aeruginosa* challenge. However for future use of this vaccine platform, selection of the appropriate recombinant antigen is critical as not all recombinant antigens expressed in this strain were immunogenic.

**Introduction**

The *Francisella tularensis* live vaccine strain (LVS) has been used to safely vaccinate millions of people worldwide and thousands of at-risk personnel in the US. However, even though this vaccine was used safely for over 50 years, immunization with LVS was discontinued as this vaccine has not been licensed by the FDA due to a number of regulatory issues. As many of these issues have been resolved, the LVS vaccine is nearing licensure evidenced by the completion of Phase II clinical trials (ClinicalTrials.gov identifier NCT01150695). Patients that had been immunized with *F. tularensis* LVS prior to this strain being deemed unavailable for human use, exhibited robust, long-term immunological memory (over 30 years post vaccination) indicated by a strong cell-mediated immune response. Given the long-term cell-mediated memory responses associated with LVS vaccination, and the safety of this vaccine strain, LVS is a superb candidate for use as a vaccine platform to deliver antigens that protect against pathogenic organisms.

Currently, there is not a licensed vaccine against the important opportunistic bacterial pathogen, *Pseudomonas aeruginosa*. *P. aeruginosa* is a leading cause of nosocomial and burn wound infections, and chronically infects those afflicted with cystic fibrosis. Treating these infections therapeutically is challenging, as many strains of *P. aeruginosa* are drug resistant. This magnifies the need for an effective vaccine. Although a vaccine targeting *P. aeruginosa* is not available for use in humans, various attempts at vaccine development have identified protective antigens. However, corresponding long-term immunity has been diminutive. Our objective here is to engineer *F. tularensis* LVS—a vaccine strain that elicits long term memory and cell-mediated immunity against *P. aeruginosa*.
immunity—to encode protective antigens of *P. aeruginosa*. This recombinant strain may provide adequate protection against *P. aeruginosa* infections.

**Results**

For use as a potential vaccine platform, encoding heterologous genes in the chromosome of *F. tularensis* LVS would be most ideal. However, a plasmid-based expression system is more practical to provide proof of concept. Therefore, we modified a stable Francisella plasmid, pFNLT88 to encode the robust groE promoter of *F. tularensis* (Fig. 1A). This plasmid, pABST was further modified to encode the *P. aeruginosa* genes *pilA*, *oprF*, and *fliC* (Fig. 1A). *pilA* encodes the major pilin protein subunit of the type IV pilus, *oprF* encodes an outer membrane porin protein, and *fliC* encodes the monomeric flagellin subunit protein of the flagellum.10-12 These genes were selected because they encode protective antigens13-15 and because the expression of these recombinant proteins could be tested using specific antibodies we had in our possession. We therefore generated the plasmids pBR, pOPRF, and pFLI, which encoded *P. aeruginosa* pilA, oprF, and fliC respectively, under the control of the *F. tularensis* groE promoter (Fig. 1A). After mobilizing these plasmids into *F. tularensis* LVS, we tested their expression by Western blotting. This analysis indicated that *F. tularensis* LVS/pBR produced PilA of *P. aeruginosa* (PilA Pa) (Fig. 2A). In addition, OprF of *P. aeruginosa* (OprF Pa) was produced by LVS/pOPRF (Fig. 2B). These recombinant proteins produced doublet bands (PilA Pa) or a band at a slightly higher molecular weight than the endogenous version (OprF Pa), likely due to the incompatibility between leader peptides of LVS and the signal peptides (Fig. 2A and B).16 Moreover, the level of OprF Pa protein expression in *F. tularensis* LVS appeared to be substantially diminished compared to those observed naturally by *P. aeruginosa* (Fig. 2B). Upon mobilization of pFLI into *F. tularensis* LVS, we observed very few
transforms (data not shown). We hypothesized that perhaps expression of FliC of *P. aeruginosa* (FliC<sub>Pa</sub>) in LVS is detrimental to this bacterium. Since groE is an especially robust promoter, we reasoned using a weaker promoter to drive expression of fliC may reduce the apparent unfavorable effect that overexpression of this heterologous gene was having on LVS. Therefore, we cloned fliC into pGRP so that this *P. aeruginosa* gene was under the control of the FTL_0580 (FGRp) promoter which produces substantially fewer transcripts than the groE promoter (Fig. 3). The resulting plasmid, pGFLI, was mobilized into *F. tularensis* LVS and expression of FliC<sub>Pa</sub> was determined by Western blotting. This Western blot indicated that *F. tularensis* LVS / pGFLI produced FliC<sub>Pa</sub> at levels seemingly comparable to the parent *P. aeruginosa* strain (Fig. 2C). However, as we observed for PilA<sub>Pa</sub> and OprF<sub>Pa</sub>, the recombinant FliC<sub>Pa</sub> appeared to be of a higher molecular weight, indicating that this protein is likely processed differently when expressed in *F. tularensis* LVS.

Since these recombinant *F. tularensis* LVS strains were capable of producing PilA<sub>Pa</sub>, OprF<sub>Pa</sub>, and FliC<sub>Pa</sub>, we wanted to test their ability to elicit production of specific antibodies. Antibodies specific for surface antigens of *P. aeruginosa* are important for opsonin-mediated phagocytosis of this pathogen, a phenomenon associated with protective immunity. Mice were immunized by intranasal (i.n.) administration with individual recombinant *F. tularensis* LVS strains (LVS / pBR, LVS / pOPRF, or LVS / pGFLI), LVS alone, or phosphate buffered saline (PBS). This immunization route was selected because previous studies showed robust mucosal and systemic antibody production in response to alternative *Pseudomonas* vaccines. Serum was collected from mice 42 days post-immunization. Mice that had been immunized with LVS / pGFLI produced robust levels of antibodies specific for *P. aeruginosa* (*P < 0.05*) compared to animals treated with PBS (Fig. 4A). However, immunization with LVS / pOPRF or LVS / pBR did not result in high levels of antibodies specific for *P. aeruginosa* (Fig. 4A and B). Therefore, even though LVS elicits a robust adaptive immune response, not all recombinant antigens expressed in this strain may be equally immunogenic. In the case of OprF, it is also possible that the lower level of expression relative to *P. aeruginosa* was not sufficient to elicit a robust response.

**Discussion**

In this study, *F. tularensis* LVS was engineered to express *P. aeruginosa* proteins. This strategy employed the use of a stable Francisella shuttle vector in which the exogenous genes were under the control of either the groE or FGRp promoter. These recombinant *F. tularensis* LVS strains were used to immunize mice to determine if the heterologously-expressed proteins could generate a robust adaptive immune response against *P. aeruginosa*. Mice that were immunized with recombinant LVS expressing FliC of *P. aeruginosa* produced a significant level of antibodies specific for *P. aeruginosa* relative to mock-immunized mice. This study establishes that *F. tularensis* LVS could potentially be used as a vaccine platform to deliver antigens that stimulate an immune response against heterologous bacteria. A recent study indicated that a mutant of *F. novicida* U112 expressing flagellin of *Salmonella typhimurium* could activate TLR5 resulting in increased inflammation, and therefore, this strain may be a potent tularemia vaccine candidate. The possibility exists that *F. tularensis* LVS / pGFLI may also provide increased protection against *F. tularensis* for similarly stimulating TLR5, however this remains to be determined.

Mice immunized with LVS expressing either PilA or OprF of *P. aeruginosa* did not stimulate robust antibody production from mice. This could be due to the meager protein levels produced by the recombinant LVS expressing these proteins (Figs. 2 and 3). Future efforts will focus on determining whether optimizing codon selection or utilizing a more robust promoter will lead to increased expression and enhanced ability to stimulate antibody production. Two candidate promoters would be those of bfr or FTL_1138. Zaiade et al showed these 2 promoters, along with groE, are the most potent of *F. tularensis*. An alternative strategy to achieve greater protein expression could be to utilize tandem promoters to maximize transcript levels. However, the
possibility exists that excessive expression of exogenous proteins could be deleterious to the host bacterium—a plausible explanation for the poor transformation efficiency we observed for pFLI.8,9 This transformation behavior suggests that, although plasmids could be deleterious to the host bacterium—a plausible explanation, future studies could take advantage of tet-off systems for the inducible repression of downstream genes. Such systems have been developed for Francisella species.24,25 However, whether or not the tetracycline-regulation for Francisella is effective inside an animal host remains to be determined. Another potential pitfall of the current study is that, for ease of manipulation, the genes encoding the exogenous proteins were harbored on plasmids. Although stable, these plasmids could have been lost in vivo, diminishing exposure of the animals to the antigens.

Future studies should focus on utilizing existing molecular tools to generate stable recombinant F. tularensis LVS bacteria encoding chromosomal copies of selected heterologous genes.6,7 Aside from adjusting expression levels by F. tularensis, more robust immune responses may be attained by altering the route of immunization or utilizing a boost following vaccination.31 It is also possible that lack of pre-protein processing may have altered the antigenicity of the recombinant proteins. Future experiments should focus on recombinantly expressing coding sequence for mature heterologous proteins in F. tularensis LVS and determining whether the resulting strains induce a more robust immune response.

Because recombinant F. tularensis LVS is capable of directing an immune response against heterologous proteins, and since immunization with this bacterium leads to over 30 years of cell-mediated immunity4, this bacterium has potential for use as a universal vaccine platform against a number of bacterial and viral infections. Genetic tools have been developed that could allow for stable, safe, and effective vaccine strains.26,30 However, viral proteins requiring glycosylation by eukaryotic host machinery may not be compatible for use with this system. Nevertheless, there is immense potential for F. tularensis LVS to express heterologous bacterial toxoids, surface proteins, and enzymes for lipid or carbohydrate biosynthetic pathways to direct the immune response against the cognate pathogens. In the more immediate future, studies should investigate whether immunization with F. tularensis / pGFLI protects against a lethal P. aeruginosa infection using the appropriate animal model such as the murine cystic fibrosis model or the burn mouse model.31,32

### Generation of recombinant vaccine strains

Plasmids and oligonucleotide primers used in the study are listed in Table 1. All general cloning was conducted using E. coli 5-α (New England Biolabs) bacteria were cultivated using LB agar incubated at 37°C for 14–24 hours. E. coli was also cultivated using LB broth incubated at 37°C with shaking. When necessary, the following antibiotics were supplemented into the media: ampicillin (100 μg/ml), kanamycin (35 μg/ml for E. coli; 10 μg/ml for F. tularensis).

### Bacterial strains utilized in this study

Bacterial strains utilized in this study are listed in Table 1. F. tularensis LVS frozen stock cultures were used to inoculate chocolate II agar plates which were incubated at 37°C with 5% CO2 for 2–4 days. P. aeruginosa bacteria were cultivated overnight at 37°C on trypticase soy agar or in trypticase soy broth with agitation. Escherichia coli 5-α (New England Biolabs) bacteria were cultivated using LB agar incubated at 37°C for 14–24 hours. E. coli was also cultivated using LB broth incubated at 37°C with shaking. When necessary, the following antibiotics were supplemented into the media: ampicillin (100 μg/ml), kanamycin (35 μg/ml for E. coli; 10 μg/ml for F. tularensis).
digested with EcoRI and NdeI, gel purified, and ligated with pABST that had been digested with these same enzymes.

The plasmid pFLI encoding fliC of *P. aeruginosa* PA14 under the control of the *F. tularensis* LVS groE promoter was constructed using the following procedures. The primers flfwd and fliCrevbam were used to amplify fliC of *P. aeruginosa* PA14. This amplicon was digested with EcoRI and NdeI, gel purified, and ligated with pABST that had been digested with these same enzymes.

The plasmid pGFLI encoding fliC of *P. aeruginosa* PA14 under the control of the *F. tularensis* LVS FGRp promoter was generated as follows. The primers fliCfwdnde and fliCrevwere used to amplify fliC of *P. aeruginosa* PA14 under the control of the *F. tularensis* LVS FGRp promoter. This amplicon was digested with EcoRI and NdeI, gel purified, and ligated with pABST that had been digested with these same enzymes.

### Plasmid maps

Plasmid maps were generated using pDRAW32.

### Electroporation

Plasmids were mobilized into *F. tularensis* LVS by electroporation as previously described. Briefly, *F. tularensis* LVS bacteria grown on chocolate agar plates were used to inoculate trypticase soy broth supplemented with 0.1% Cysteine HCl (TSBc). This culture was incubated overnight at 37°C with shaking until bacteria reached stationary phase. This starter culture was diluted 1:10 in fresh TSBc and incubated at 37°C with shaking until bacteria reached double their optical density (about 3-4 hours). For each electroporation, 1 ml of culture was washed 3 times in 500 mM sucrose. Subsequently, pellets were suspended in 50 μl of 500 mM sucrose, plasmid DNA was added (approximately 1-3 μg DNA in 2.5 μl), and then this suspension was transferred to a 0.2 cm gap electrocuvette. Electrocuvettes were pulsed at 2.5 kV, 150 μF, and 25 μF. Cells were then recovered in 1 ml of TSBc and incubated at 37°C with shaking for at least 1 hour before plating on chocolate II agar containing kanamycin.

### Western blotting

Western blotting was conducted in a similar fashion as described previously. Bacterial cells were normalized to the same density (optical density at 600 nm), pelleted, and suspended in Laemmli buffer with 2.5% β-mercaptoethanol. This material was sonicated, subjected to SDS-PAGE, and then electroblotted onto nitrocellulose paper. After the membrane was blocked (phosphate buffered saline containing 0.5% casein, 0.5% Bovine serum albumin, 100 mg/L Phenol Red, and 0.2% Sodium Azide, pH 7.4), the blot was probed with mouse monoclonal 5.44 specific for PilA of *P. aeruginosa* 1244 (a gift from Peter Castric), or rabbit serum specific for OprF (a gift from Hiroshi Nikaido), or FliC (a gift from Reuben Ramphal). Alkaline phosphatase-labeled secondary antibodies (Pierce) were used, and bands were visualized after adding naphthol as-mx phosphate

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**Table 1.** Strains, plasmids, and primers used in this study. Primer sequences are written 5’→3’

| Strains | Description | Source or Reference |
|---------|-------------|---------------------|
| *F. tularensis* | LVS | *F. tularensis* subsp. holarctica live vaccine strain | Karen Elkins |
| *E. coli* | S-α | fhuA2 Δ(argF-lacZ)/U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 | New England Biolabs |
| *P. aeruginosa* | 1244 | Wild type | Peter Castric |
|       | PA14 | Wild type | Costi Sifri |
| Plasmids | pFNLTP8 | Francisella shuttle plasmid, Km’ | Ref. 8 |
|       | pABST | pFNLTP8 with *F. tularensis* LVS groE promoter | This study |
|       | pBR | pABST with *P. aeruginosa* 1244 pilA under the control of the *F. tularensis* LVS groE promoter | This study |
|       | pBSPF | pABST with *P. aeruginosa* PA14 oprF under the control of the *F. tularensis* LVS groE promoter | This study |
|       | pFLI | pABST with *F. aeruginosa* PA14 flic under the control of the *F. tularensis* LVS groE promoter | This study |
|       | pGFLI | pGRP under the control of the *F. tularensis* LVS FGRp promoter | This study |

| Primers | groE1 | ACGTGTTACCGAGGCTGTTGACAAAAAAC | This study |
|         | groE2 | CATGGAATTCACAATCTCTCCCTTTGTTAATTATTATG | This study |
|         | pilA1 | ACGTGAATTCATGAAAGCTCAGAAGGTTTTAC | This study |
|         | pilA2 | CATCCTATGTTAGGATTCGGGCAATTAGC | This study |
|         | oprFfwd | CATCGAATTCTAATGCATACAGAAGGTTTTAC | This study |
|         | oprFrev | CATCGATATGTTGGTCGCTAAGGTTTTAC | This study |
|         | fliCfwd | CATCGAATTCTCCAGTGTTAGGCTAAGGTTTTAC | This study |
|         | fliCrev | CATCGAATTCTCCAGTGTTAGGCTAAGGTTTTAC | This study |
|         | fliCfwdbam | CATCGAATTCTCCAGTGTTAGGCTAAGGTTTTAC | This study |
|         | fliCrevbam | CATCGAATTCTCCAGTGTTAGGCTAAGGTTTTAC | This study |
(Siga-Aldrich) and fast red tr salt zinc chloride (MP Biomedicals, LLC).

**Animal immunizations**

All experiments involving mice were conducted at the University of South Carolina and were approved by this institution’s animal care and use committee. Female 5-6 week old Balb/c mice (Jackson labs) were immunized (i.n.) using F. tularensis LVS, F. tularensis LVS / pBR, F. tularensis LVS / pGFLL, F. tularensis LVS / pOPRF, or PBS as a control in a similar fashion to previously conducted studies. Blood was extracted from the tail vein of mice on day 42 post-immunization. The blood was allowed to clot at room temperature, and serum was extracted following centrifugation.

**ELISA**

Serum antibody concentrations were determined by ELISA in a similar manner as previously described. Approximately 2 × 10^8 CFU P. aeruginosa bacteria suspended in 200 μl phosphate buffered saline were distributed into each well of a microtitre plate (96-well). Plates were covered and stored overnight at 4°C to allow bacteria to adhere. After this incubation, the remaining liquid was discarded and the wells were washed twice with PBS containing 0.05% Tween-20 (PBSt). Each well was blocked with 200 μl of PBS containing 1% bovine serum albumin (PBSb) at room temperature for 1 hour. After 2 washes with PBSt, plates were covered and stored at 4°C until used.

To determine serum antibody concentrations, prepared ELISA plates were washed twice with PBSt and subsequently serially diluted mouse serum samples (diluted in PBSb) were added to the wells. Control wells were treated similarly but did not contain diluted mouse serum. After an overnight incubation at 4°C, this plate was washed twice with PBSt. The secondary antibody (goat anti-mouse Ig [heavy and light chain, HRP-conjugated; Southern Biotech]) was diluted (10^-4) in PBSb and was added to each well. After a 90 minute incubation at 37°C, the plate was washed 3 times with PBSt and subsequently 200 μl of 3,3',5,5'-tetramethylbenzidine was added to each well. The plate was incubated in the dark, and the reaction was stopped by adding 50 μl of 1 M HCl to each well. The absorbance of each well at 450 nm (OD450) was measured using an Eppendorf PlateReader AF2200. The mean OD450 of control wells plus 3 standard deviations was used to calculate the cutoff for antibody concentrations. The antibody concentration was determined to be the inverse of the lowest dilution of serum producing a higher OD450 than the cutoff. Samples in which the antibody concentration was below the limit of detection were assigned a value of the inverse of the lowest dilution assayed minus 1.

A statistical analysis of the antibody titers was conducted using GraphPad Prism software. Data were analyzed using a Kruskal-Wallis with a Dunn’s multiple comparisons test to determine statistically significant differences.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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