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In Vivo *Himar1*-Based Transposon Mutagenesis of *Francisella tularensis*

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***Francisella tularensis* is the intracellular pathogen that causes human tularemia. It is recognized as a potential agent of bioterrorism due to its low infectious dose and multiple routes of entry. We report the development of a *Himar1*-based random mutagenesis system for *F. tularensis* (*HimarFT*). In vivo mutagenesis of *F. tularensis* live vaccine strain (LVS) with *HimarFT* occurs at high efficiency. Approximately 12 to 15% of cells transformed with the delivery plasmid result in transposon insertion into the genome. Results from Southern blot analysis of 33 random isolates suggest that single insertions occurred, accompanied by the loss of the plasmid vehicle in most cases. Nucleotide sequence analysis of rescued genomic DNA with *HimarFT* indicates that the orientation of integration was unbiased and that insertions occurred in open reading frames and intergenic and repetitive regions of the chromosome. To determine the utility of the system, transposon mutagenesis was performed, followed by a screen for growth on Chamberlain's chemically defined medium (CDM) to isolate auxotrophic mutants. Several mutants were isolated that grew on complex but not on the CDM. We genetically complemented two of the mutants for growth on CDM with a newly constructed plasmid containing a nourseothricin resistance marker. In addition, uracil or aromatic amino acid supplementation of CDM supported growth of isolates with insertions in *pyrD*, *carA*, or *aroE1* supporting the functional assignment of genes within each biosynthetic pathway. A mutant containing an insertion in *aroE1* demonstrated delayed replication in macrophages and was restored to the parental growth phenotype when provided with the appropriate plasmid in *trans*. Our results suggest that a comprehensive library of mutants can be generated in *F. tularensis* LVS, providing an additional genetic tool to identify virulence determinants required for survival within the host.**

Francisella tularensis is the etiologic agent of human tularemia. Four subspecies of *F. tularensis* have been recognized, including (i) the virulent type A *F. tularensis* subsp. *tularensis*, (ii) the less virulent type B *F. tularensis* subsp. *holarctica*, (iii) *F. tularensis* subsp. *mediasiatica*, and (iv) *F. tularensis* subsp. *novicida*. The *F. tularensis* LVS (live vaccine strain) is derived from *F. tularensis* subsp. *holarctica.* This strain demonstrates an attenuated phenotype in humans but remains virulent for mice, making it a potential model system to identify virulence factors (13, 23). Although *Francisella* replicates in several cell types including macrophages (4, 22, 25), hepatocytes (17), and amoebae (1), the virulence determinants that contribute to its intracellular lifestyle remain an active area of investigation. Approaches to identify and functionally characterize specific genes involved in intracellular maintenance and replication will contribute toward a general understanding of *Francisella* and host biology.

There has been steady progress toward the development and use of genetic methods to manipulate *Francisella*. Chemically induced or spontaneous mutants have been reported for both *Francisella novicida* (40) and *F. tularensis* LVS (10, 50). Tn*10*- or Tn*1721*-based transposon shuttle mutagenesis has been performed in *F. tularensis* subsp. *novicida* (5, 7–9, 18, 27, 41), but the observed instability of the transposons is problematic (35). Recently, transposon-transposase complexes were used to construct stable Tn*5*-derived insertion mutants in *F. tularensis* LVS in vivo (31). The functional aspects of candidate virulence genes have been tested using allelic replacement strategies (26, 35), and genetic strategies to complement attenuated mutants to wild-type have been developed (7, 36, 37, 42). The recent availability of genomic sequence information and chip arrays provides additional opportunities to identify genomic and transcript expression differences between subspecies and strains. As the mechanisms of pathogenesis remain poorly understood, particularly for the highly virulent type A strains of *F. tularensis*, random mutagenesis strategies may facilitate the discovery of new genes involved in maintaining an intracellular lifestyle. The identification of virulence factors should, in turn, facilitate the rational design of therapeutics or vaccines.

In previous work we constructed shuttle plasmids with expanded capabilities for use in *Francisella* (37). In this study, we report the utilization of a conditionally replicating derivative for delivery of a modified *Himar1* (*HimarFT*) into *F. tularensis* LVS in vivo. Our results demonstrate that *HimarFT*-based mutagenesis of *F. tularensis* LVS results in random, single, stable insertions at high efficiency, providing a new genetic tool for the potential identification of coding regions important for pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. *F. tularensis* LVS was routinely grown at 37°C in modified Mueller-Hinton (MH) broth or on agar (Difco Laboratories) as previously described (37). In some experiments, cysteine heart agar (Difco) with 5% defibrinated horse blood (Becton Dickinson) was used. Screening for auxotrophic mutants was performed with Chamberlain's chemically defined medium (CDM) (15). Strains containing temperature-sensitive plasmid derivatives were grown at 30°C (permissive temperature) or 40°C (nonpermissive

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

Strain, plasmid, or primer	Description[a]	Reference or source
Strains		
F. tularensis		
LVS	*F. tularensis* subsp. *holartica* live vaccine strain	K. L. Elkins
LVS *carA*::*HimarFT*	LVS containing a *HimarFT* insertion within *carA*	This study
LVS *pyrD*::*HimarFT*	LVS containing a *HimarFT* insertion within *pyrD*	This study
LVS *aroE1*::*HimarFT*	LVS containing a *HimarFT* insertion within *aroE1*	This study
E. coli		
DH5α	F$^-$ ϕ80*lacZ*ΔM15 *endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169*	Invitrogen
DH5αλ*pir*	as DH5α, lysogenized with λ*pir* phage; used as host for *Himar1* rescue	A. Camilli
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (Sm^r) *endA1 nupG;* used as host for plasmids derived from pCR2.1-TOPO	Invitrogen
Plasmids		
pCR2.1-TOPO	3.9-kb plasmid for cloning PCR products; Km^r Ap^r	Invitrogen
pAG36	6.1-kb plasmid used as the source of the nourseothricin resistance marker; Ap^r Nt^r	Euroscarf
pMiniHimar	4.0-kb plasmid containing the *magellan4* transposon constructed with a neomycin phosphotransferase (*npt*) promoter upstream of *npt* and a mycobacterial-specific promoter upstream of the transposase (*tnp*); Km^r	49
pFNLTP1	6.9-kb plasmid obtained by spontaneous deletion of pTOPO/FNL10; Km^r Ap^r	37
pFNLTP8	6.9-kb pFNLTP1 derivative with EcoRI, NdeI, NotI, NheI, SmaI, and SalI restriction enzyme sites (MCS4) cloned between the KpnI and BamHI sites; Km^r Ap^r	37
pFNLTP9	6.9-kb temperature-sensitive derivative of pFNLTP1 with a mutation in amino acid 120 (M120I) of RepA; Km^r Ap^r	37
pFNLTP11	6.9-kb derivative of pFNLTP9 with NdeI, EcoRI, SmaI, NotI, NheI, and XhoI restriction enzymes sites (MCS2) cloned between the KpnI and BamHI sites; Km^r Ap^r	This study
pFNLTP16	5.7-kb derivative of pFNLTP11 lacking kanamycin resistance used for delivery of *Himar1* into *Francisella*; Ap^r	This study
pFNLTP16 H1	9.7-kb derivative of pFNLTP16 combined with pMiniHimar using NotI; Km^r Ap^r	This study
pFNLTP16 H2	9.9-kb derivative of pFNLTP16 containing *Himar1* modified with the *F. tularensis* LVS *acpA* promoter in the PvuI site upstream of *tnp*; Km^r Ap^r	This study
pFNLTP16 H3	10-kb derivative of pFNLTP16 containing *HimarFT* (*Himar1* modified with the *F. tularensis* LVS *groEL* promoter in the BclI site upstream of *npt*); Km^r Ap^r	This study
pFNLTP16 H4	10.2-kb derivative of pFNLTP16 containing *Himar1* modified with the *F. tularensis* LVS *groEL* and *acpA* promoters upstream of *npt* and *tnp*, respectively; Km^r Ap^r	This study
pFNLTP25 H3	11.3-kb derivative of pFNLTP16 H3 containing nourseothricin acetyltransferase at the SmaI site outside of the transposon; Km^r Ap^r Nt^r	This study
pFNLTP23	7.0-kb derivative of pFNLTP8 lacking kanamycin resistance and containing nourseothricin acetyltransferase flanked with XbaI at NheI; Ap^r Nt^r	This study
pFNLTP23 *pyrDF*	1.8-kb fragment containing *pyrDF* and 344 bp upstream sequence cloned into pFNLTP23 at NotI for complementation of *pyrD*::*HimarFT*; Ap^r Nt^r	This study
pFNLTP23 *aroE1*	1.2-kb fragment containing *aroE1* and 319 bp upstream sequence cloned into pFNLTP23 at NotI for complementation of *aroE1*::*HimarFT*; Ap^r Nt^r	This study
Primers[b]		
A1	5′-ATTTCCGTGTCGCCCTTATTC-3′; forward primer for β-lactamase (*bla*) probe	
A2	5′-TTATCCGCCTCCATCCAGTC-3′; reverse primer for β-lactamase (*bla*) probe	
A3	5′-ATAAGAATGCGGCCGCTTGGTGTAGCATTCATTGCC-3′; forward primer for *aroE1*	
A4	5′-ATAAGAATGCGGCCGCAACTAGCCTAGCAACTATGC-3′; reverse primer for *aroE1*	
K1	5′-GCTATTCGGCTATGACTG-3′; forward primer for kanamycin marker (*npt*) probe	
K2	5′-CAGCAATATCACGGGTAG-3′; reverse primer for kanamycin marker (*npt*) probe	
K3	5′-GCTTCCTCGTGCTTTACGG-3′; *npt* primer for *Himar1* insertion sequencing	
K4	5′-GAGGATCTCGTCGTGACC-3′; forward primer for *npt* removal from pFNLTP11 by inverse PCR	
K5	5′-TAACCAATAGGCCGAAATCG-3′; reverse primer for *npt* removal from pFNLTP11 by inverse PCR	
N1	5′-TCTAGAACCAGCTGAAGCTTCGTACGC-3′; nourseothricin acetyltransferase forward primer	
N2	5′-TCTAGAGCATAGGCCACTAGTGGATCTGAT-3′; nourseothricin acetyltransferase reverse primer	
R1	5′-TGCCACCTAAATTGTAAGCG-3′; R6K primer for *Himar1* insertion sequencing	
P1	5′-ATAAGAATGCGGCCGCGGTCTTAATCTTATTATATGCGG-3′; forward primer for *pyrDF*	
P2	5′-ATAAGAATGCGGCCGCGTATGTCGCTAAAACTTACGC-3′; reverse primer for *pyrDF*	

[a] Km^r, Ap^r, and Nt^r indicate resistance to kanamycin, ampicillin, and nourseothricin, respectively.

[b] The primers for the promoters *groEL* and *acpA* are as described (37) except BglII and PvuI restriction enzyme sites were added in this study, respectively. Underlined regions are restriction enzyme sites for NotI (A3, A4, P1 and P2) or XbaI (N1 and N2).

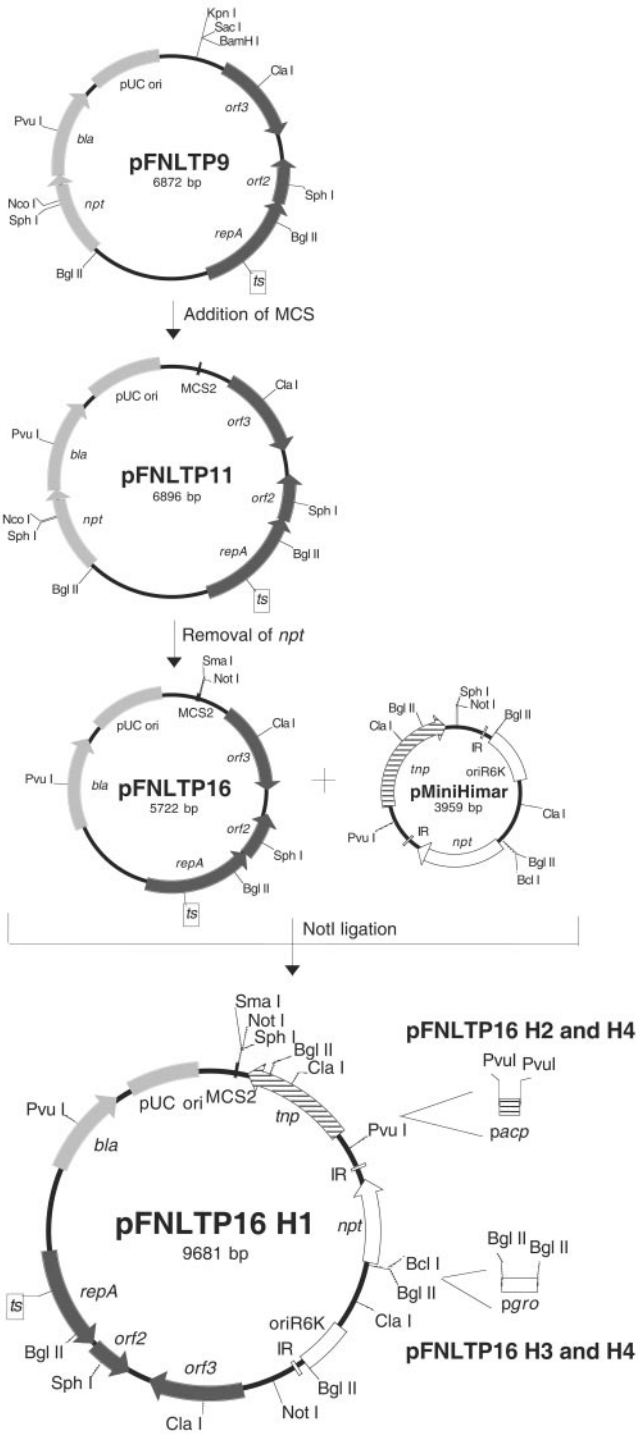

FIG. 1. Construction of pFNLTP16 derivatives containing modified and unmodified pMiniHimar to optimize transposase and kanamycin resistance. The pFNLTP16 delivery vehicle was derived from pFNLTP9 (37), a temperature-sensitive derivative of pFNLTP1. Unique restriction sites (multiple cloning site 2 [MCS2]; NdeI, EcoRI, SmaI, NotI, NheI, and XhoI between the KpnI and BamHI sites) were added to pFNLTP9 to generate pFNLTP11. Inverse PCR was performed on pFNLTP11 to generate pFNLTP16, deleting *npt*. The pUC *ori* and β-lactamase gene (*bla*) encoding ampicillin resistance are from pCR2.1-TOPO (light gray arrow or box) (Invitrogen), while *repA*, *orf2*, and *orf3* are from pFNL10 (dark gray arrows) (46). The pFNLTP16 vehicle was combined with unmodified pMiniHimar using NotI to generate pFNLTP16 H1. The transposase (*tnp*; striped arrow) is provided in *cis* with (pFNLTP16 H2 and H4) or without (pFNLTP16 H1 and H3) the addition of the *acpA* promoter (p*acp*; striped small box) upstream at PvuI. *Himar1*, shown in white between the inverted repeats (IR), contains *npt* and the R6K origin. Expression of *npt* in pFNLTP16 H3 and H4 is under the control of the *F. tularensis* LVS *groEL* promoter (p*gro*).

temperature). When required, medium was supplemented with kanamycin (10 μg ml^{-1}) or nourseothricin (5 μg ml^{-1}). Selection for nourseothricin resistance was performed at 30°C. *Escherichia coli* was grown aerobically in Luria-Bertani (LB) medium (Difco) at 37°C, supplemented with kanamycin (50 μg ml^{-1}), ampicillin (100 μg ml^{-1}), or nourseothricin (50 μg ml^{-1}). Kanamycin and ampicillin were purchased from Sigma-Aldrich (St. Louis, Mo.) or United States Biochemical Corporation (Cleveland, Ohio). Nourseothricin was purchased from WERNER BioAgents (Jena, Germany).

DNA manipulation and transformation. Purification and manipulation of plasmid or genomic DNA, electroporation of *F. tularensis* LVS, and chemical transformation of *E. coli* were performed as described previously (37). Custom oligonucleotide primers (Table 1) were synthesized by Operon (Huntsville, AL). DNA maps were constructed using MacPlasmap Pro (CGC Scientific, Inc., Ballwin, Mo.).

To determine insertion locations in *Francisella*, genomic DNA was isolated from each mutant strain, digested with SpeI, and treated with T4 DNA ligase. *HimarFT*-containing fragments were recovered as plasmid DNA in *E. coli* DH5α*λpir* (14). Analysis of genomic DNA adjacent to insertions was performed as previously described (37). The *HimarFT* insertion site sequences were compared against the *F. tularensis* LVS genome at http://bbrp.llnl.gov/bbrp/bin/f.tularensis_blast and the *F. tularensis* Schu S4 genome available at http://artedi.ebc.uu.se/Projects/Francisella/blast/.

Southern blot analysis. Genomic DNA (0.5 μg) was digested with SpeI overnight, resolved on a 0.7% agarose gel, and transferred to a positively charged nylon membrane (Roche Diagnostics Corporation, Indianapolis, IN) using an LKB 2016 VacuGene Vacuum Blotting System (Pharmacia). Blots were probed with DNA fragments randomly labeled using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche); probes detected either the *HimarFT* transposon with a 634-bp fragment recognizing the neomycin phosphotransferase (*npt*) gene responsible for kanamycin resistance or the pFNLTP16 vehicle with a 624-bp fragment recognizing the β-lactamase (*bla*) gene responsible for ampicillin resistance. Gel processing, probe labeling, and detection were performed as recommended by the manufacturer.

Construction and transposition of *Himar1* derivatives. All *Himar1* derivatives are listed in Table 1. The vehicle pFNLTP16 is a derivative of pFNLTP9 (37) in which *npt* was removed by inverse PCR and multiple cloning sites were added (Fig. 1). The pMiniHimar plasmid containing *Himar1* was linearized and ligated to pFNLTP16 to generate pFNLTP16 H1 (Fig. 1). Derivatives of pFNLTP16 H1 were generated by cloning promoters for *groEL* (p*gro*) and/or *acpA* (p*acp*) (21, 48) upstream of *npt* and/or *tnp*, respectively (Fig. 1). A derivative of pFNLTP16 H3 containing a 1.3-kb fragment encoding nourseothricin resistance, including the translation elongation factor promoter and terminator (24), was amplified from pAG36 (Euroscarf) to construct pFNLTP25 H3.

Several transposition conditions were tested to determine the optimal growth medium and length of time for outgrowth. For optimal transposition, 100 ng of each transposon-containing plasmid was electroporated into newly prepared electrocompetent *F. tularensis* LVS. After outgrowth at 30°C on a shaker for 5 h, dilutions were plated on MH medium containing kanamycin and incubated at 40°C to select for *Himar1* integration and loss of plasmid (one-step protocol) or incubated at 30°C to obtain replicating plasmid before secondary selection for plasmid loss at 40°C (two-step protocol). In addition, dilutions were plated onto MH medium with or without kanamycin to determine total CFU transformed or total potential recipients, respectively. Colonies grown for 3 or 4 days at 40°C were picked and struck onto MH medium containing kanamycin and grown at 37°C to recover individual clones containing *Himar1* insertions in the genome.

Complementation of *HimarFT* auxotrophic mutants. The transposon insertion was mapped in each auxotrophic mutant identified. Wild-type *aroE1* or *pyrDF* and predicted promoter regions were amplified from *F. tularensis* LVS genomic DNA and cloned into pFNLTP23 (see Fig. 4), a derivative of pFNLTP8 (37) containing the gene encoding nourseothricin acetyltransferase. After electroporation into the respective *F. tularensis* LVS *HimarFT* mutant strains, the ability to restore growth on CDM was determined. To functionally complement *carA*, *pyrD*, or *aroE1* mutant strains, uracil (50 μg ml^{-1}) or a mixture of phenylalanine

TABLE 2. *Himar1* derivatives and frequency of transposition into *F. tularensis* LVS[a]

Himar1 derivative	No. of transformants[b]	No. of insertions[c]	Avg no. of insertions/avg no. of transformants	Avg no. of insertions/avg total no. of cells
pFNLTP16 H1	0	0	0	0
pFNLTP16 H2	0	0	0	0
pFNLTP16 H3	$8.73 \times 10^5 \pm 5.03 \times 10^5$	$1.04 \times 10^5 \pm 5.03 \times 10^4$	0.12	1.82×10^{-5}
pFNLTP16 H4	$7.35 \times 10^5 \pm 6.14 \times 10^4$	$1.13 \times 10^5 \pm 1.55 \times 10^4$	0.15	1.97×10^{-5}

[a] Results shown are averages from four separate experiments. The average total number of cells recovered after electroporation and 5 h of outgrowth was $5.73 \times 10^9 \pm 2.38 \times 10^9$ CFU ml^{-1}. Both plasmids pFNLTP16 and pMiniHimar resulted in no viable CFU ml^{-1} recovered as transformants or insertions.
[b] Transformants are the average number of viable CFU ml^{-1} recovered after growth on selective medium at the permissive temperature.
[c] Insertions are the average number of viable CFU ml^{-1} recovered after growth on selective medium at the nonpermissive temperature.

and tryptophan (100 μg ml^{-1} each) was added to CDM. The intracellular growth phenotype was determined by measuring bacterial replication over 3 days in the murine BALB/c macrophage cell line J774A.1 as described previously (37).

Nucleotide sequence accession numbers. Sequence information for pFNLTP16 H3 and pFNLTP23 is available from the GenBank database under accession numbers DQ236098 and DQ266433, respectively.

RESULTS

Construction of a *Himar1* transposon for use in *Francisella*. Initial attempts to isolate *Himar1* insertions in *Francisella* using pMiniHimar (Table 1), a plasmid unable to replicate in *Francisella*, were unsuccessful. This result could be due to failures in (i) transposon delivery, (ii) expression of the transposase, (iii) expression of the selection marker, or (iv) a combination of these factors. To address delivery of the transposon, *Himar1* derivatives were cloned into the temperature-sensitive shuttle plasmid pFNLTP16 (Fig. 1), a derivative of pFNLTP9 in which *npt* had been removed. This plasmid can be efficiently electroporated into *Francisella* species and maintained at a permissive (30°C) but not at a nonpermissive (40°C) temperature (37). To ensure transcriptional initiation of the selectable marker and transposase, the *Francisella* promoter for *groEL* (p*gro*) (21) was cloned upstream of *npt* (pFNLTP16 H3 and H4), and/or the promoter for *acpA* (p*acp*) (48) was cloned upstream of *tnp* (pFNLTP16 H2 and H4) (Fig. 1). Previous experiments demonstrated that both p*gro* and p*acp* drive the expression of *gfp* cloned into pFNLTP6 in *F. tularensis* LVS (37).

Optimization of *Himar1* transposition in *F. tularensis* LVS. One-step and two-step transposition protocols were tested using various pFNLTP16 *Himar1* derivatives, including those that had been modified by the addition of one or both *Francisella* promoters. All derivatives were electroporated into *F. tularensis* LVS to determine if Km^r colonies could be recovered at 40°C in a one-step procedure. Transposition was not detected with pFNLTP16, pMiniHimar, pFNLTP16 H1, or pFNLTP16 H2. However, Km^r isolates were recovered at high efficiency using the pFNLTP16 H3 or H4 derivatives (Table 2). The efficiency of plating at the nonpermissive temperature was approximately 12 to 15% of that observed at the permissive temperature (Table 2). Loss of plasmid DNA was investigated using a derivative of pFNLTP16 H3 that contains the nourseothricin resistance cassette as a selectable marker (pFNLTP25 H3) (Table 1). After electroporation and growth at the nonpermissive temperature, 100 kanamycin-resistant isolates were picked onto MH medium containing either nourseothricin or kanamycin. All isolates retained kanamycin resistance, but none was able to grow on medium with nourseothricin, consistent with *Himar1* transposition and loss of the plasmid vehicle. Isolates from an electroporation plated at permissive temperature grew on medium containing kanamycin or nourseothricin, a result consistent with plasmid maintenance (data not shown).

To determine if the frequency of transposition could be further improved, the procedure was repeated for all transposon derivatives using a two-step method. Strains were first selected for inheritance and maintenance of plasmid DNA at 30°C. Cultures were then shifted to the restrictive temperature (40°C) to inhibit subsequent plasmid replication. As in the one-step protocol, Km^r colonies were obtained only with the pFNLTP16 H3 and H4 derivatives after incubation at 40°C. Plasmid DNA, however, remained detectable in these isolates. The presence of plasmid DNA may be due to a residual plasmid-containing subpopulation that should be lost by subsequent replication cycles at 40°C. Since the retention of plasmid-encoded Km^r could be problematic in subsequent screening steps, we concluded that a one-step protocol using pFNLTP16 H3 was optimal for the delivery and transposition of *HimarFT* into *F. tularensis* LVS.

Verification of *HimarFT* transposition in *F. tularensis* LVS. Southern blot analysis was performed on genomic DNA from 33 random Km^r colonies recovered after a one-step transposition protocol with pFNLTP16 H3. Hybridization of SpeI-digested genomic DNA with a probe specific to *npt* present on *HimarFT* resulted in single bands of various sizes, as shown for 15 representative isolates (Fig. 2A). No signal was obtained with a probe specific to the β-lactamase gene (*bla*) present on pFNLTP16 for 31 of these random isolates (data not shown). DNA from two isolates hybridized to the plasmid-specific probe, suggesting that plasmid DNA was incompletely resolved during subsequent replication.

Rescue of *HimarFT* and DNA sequence analysis of insertions. *HimarFT* insertion sites were mapped by ligation of SpeI-digested genomic fragments and recovery of plasmid DNA in *E. coli* DH5αλ*pir*. Nucleotide sequence analysis was performed with primers annealing to *HimarFT* and reading into flanking genomic DNA. When insertion locations were mapped to the *F. tularensis* LVS genome (Fig. 2B), a random distribution of *HimarFT* insertion was observed with no apparent regional bias. A variety of insertions occurred with no observed preference for the open reading frame (ORF) or transposon orientation (Fig. 2C). Of the 31 rescued genomic insertions, 19 possessed *HimarFT* insertions in a predicted ORF (Table 3). *HimarFT* inserted into genes involved in secretion, transport, energy production, metabolism, cell division, and protein turnover. Eight insertions mapped to intergenic regions, and two were located in repetitive regions of the *F. tularensis* LVS

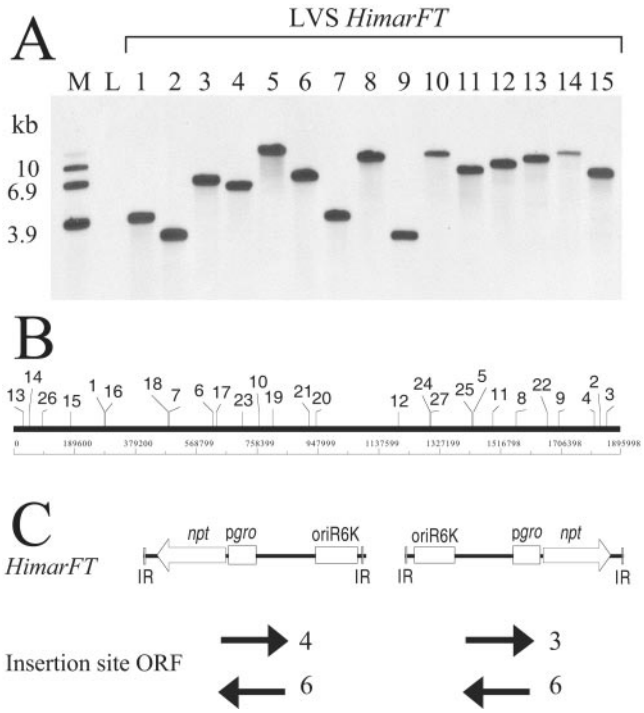

FIG. 2. Transposition of *HimarFT* into *F. tularensis* LVS. (A) Southern blot analysis. Genomic DNA from Km^r colonies selected at the nonpermissive temperature was prepared, digested with SpeI, separated on an agarose gel, and transferred to a nylon membrane. The membrane was hybridized to a probe specific to the kanamycin resistance marker present on the *HimarFT* transposon. Isolates 1 to 15 are representative of the 33 random isolates screened for *HimarFT* insertion (numbers 13 to 15 were recovered as auxotrophic strains). Wild-type *F. tularensis* LVS (lane L) was included as a negative control. Plasmid markers (lane M) included pFNLTP1 (6.9 kb), pFNLTP16 H1 (10 kb), and pCR2.1 TOPO (3.9 kb). (B) Map of the *F. tularensis* LVS genome with identification of the *HimarFT* insertion site locations. (C) Graphical representation of the orientation of *HimarFT* insertion. *HimarFT* was found in both orientations in the *F. tularensis* LVS genome with no bias to the orientation of the ORF (arrows) at the insertion site based on the *F. tularensis* Schu S4 genome annotation. The number of isolates containing insertions into ORFs in the indicated orientation is shown adjacent to each arrow.

genome. The remaining two isolates likely represent aberrant transposition events. We detected one deletion and one duplication of flanking genomic sequence. The typical TA insertion site for *Himar1* transposons was present in all but one isolate.

Stability of *HimarFT* insertions in *F. tularensis* LVS. Five *HimarFT* mutants were serially passaged in MH broth without kanamycin for 5 days. After ~40 generations, cultures passaged in the absence of selective pressure maintained the kanamycin marker. A Southern blot comparing the genomic DNA isolated from cultures grown under kanamycin selection and those isolated after 5 days without kanamycin confirmed that the insertion site was identical (Fig. 3). These results indicate that once transposition and plasmid loss has occurred, the kanamycin marker remains stably integrated within the genome.

Phenotypic screen for auxotrophic mutants after *HimarFT* transposition. To test the utility of *HimarFT*, a one-step mutagenesis protocol was performed, and Km^r colonies were screened for growth by replica plating onto Chamberlain's CDM. From approximately 6,500 clones, three isolates were obtained that failed to grow on CDM. Each strain possessed a single insertion of *HimarFT*, as shown by Southern blot analysis with the *npt* probe (Fig. 2A, lanes 13 to 15). A hybridization signal was undetectable with the *bla* probe (data not shown). *HimarFT* insertions resulting in auxotrophy mapped to *carA* (required for carbamoyl phosphate synthesis, an intermediate in arginine and pyrimidine synthesis), *pyrD* (required for pyrimidine synthesis), and *aroE1* (required for the shikimate pathway involved in aromatic amino acid, ubiquinone/menaquinone, and folate synthesis, respectively) based on the annotated Schu S4 genome (Table 3).

TABLE 3. Compilation of *HimarFT* insertions within open reading frames[a]

Location of insertion	Putative function
FTT1495c	Hypothetical membrane protein
FTT0194c	Conserved hypothetical membrane protein
FTT1738c	*kdpB*, K-transporting ATPase B chain
FTT1425c	*naoX*, NADH oxidase
FTT0432	*speA*, putative arginine decarboxylase
FTT1591	Lipoprotein
FTT0088	*pilT*, type IV pilus nucleotide-binding protein
FTT1171c	*hsdM*, DNA methyltransferase subunit M
FTT0491c	*gph*, phosphoglycolate phosphatase
FTT0948c	Aldo/keto reductase
FTT1133	*pilB*, type IV pilus binding protein
FTT0679c	GTP-binding protein
FTT0745c	Hypothetical protein
FTT1339c	Sulfate permease family protein
FTT1769c	*clpB*, ClpB protein
FTT0742	Hypothetical lipoprotein
FTT1663	*carA*, carbamoyl-P synthase small chain
FTT1647c	*pyrD*, dihydroorotate dehydrogenase
FTT0238	*aroE1*, shikimate 5-dehydrogenase

[a] Location of *HimarFT* insertion and putative functions according to the *F. tularensis* subsp. *tularensis* (FTT) designation of the Schu S4 genome.

Complementation of the auxotrophic *HimarFT* mutants. To genetically complement *HimarFT* insertion strains, we constructed a plasmid expressing nourseothricin resistance as the selective marker (pFNLTP23). Nourseothricin is an aminoglycoside that inhibits a broad spectrum of organisms, including bacteria, protozoa, yeasts, viruses, and plants. Treatment with nourseothricin is postulated to inhibit protein synthesis by inducing miscoding events. It is an attractive marker for research because it is not used in human or veterinary medicine

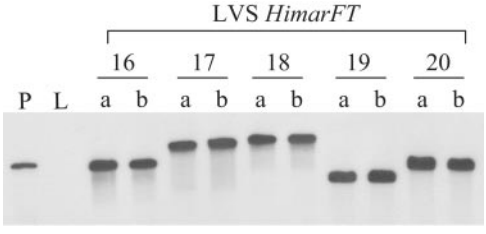

FIG. 3. Southern blot analysis of five *HimarFT* insertion mutants (isolates 16 to 20) and wild-type *F. tularensis* LVS (L) with a probe specific to the kanamycin resistance marker on *HimarFT*. Genomic DNA was prepared from the mutants after initial isolation (a) or after approximately 40 generations in the absence of kanamycin selection (b).

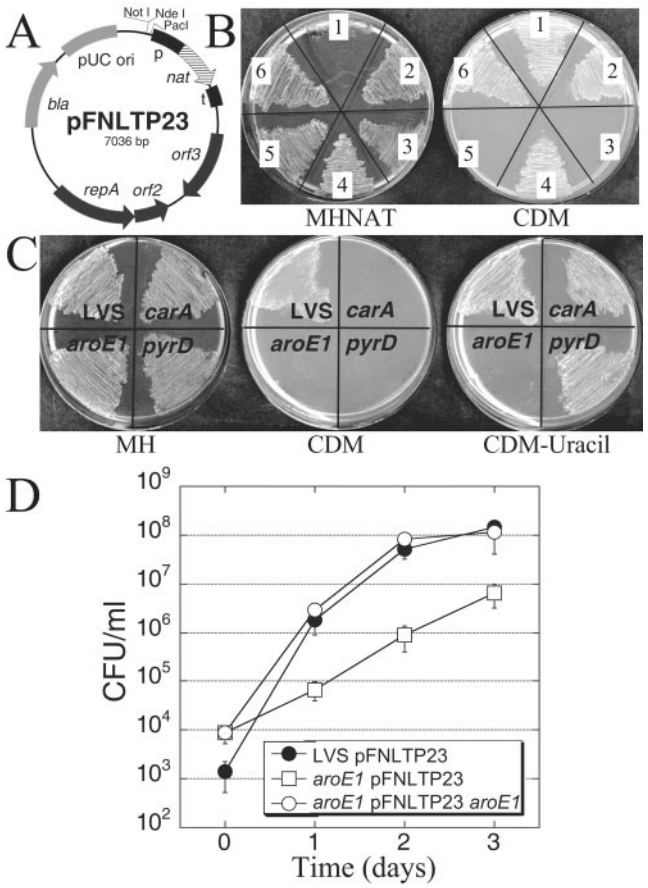

FIG. 4. (A) Map of complementation plasmid pFNLTP23 containing nourseothricin acetyltransferase (*nat*; striped arrow) with the promoter (p; black box) and terminator (t; black box) for the translation elongation factor. The pUC *ori* and β-lactamase gene (*bla*) are from pCR2.1-TOPO (light gray arrow or box) (Invitrogen), while *repA*, *orf2*, and *orf3* are from pFNL10 (dark gray arrows) (46). (B) Genetic complementation of two auxotrophic mutants. Growth is illustrated on medium containing nourseothricin (MHNAT) or on CDM. Culture designations 1 to 6 are LVS, LVS pFNLTP23, *pyrD*::*HimarFT* pFNLTP23, *pyrD*::*HimarFT* pFNLTP23 *pyrDF*, *aroE1*::*HimarFT* pFNLTP23, and *aroE1*::*HimarFT* pFNLTP23 *aroE1*, respectively. (C) Functional complementation of two auxotrophic mutants using CDM supplemented with uracil. Growth is illustrated on MH medium, CDM, and CDM containing uracil. In panels C and D the insertionally inactive gene name is used for space considerations (i.e., *aroE1* is *aroE1*::*HimarFT*). (D) Growth of *F. tularensis* LVS pFNLTP23, *aroE1* pFNLTP23, and *aroE1* pFNLTP23 *aroE1* in the murine BALB/c macrophage cell line J774A.1. Macrophage monolayers were infected at a multiplicity of infection of 1 for 2 h, washed, and incubated for a total of 3 days. Cells were lysed at different times, and bacteria were enumerated. Experiments were performed in triplicate (results are means ± standard deviations). The growth of *aroE1*::*HimarFT* pFNLTP23 on days 1 to 3 was significantly different from LVS pFNLTP23 to $P < 0.03$.

and has not been shown to display cross-resistance (24). The MIC of nourseothricin for *F. tularensis* LVS is approximately 2 to 5 μg ml^{-1} when it is incorporated in complex medium (data not shown). No cross-resistance to kanamycin was observed in strain LVS.

DNA fragments containing the coding sequences and putative promoter regions for the *HimarFT*-interrupted ORFs were amplified from the genome and cloned into pFNLTP23 for complementation (Fig. 4A). *F. tularensis* LVS containing pFNLTP23 grew on defined medium (Fig. 4B, sector 2), while the auxotrophic mutants containing pFNLTP23 were unable to grow (Fig. 4B, sectors 3 and 5). When *pyrDF* and *aroE1* (including ~300 bp upstream) were provided in *trans*, they restored the ability of *pyrD*::*HimarFT* and *aroE1*::*HimarFT*, respectively, to grow on CDM (Fig. 4B, sectors 4 and 6). We were unable to genetically test complementation of *carAB*::*HimarFT* as several attempts to clone this fragment in pFNLTP23 were unsuccessful. However, the addition of uracil to CDM functionally complemented both *carAB* and *pyrD* insertion strains (Fig. 4C), consistent with the predicted function of these genes in pyrimidine biosynthesis (12). Similarly, *aroE1*::*HimarFT* grew on CDM supplemented with phenylalanine and tryptophan (data not shown).

To determine if any of the auxotrophic mutants possessed intracellular replication defects, a growth analysis was performed in J774A.1 macrophages. Strains with insertions in *pyrD* or *carA* were indistinguishable from LVS when replication in macrophages was assessed (data not shown). In contrast, *aroEI*::*HimarFT* pFNLTP23 exhibited a distinct delay in replication compared to LVS pFNLTP23 (Fig. 4D). This delay is specific to intracellular growth since no growth difference was seen at 37°C in MH broth between LVS and *aroEI*::*HimarFT* with or without pFNLTP23 (data not shown). The replication defect of *aroEI*::*HimarFT* pFNLTP23 in J774A.1 macrophages was complemented with the cloned gene in *trans* (Fig. 4D).

DISCUSSION

Himar1 has been useful for in vitro (2, 3, 45) and in vivo (6, 49, 51, 54) transposon mutagenesis of a variety of bacteria. Moreover, since *Himar1* does not require host-specific factors for transposition and displays a lack of site specificity, it seemed ideal for mutagenesis of the AT-rich *Francisella* genome (32–34). The modified *Himar1* transposon developed in this study (*HimarFT*) allows efficient in vivo random mutagenesis of *F. tularensis* LVS. Analysis of 31 insertions from a single transposition reaction suggested that *HimarFT* could transpose randomly within the *F. tularensis* LVS genome with no known sequence specificity apart from the TA dinucleotide previously reported to be required for all *mariner* transposition events (19, 33). The frequency of insertion for *HimarFT*, either 15% of transformed cells or 2×10^{-5} of potential recipient organisms, is comparable to other reported in vivo random mutagenesis systems in *Francisella* (31) or in other bacteria (49, 51, 54). This frequency allows saturation mutagenesis to be conducted in *Francisella* from a single electroporation, an advantage over transposon-transposase complexes (31).

Implementing a transposon mutagenesis strategy in diverse organisms may require optimization of several parameters, including expression of the transposase and antibiotic resistance marker for selection. Alteration or substitution of promoter sequences has been used in prior studies to eliminate host restriction (49, 51, 54). Recent studies indicate that this could also be a limitation encountered in *Francisella*. Interestingly, detection of EZ::TN insertions in the *Francisella* genome appears to be limited to transcriptionally active regions, perhaps due to poor expression of the resistance marker in single copy. In *HimarFT*, the orientation of the *groEL* promoter rel-

ative to *npt* and *tnp* may result in sufficient expression of both genes, as the addition of the *acpA* promoter upstream of *tnp* does not affect the frequency of transposition. This configuration may actually limit *tnp* expression to a single event per plasmid.

Although single, stable genomic insertions were identified in most cases, spurious events were also detected using *HimarFT* as constructed. Isolates were obtained that possessed rearrangements adjacent to the insertion or parts of the delivery vehicle. This is not specific to *Francisella* or *HimarFT* but is common for transposon mutagenesis (6, 11, 16, 20, 38, 53). Additionally, two isolates appeared to maintain plasmid sequences, due possibly to reversion of the temperature-sensitive mutation within RepA. We anticipate that spurious events represent only a minority of the *HimarFT*-containing clones in a genomic library, some of which may not generate a phenotype during subsequent screening steps.

Auxotrophy is one phenotype that has been considered for the development of a suitable attenuated vaccine candidate. Both the shikimate and the purine biosynthetic pathways have been proposed as targets to generate vaccine strains in *Francisella* (30, 47). The attenuation of growth in vivo while maintaining the expression of protective antigens is important in considering immunization strategies (28, 43). Mutants of the *aro* pathway in *Listeria monocytogenes* are attenuated in virulence in epithelial cell culture and in mice (52), while *Mycobacterium tuberculosis* requires this pathway for viability (44). Disruption of *carA* or *pyrD* did not result in any phenotypic change for entry or replication within macrophages in *F. tularensis* LVS. In contrast, disruption of *aroE1* results in delayed or reduced replication in *F. tularensis* LVS, a characteristic fully reversible when *aroE1* is provided in *trans*. Further analysis of LVS *aroE*::*HimarFT* in mice will be necessary to better delineate the in vivo phenotype and its potential as a live vaccine.

Isolation of auxotrophs in other bacteria often results in 1 to 2% recovery (6, 29). We had expected to obtain more auxotrophs in our screen, consistent with the *F. tularensis* Schu S4 genomic analysis that identified approximately 350 enzymes postulated to participate in metabolism (34). Although ~6,500 clones were analyzed on CDM, replica plating resulted in crowding on the assay medium, reducing the ability to detect loss of growth. We arrayed the library in a 96-well format and repeated the screen for growth on CDM. From 5,467 clones, eight isolates grew on complex medium and reproducibly failed to grow on CDM (0.15%) (data not shown). All insertions mapped within genes annotated as components of biosynthetic pathways. Additionally, different insertions in *carB* and *aroE1* were identified in the second screen. A similar frequency of auxotrophy (0.5%) was reported in a screen of *Xenorhabdus nematophila* transposon mutants on a defined medium supplemented with all 20 L-amino acids (39). The complexity of the CDM and the exclusion of essential genes from our screen may influence the total number of auxotrophs that could be isolated by insertional mutagenesis.

The in vivo *HimarFT* mutagenesis system utilized in this study for *Francisella* expands the array of newly developed tools to analyze gene function in this intracellular pathogen. Continued development of random mutagenesis strategies should accelerate the discovery of determinants required for virulence and replication and perhaps guide future work toward a defined vaccine strain.

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