

Use of Transposon-Transposase Complexes To Create Stable Insertion Mutant Strains of *Francisella tularensis* LVS

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***Francisella tularensis* is a highly virulent zoonotic bacterial pathogen capable of infecting numerous different mammalian species, including humans. Elucidation of the pathogenic mechanisms of *F. tularensis* has been hampered by a lack of tools to genetically manipulate this organism. Herein we describe the use of transposome complexes to create insertion mutations in the chromosome of the *F. tularensis* live vaccine strain (LVS). A Tn5-derived transposon encoding kanamycin resistance and lacking a transposase gene was complexed with transposase enzyme and transformed directly into *F. tularensis* LVS by electroporation. An insertion frequency of $2.6 \times 10^{-8} \pm 0.87 \times 10^{-8}$ per cell was consistently achieved using this method. There are 178 described Tn5 consensus target sites distributed throughout the *F. tularensis* genome. Twenty-two of 26 transposon insertions analyzed were within known or predicted open reading frames, but none of these insertions was associated with the Tn5 target site. Analysis of the insertions of sequentially passed strains indicated that the transposons were maintained stably at the initial insertion site after more than 270 generations. Therefore, transformation by electroporation of Tn5-based transposon-transposase complexes provided an efficient mechanism for generating random, stable chromosomal insertion mutations in *F. tularensis*.**

Francisella tularensis is a gram-negative bacterial pathogen and is the etiologic agent of tularemia. The manifestations of tularemia depend on the initial route of inoculation, but all modes of contact can result in sepsis and disseminated disease, with organisms found in the liver, spleen, lymph nodes, kidney, and lungs (6, 20, 24, 25). Skin contact results in ulcer formation at the site of inoculation, where the organisms multiply and spread to the draining lymph nodes. Inhalation of *F. tularensis* leads to bronchial hemorrhaging, mediastinal lymphadenopathy (27), and pneumonia without a corresponding productive cough. Ingestion of the organisms can result in oropharyngeal tularemia, where patients typically develop exudative ulcerative pharyngitis and pharyngeal lymphadenopathy (1, 4).

F. tularensis strains are divided into two groups, A and B, which are distinguished by acid production from glycerol and by citrulline ureidase activity. The two groups of organisms exhibit similar pathogenesis; however, group A strains are considered to be highly virulent for humans and other animals, whereas group B strains typically cause milder disease (28). A live attenuated vaccine strain (LVS) derived from a group B *F. tularensis* strain has been developed and used to vaccinate laboratory workers (7). This vaccine provides significant protection against the highly virulent strains initiated by skin contact (21) and inhalation (13, 22). However, the basis for attenuation of this strain in humans is not known, and it has not been licensed for general public use in the United States.

F. tularensis can survive within macrophages, a feature that is thought to be important in the pathogenesis of this organism

(5, 12, 17, 26). *F. tularensis* subsp. *novicida*, a related organism historically referred to as *Francisella novicida*, also survives within macrophages, but it is an animal pathogen that does not infect humans. Two different genetic loci, termed *mglAB* for macrophage growth locus (2) and *iglABCD* for intracellular growth locus (11), have been identified in *F. tularensis* subsp. *novicida* that contribute to its survival in macrophages. The function of the *igl* gene products in promoting this survival has not been determined. *MglA* of *F. tularensis* subsp. *novicida* has recently been shown to function as a positive regulator for at least seven different genes (16) that are normally induced in macrophages, including *iglC*. An *F. tularensis* subsp. *novicida* mutant lacking *mglA* is unable to survive in cultured macrophages and is attenuated in mice (16). Homologues of these genes are present in both group A and B *F. tularensis* strains, and they presumably serve similar functions in these organisms.

Apart from the described macrophage survival phenotype and the identification of a limited number of genetic loci that contribute to intracellular survival (11), very little is known about the molecular mechanisms that support *F. tularensis* pathogenesis and virulence. The lack of tools for the genetic manipulation of *F. tularensis* has made it difficult to identify and dissect the bacterial products and processes that contribute to this organism's extraordinary virulence and pathogenesis. Mechanisms of transformation and allelic exchange have only recently been described (3, 8, 15), and most of these procedures have been developed in *F. tularensis* subsp. *novicida*. Lauriano et al. (15) recently described the use of allelic exchange to generate targeted insertion mutations in *F. tularensis* LVS. They also reported that Tn10- and Tn1721-based transposon insertions were unstable in *F. tularensis* and that bacterial gene-encoded transposase-complementing activity may function to promote the movement of these transposons.

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TABLE 1. Transposon insertion frequencies

Input (CFU)	No. of mutants	Mutants/CFU
2.8×10^{10}	344	1.0×10^{-8}
4.9×10^9	62	1.2×10^{-8}
1.1×10^9	50	4.5×10^{-8}
2.6×10^9	207	7.0×10^{-8}
5.7×10^{10}	1,097	1.9×10^{-8}
2.3×10^{10}	550	1.0×10^{-8}
1.4×10^{10}	210	1.5×10^{-8}

Herein we describe a procedure that uses a Tn5 derivative to create insertion mutations in *F. tularensis* LVS which, unlike Tn10 and Tn1721, were stably maintained at the initial insertion site.

F. tularensis LVS was obtained from the Centers for Disease Control and Prevention, Atlanta, Ga., and were propagated at 37°C on chocolate medium supplemented with 1% IsoVitalax (BBL). Multiple colonies from an overnight culture of LVS grown on chocolate agar were picked and swabbed onto fresh plates to achieve confluent growth, and they were then incubated for 16 h. Cells from one plate of freshly confluent LVS were suspended in 6 ml of wash buffer, consisting of 0.5 M sucrose and 10% glycerol, and then were centrifuged at $16,000 \times g$ for 3 min. The pellet was suspended in wash buffer and centrifuged again for a total of four washes. The pellet resulting from the final centrifugation was suspended in wash buffer to a total volume of 100 μ l.

A procedure initially described by Goryshin et al. (9) to create mutations in *Salmonella*, *Proteus*, and *Escherichia* species was used to produce similar transposon insertion mutations in *F. tularensis* LVS. One microliter of EZ::TN <kan-2> transposome complex (Epicentre) containing 0.1 pmol of transposon and 1 U of transposase was added to 100 μ l of the washed cell suspension, consisting of 10^9 to 10^{10} cells in 0.5 M sucrose and 10% glycerol. The contents were then mixed and transferred to a 0.1-cm-gap electroporation cuvette.

The EZ::TN <kan-2> transposome contains a derivative of Tn5 that lacks a transposase gene and has the transposase enzyme bound to the inverted repeat ends of the transposon. The transposase is stably associated with the transposon but is inactive in the absence of Mg^{2+} . Magnesium ions present inside the bacterium activate the transposase following transformation, facilitating transposition into the *Francisella* chromosome. Thus, transposition is dependent simply upon activation of the enzyme and not the expression of a foreign transposase gene.

The transposome was introduced into *F. tularensis* LVS by electroporation using a Bio-Rad Gene Pulsar set at 2.5 kV, 25 μ F, and 200 Ω . Immediately following electroporation the cells were suspended in 1 ml of brain heart infusion broth (BBL) supplemented with 50 μ g of hemin/ml, incubated for 1 h at 37°C, and then plated on chocolate agar containing 10 μ g of kanamycin/ml.

We consistently achieved an insertion frequency of $2.6 \times 10^{-8} \pm 0.87 \times 10^{-8}$ (Table 1), as determined by the number of antibiotic-resistant colonies divided by the total number of potential recipient organisms. The insertion frequency was not appreciably affected by the number of bacteria, nor was it

TABLE 2. Identified transposon insertion sites

Strain designation	Predicted insertion
A1	Extragenic
A2	Deacylase
A3	Guanosine polyphosphate pyrophosphohydrolases
A4	Hydrolase of HD superfamily
A5	Metal-dependent hydrolase
A6	Extragenic
A8	Hypothetical protein gi34496297
A9	Extragenic
A10	<i>Francisella</i> transposase (14)
B1	ATPase
B2	Extragenic
B4	Eflux pump protein
B5	RNA polymerase β subunit
B6	Transcription terminator
B7	Predicted membrane protein of unknown function, pfam03956
B8	Phosphate acetyltransferase
B9	Adenylosuccinate synthase
B10	UsoAp of <i>Emericella nidulans</i>
C1	Pyruvate phosphate dikinase
C2	Methyltransferase
C3	Isocitrate dehydrogenase
C4	Chloride channel protein Eric
C6	Anthranilate synthase
C7	Tryptophan synthase alpha chain
C8	Multidrug resistance protein
C10	Integral membrane protein

improved by increasing the concentration of transposome complexes. Recovering the organisms with cold or prewarmed media following electroporation as described for other organisms (23) also did not alter the frequency with which we isolated antibiotic-resistant organisms.

Ideally a transposon will insert randomly throughout a genome in order to be a useful tool for creating insertion mutation libraries. The transposon insertion sites were mapped by directly sequencing the transposon-chromosome junctions by using oligonucleotide primers that hybridize within the transposon. Primers Kan-2 FP-1 (ACCTACAACAAAGCTCTCA TCAACC) and JF119 (GGATCAGATCACGCATCTTC) hybridize 70 and 150 bp, respectively, from the end of the transposon adjacent to the 3' end of the Kan resistance gene, and primer KAN RP-1 (GCAATGTAACATCAGAGATTTT GAG) hybridizes 43 bp from the other end of the transposon. Individual kanamycin-resistant colonies were picked and restreaked on selective media. Chromosomal DNA was prepared from strains by using the MasterPure DNA purification kit according to the manufacturer's instructions (Epicentre).

The precise transposon-chromosome junction sites were determined in a total of 26 kanamycin-resistant colonies from each of three different transformations. One microgram of chromosomal DNA was mixed with 100 pM primer, and DNA sequence was generated by the University of North Carolina Genome Analysis Center. The transposon insertion site was determined by aligning the sequence to the *F. tularensis* LVS strain genome database produced by the Biology and Biotechnology Research Program Sequencing Group at Lawrence Livermore National Laboratory (<http://bbrp.llnl.gov/bbrp/html/microbe.html>).

Tn5, the transposon upon which EZ::TN is based, has a reported insertion bias for the sequence A-GNTYWRANC-T (where W is A or T, R is A or G, Y is C or T, and N is any base) (10). The *F. tularensis* 1.8-Mbp LVS genome has 178 of these

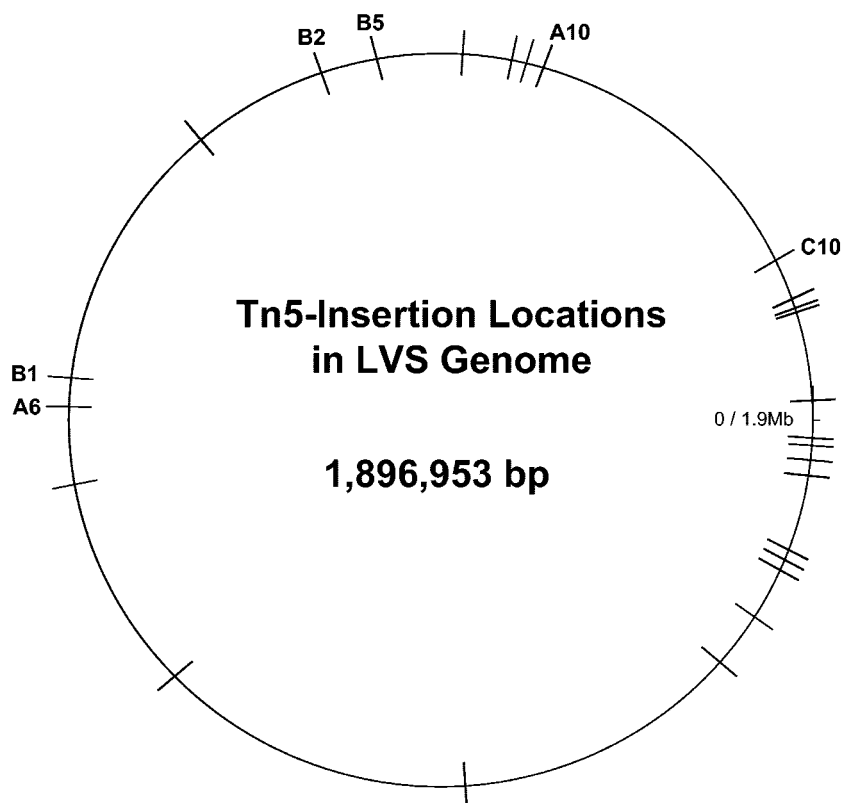


FIG. 1. Graphic representation of the chromosomal positions of the 27 identified insertion sites. The insertions assessed for stability and depicted in Fig. 2 are labeled.

insertion bias sequences, but none of the 26 insertions that we analyzed occurred within one of these sites. Of the 26 junction sites sequenced, 21 were within potential open reading frames and 1 was in the transcription termination sequence of an open reading frame, which is consistent with the observation that, apart from the Tn5 insertion bias site, this transposon has a propensity for inserting into actively transcribed DNA and regions of high superhelical density (18, 19). One insertion, B5, was in a gene encoding the RNA polymerase β subunit; this is an essential gene. The insertion is located 12 bases from the 3' end of the gene. *F. tularensis* LVS does not possess a second copy of this gene; thus, it is likely that the B5 mutant still produces a functional RNA polymerase β subunit. Ironically, one of the insertions (strain A10; Table 2 and Fig. 1) was within a gene reported to encode a *Francisella* transposase (14). The insertions did not appear to cluster within a specific chromosomal segment (Fig. 1).

Insertions in potential reading frames were examined further by translating the sequence and blasting the protein database to tentatively identify the products of interrupted genes. The majority, but not all, of these mutations occurred in genes that encoded proteins with significant homology to proteins with known functions or conserved hypothetical proteins (Table 2).

Lauriano et al. reported that insertion mutations created by derivatives of the Tn10 and Tn1721 transposons in the closely related organism *F. tularensis* subsp. *novicida* were unstable and subject to movement to other chromosomal locations,

possibly through the activity of a host-encoded transposase (15). We picked five transposon insertion mutation strains representing insertions within and outside of potential reading frames, including the strain with the insertion in the reported *Francisella* transposase gene. These strains were passed daily for 10 days on kanamycin-containing media, and chromosomal DNA was prepared from cells on passages 0, 5, and 10. Southern blots were performed by digesting 5 μ g of chromosomal DNA with EcoRI, which does not cleave the transposon. Digested DNA segments were separated by agarose gel electrophoresis, transferred to nylon, and probed with digoxigenin-labeled Tn5 probe. The Tn5 probe hybridized to identically sized fragments in each of the DNA samples prepared from different passages of the same insertion strain (Fig. 2). Given that a single colony is composed of at least 10^8 organisms, a single passage equates to at least 27 generations of growth. Thus, after 10 passages the analyzed insertions were stable for a minimum of 270 generations.

Electroporation of a Tn5-derived transposon-transposase enzyme complex into *F. tularensis* LVS resulted in the creation of random insertion mutant strains at a frequency that is sufficient to generate mutant libraries of this organism. These insertions are genetically stable and are apparently unaffected by the activities of any potential chromosomally encoded transposases. Thus, this scheme will facilitate the use of genetic approaches to study the mechanisms of *F. tularensis* physiology and pathogenesis.

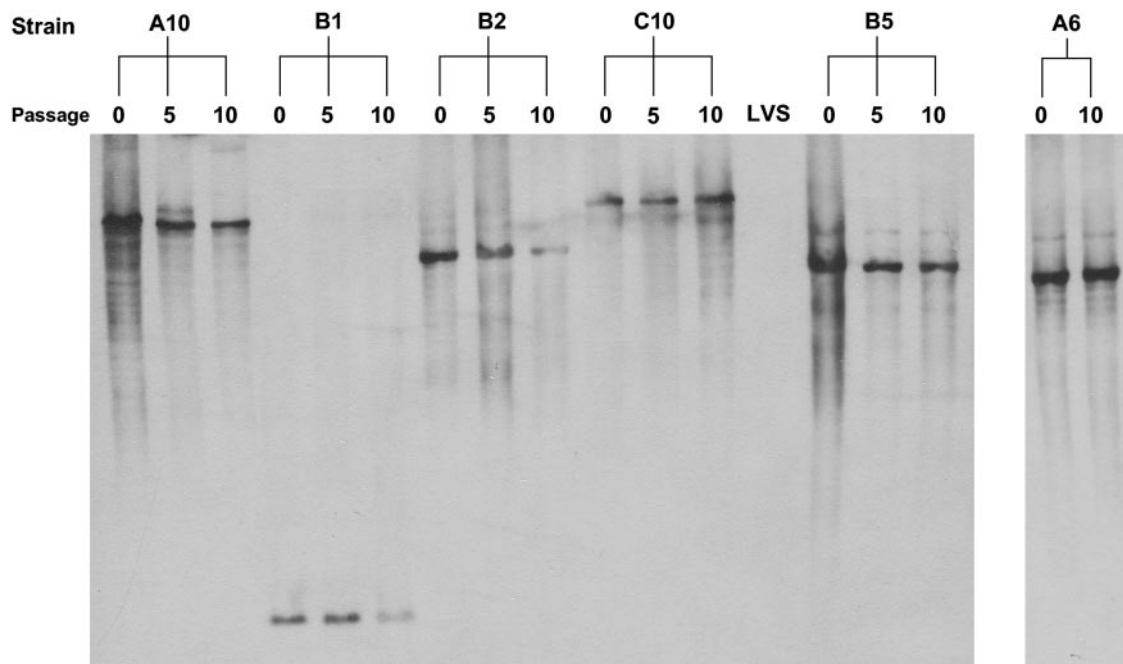


FIG. 2. Southern blot of five insertion mutants (A6, A10, B1, B2, B5, and C10) and wild-type *F. tularensis* LVS probed with labeled Tn5. Chromosomal DNA was prepared from mutants after 0, 5, and 10 passages, digested with EcoRI, and probed. All insertions analyzed in this manner retained the transposon at the initial insertion site.

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