AN ABSTRACT OF THE THESIS OF

Lindsay A. Flax for the degree of Master of Science in Microbiology presented on September 11, 2006.

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Abstract approved:

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Francisella tularensis is a gram-negative facultative intracellular coccobacillus that primarily infects macrophages. The causative agent of tularemia, this bacterium is considered among the most infectious organisms known, requiring fewer than ten organisms to cause disease. Although ubiquitous in nature, transmission to humans is rare but can occur via insect bites, direct contact with infected animals, ingestion of contaminated water, or through the inhalation of aerosols. There are several species of *Francisella*, however the majority of infections are caused by *F. tularensis*. Species of *F. tularensis* are further classified into two groups according to pathogenesis, the Type A highly virulent strain and the Type B, less pathogenic strains. Type A pathogens cause a variety of clinical manifestations including several glandular infections and the most life threatening, pneumonic tularemia. Given the many routes of transmission, low infectious dose and severity of the illness, *F. tularensis* has become a concern for potential development of the bacteria into a bioweapon and has been classified as a Category A pathogen by the Centers for Disease Control and Prevention (CDC).

Previous studies have attempted to investigate the pathogenicity of *F. tularensis* using a variety of genetic manipulation techniques. However due to the

unique challenges of applying current genetic techniques in *F. tularensis*, few genes important for *Francisella* virulence have been identified. This study aims to develop a random transposon mutagenesis library and primary screening assay to rapidly identify virulence factors associated with intra-macrophage survival. A potential library was generated using plasmid pFT-mariner, a *Francisella* mutagenesis vector constructed for this study. This plasmid utilizes a eukaryotic mariner himar-1 transposase and transposon cassette. An arabinose inducible promoter that regulates transposase activity, controls transposition of the kanamycin flanked transposon cassette. The pFT-mariner plasmid was introduced into *F. tularensis* live vaccine strain (LVS) through conjugation and resulted in several potential library founder clones. Founder clones were screened by polymerase chain reaction (PCR) and found to contain pFT-mariner components in several generations of passed bacteria. Select clones were incubated with arabinose to induce transposon integration into the genome. A counter-selection method was used to eliminate the pFT-mariner plasmid. DNA from potential library clones was screened by PCR to detect the integration of the transposon and to verify the loss of the remaining plasmid. Following confirmation of transposition, several methods were used to try to determine the site of insertion. To screen for pathogenicity, any identified mutants would be applied to a macrophage infection assay and compared to a *F. tularensis* LVS infection.

This study generated multiple potential library founder clones and developed a rapid screening assay for intra-macrophage survival of *F. tularensis* LVS. However in our investigation we encountered several difficulties; while we were able to detect transposon integration immediately following transposase induction, these failed to be

identified again in subsequent investigation. Ultimately, similar to previously reported mutagenesis attempts our potential library of transposon mutants was determined to be unstable. Thus, future transposon mutagenesis efforts should focus on verifying stability of the vector and transposon.

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Master of Science thesis of Lindsay A. Flax presented on September 11, 2006.

APPROVED:

Major Professor, representing Microbiology

Chair of the Department of Microbiology

Dean of the Graduate School

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Lindsay A. Flax, Author

Developing a System of Mutagenesis in *Francisella tularensis* LVS

by
Lindsay A. Flax

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Chapter 1

INTRODUCTION

GEOGRAPHIC DISTRIBUTION

The original isolation of *Francisella tularensis* occurred during a plague-like outbreak in Tulare County, California in 1911 [1]. Although isolated in the United States, the bacterium is endemic throughout North America, parts of Europe and in Asia [2-4]. The majority of tularemia cases occur in Scandinavian and former Soviet countries [5]. Outbreaks have occurred globally, the largest recorded was in 1966 in a farming region of Sweden [6].

Human cases have occurred in every state of the United States except Hawaii, mostly in south-central and western states [7]. Although the pathogen appears ubiquitous in nature, incidence of human tularemia within the US is uncommon with approximately 1400 cases occurring in the last decade [8, 9]. The causative agent of tularemia is the bacterium, *F. tularensis* [10]. The large geographic distribution is a direct result of the various habitats that *F. tularensis* can persist in, including water, soil and vegetation [3, 4, 11]. Both terrestrial and aquatic animals can maintain *F. tularensis* acting as natural reservoirs including voles, mice, water rats, squirrels, and rabbits [5, 12-14]. These animals acquire the bacteria via ticks, mosquitoes, and other environmental contact. *F. tularensis* outbreaks have been noted to occur correlating to outbreaks of similar pathogens including *Coxiella burnetii* and *Legionella pnuemophila* [15]. Several outbreaks have been associated with contaminated water, possibly due to the ability of *F. tularensis* to survive in amoeba, mainly *Acanthamoeba castellanii* [15]. It has been shown that not only does the active

amoeba support this bacteria but for long-term survival it also persists in the amoebal cyst [16, 17]. Examining the occurrence within the US, greater than 200 species of animals including mammals, reptiles, and fish have been reported to experience tularemia infections [8].

TRANSMISSION

Among the methods by which a person becomes infected are ingesting contaminated food or water, handling infected animal remains, bites from infected insects and arthropods or through inhalation of aerosolized bacteria [13, 14, 18]. Most cases of *F. tularensis* leading to infection are transmitted via arthropods, mainly *Ixodid* ticks [3, 8]. All humans regardless of age and gender are susceptible to tularemia, although certain occupations and activities can increase risk. Hunting, butchering, and farming are all associated with exposure to tularemia. Among those at greatest risk are laboratory workers, who contract the disease through accidental inoculation or inhalation.

DISEASE PATHOGENESIS

F. tularensis can be contracted through the skin, gastrointestinal tract, mucosal surfaces, and through the lungs. The route of entry directly correlates to the clinical manifestations and the severity of the disease [12]. Since the primary cell infected by *F. tularensis* is the macrophage, it is logical that the major organs affected are heavily populated with macrophages including lungs, pleural space, spleen, liver, kidney and lymph nodes [19-22]. Untreated subcutaneous skin or mucous membrane infections can migrate to lymph nodes, replicate and disseminate throughout the

body. Tissues at the site of a *F. tularensis* subcutaneous infection release a necrotic discharge before forming a granulomatous legion [19, 22]. Tularemia can present in many forms including; ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic, typhoidal, and septic tularemia [19-24]. The glandular infections often arise from handling contaminated animal remains or bites from an arthropod vector. Glandular infection refers to enlargement of local lymph nodes, while ulceroglandular is similar but forms an ulcer pustule on the skin surface. Ingesting contaminated food or water and occasionally inhaling droplets can also lead to oropharyngeal tularemia.

Respiratory infections cause acute pneumonic symptoms that are the most harmful. Exposure through inhalation in human cases has shown hemorrhagic swelling of the bronchial tubes, which can progress to bronchopneumonia [25]. Pathological findings of an inhalation exposure have shown the alveolar spaces filled with exudate as well as pleuritis with lymphoadenopathy [22, 23]. Formation of granulomas and fibrosis were observed in monkeys following challenge with a highly virulent *F. tularensis* strain [26].

FRANCISELLA SPECIES

Several biovars of *F. tularensis* exist and are classified into two groups, the highly infectious Type A pathogen or the less pathogenic Type B strains [27]. Reportedly the most dangerous strain is *F. tularensis* subspecies *tularensis*, a Type A pathogen [12, 28]. The other less virulent Type B strains include *F. tularensis* subspecies *novicida* and *F. tularensis* subspecies *holarctica*, which are typically found in Eurasia. Although considered less pathogenic, Type B strains still produce

mild disease symptoms in humans and remain highly virulent in mice [29]. A live vaccine strain (LVS) was originally developed from *F. tularensis* subspecies *holarctica* to vaccinate laboratory workers but the basis for attenuation in humans is largely unknown and thus the vaccine is not approved for use in the US [30].

BIOWEAPON

The infectious inhalation dose of *F. tularensis* has been determined to be between 10 and 25 organisms to cause severe disease [15]. Systemic spread and low infectious dose lead to significant morbidity and mortality and are among the reasons this bacterium has been considered a potential for weaponization. Previous reports suggest that *F. tularensis* was developed into a weapon during the 1930s and 1940s by several countries including the US, Russia and Japan [28]. Claims have been made by a prominent Soviet military scientist, that as late as the 1990's biological weapons using *F. tularensis* were being developed [31]. These strains include modifications resulting in increased antibiotic resistance and other enhanced virulence mechanisms that are pathogenic even in vaccinated individuals [12]. The lack of identified virulence genes, various routes of transmission and ease of dissemination have caused the CDC to classify this bacterium a Category A pathogen.

MECHANISM OF SURVIVAL

There are three methods described that bacteria utilize to survive phagocytic cell defenses including escape, adaptation, and avoidance. Being able to escape phagosome-lysosome fusion is the primary survival strategy of bacteria such as

Shigella and *Listeria* [32]. However, *Coxiella* and *Salmonella* represent bacteria that adapt to the harsh environment created in the phago-lysosome [32-34]. Avoidance is the third identified approach to endure in a host immune cell. *Chlamydia* and *Legionella* are organisms that block fusion of the phagosome and lysosome and thus prevent phagosomal acidification to maintain survival [35-37].

The macrophage is the primary target cell for *F. tularensis*, although non-phagocytic cells such as epithelial cells, fibroblasts and hepatocytes can also become infected [38, 39]. *F. tularensis* is thought to use a combination of strategies to invade, replicate, and escape a host macrophage [40-42]. Studies have begun to address at which stages of phagosome maturation *F. tularensis* evades degradation by what mechanism.

Recent findings have determined that *F. tularensis* enters quiescent macrophages via the formation of asymmetrical pseudopod loops [43]. Seconds after internalization, the *Francisella* containing phagosome remodels to an early endosomal stage [40, 41]. During normal phagosome maturation the particle containing phagosome fuses with the lysosome and acidifies to degrade the particle. Evidence supports that phagosomal pH is slightly different between phagosomes containing live and killed *F. tularensis*. Modified alkaline phagosomes block *F. tularensis* replication, suggesting acidification is required for proper bacterial growth. However, directly measuring the phagosome pH following infection has suggested that the pH does not decrease but equilibrates to approximately 6.7 and thus arrests phagosome maturation [42]. Therefore, *F. tularensis* is thought to neutralize the

phagosomal environment through disruption of the membrane within 3-4 hours of infection. *Francisella* successfully escapes into the cytosol to replicate within 8-12 hours, the exact mechanism of escape is still to be elucidated [43].

The ability of *F. tularensis* to survive within the harsh macrophage environment has been attributed in part to previously identified virulence factors. On the described *Francisella* pathogenicity island are *mglA*, *iglABCD*, and *pdpD* genes all of which seem to be required for intracellular survival [44-46]. MglA has been described as a transcriptional regulator, coordinately regulating *iglC* and *pdpD* [16]. Studies have demonstrated that both IglA and IglC proteins are necessary for intra-macrophage replication [47, 48]. Other noteworthy observations of a *Francisella* infected macrophage are the significant down-regulation of pro-inflammatory responses including superoxides, TNF-α and other cytokine production [15]. It also seems that *F. tularensis* has a modified lipopolysaccharide (LPS), reportedly 1000-fold less reactive than other gram-negative bacteria making it unresponsive to Toll-like receptor 4 (TLR4) and therefore signals induced through the normal TLR4 pathway [49, 50]. Moreover, when the bacteria escapes the macrophage it induces apoptosis which is an non-inflammatory event [51]. Taken together these features represent a unique method of intracellular survival and escape of *F. tularensis*.

GENETIC IDENTIFICATION

F. tularensis is a member of the γ-proteobacter family *Francisellaceae* that has been grouped based on both lifestyle and phylogeny with intracellular bacteria *Coxiella burnetii* and *Legionella* [28] [52]. The recently published genome sequence of *F. tularensis* revealed a 1.8Mb genome with a G+C content of 32.9%, consisting of 1804 predicted coding sequences [10, 28]. Through homology no new virulence genes of *F. tularensis* have been identified and interestingly those previously found, *iglC* and *pdpD* cannot be linked to other species through sequence homology [28]. However, uncovered in the genome were surface related potential virulence factors including all the associated genes needed for a Type VI pilli, and *capB* and *capC* genes required for capsule production in other bacterial species [28]. Despite the few probable virulence genes found through the recently sequenced genome, over 30% of open reading frames remain classified as hypothetical proteins [28]. Thus, it is likely that there are several factors still to be identified.

GENETIC MANIPULATION

Prior to this study several attempts had been made to uncover virulence genes through the generation of a transposon library. However traditional genetic techniques are not efficient in *Francisella* species. The ability to introduce foreign DNA into *Francisella* has been limited due to low transformation efficiency and a lack of replicating vectors. Systems designed to mutagenzie *Francisella* species have typically used bacterial transposons, including Tn10, Tn1721, TnMax2, and recently Tn5 [53-55]. These attempts have proved unstable, possibly due to transposon re-

activation by resident *Francisella* transposases. Of the few investigations claiming stable insertion of a transposon element, no complete mutagenesis library has been generated [55]. To date several plasmids have been developed that can be transformed into *F. tularensis* and have been shown to stably replicate without adverse effects. These plasmids are useful for complementation but would still require repeated introduction if used to generate a library [54, 56]. Thus, the development of a system that can be efficiently introduced and stably maintained in *Francisella* would provide a new genetic tool to study this highly infectious bacteria.

This investigation uses a novel plasmid construction that does not require repeated introduction into the *F. tularensis* LVS genome but instead utilizes a single parent clone to generate a library. The second unique advantage of this system is using a mariner family transposon, which has eukaryotic origins. Although eukaryotic in origin, the mariner-himar1 transposon has been previously demonstrated to facilitate *in vivo* transposition events in different bacterial species [57]. In addition, the himar1 transposon has been shown to be specific for initial activation to the himar family transposase, suggesting that using this eukaryotic system in a bacterial genome avoids potential transposon re-activation by remnant bacterial transposase genes present in the *F. tularensis* genome. Furthermore, the insertion sequence requirement for this himar1 transposon is a TA dinucleotide [57]. Thus, using the mariner-himar1 system is particularly advantageous in *F. tularensis*, which has a genome composed of 70% AT nucleotides [15]. For this study the plasmid was constructed such that the transposase is under control of an arabinose inducible promoter, which allows for

regulation of transposition. This design should permit efficient generation of a stable transposon mutant library in *F. tularensis* LVS to aid in identification of genes involved in pathogenicity.

SPECIFIC AIM: To develop a random mutagenesis system in *Francisella tularensis* LVS. The library will be generated through an inducible mariner-himar1 transposon system and aid in identifying genes important for intra-macrophage survival.

Chapter 2

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. All bacterial strains and plasmids used are listed in Table 1. *F. tularensis* LVS strain 11 obtained from FDA has been described previously [58]. LVS was maintained on chocolate agar media containing 3.6% (wt/vol) Difco GC medium base (Becton Dickinson, Franklin Lakes, NJ) with 1% (w/v) of DCM Hemoglobin Bovine Freeze-dried (Becton Dickinson, Franklin Lakes, NJ) and supplemented with 1% (vol/vol) IsoVitaleX (Becton-Dickinson, Franklin Lakes, NJ) and 0.1% (wt/ vol) glucose. All LVS bacteria plated on chocolate agar were grown at 37ºC with 5% CO_2. Liquid stocks of LVS were cultured in Mueller-Hinton media (Becton-Dickinson, Franklin Lakes, NJ) containing 0.025% (vol/vol) ferric pyrophosphate ($Fe\text{-}PP_i$), 0.1% (wt/vol) glucose, and 2% (vol/vol) IsoVitaleX; this media was designated MH+. Mueller-Hinton broth without glucose was used for arabinose induction and denoted MH-. LVS bacteria in liquid culture were grown shaking at 37ºC in the absence of CO_2. Frozen stocks were made by resuspending bacteria in 20% glycerol and MH+ broth; vials were stored at -80ºC. All LVS was manipulated and maintained in biosafety level-2 conditions [59].

Escherichia coli strain β2155, which is auxotrophic for diaminopimelic acid (DAP) was grown on Luria Bertani agar (BIO101, Carlsbad, CA) plates supplemented with 50µl of 0.3% (wt/vol) DAP, top-spread and put at 37ºC overnight

[60]. Liquid cultures were grown in 5mL volumes of LB broth also supplemented with DAP, and rotating at 37ºC overnight.

Vibrio cholerae strain 0395N1 stock cultures were maintained on Luria Bertani (LB) agar plates containing 100µg ml^{-1} streptomycin sulfate (EMD Biosciences, Gibbstown, NJ) grown at 37ºC overnight. Frozen stocks kept at -80ºC were prepared in 20% (vol/vol) glycerol 80% (vol/vol) LB liquid media. Prior to experimentation liquid cultures with streptomycin were grown in LB rotating at 37ºC overnight.

Conjugal Transfer of Plasmid.

Competent DAP^- *E. coli* were grown aerobically to mid-log phase and prepared for electroporation with a series of 20% glucose washes. The transformation conditions were as previously reported [54]. Once the plasmid, pFT-mariner had been successfully electroporated bacteria were plated on LB agar plates supplemented with DAP.

For bacterial conjugation conditions were used as previously described [61]. The DAP^- *E. coli* donor strain containing the plasmid, pFT-mariner was used to transfer the plasmid to *F. tularensis* LVS [62]. Bacteria were grown in liquid culture of MH+ broth for two days prior to conjugation. *E. coli* were grown on an LB plate supplemented with 0.3% (wt/vol) DAP overnight at 37ºC. Bacteria were both pelleted and washed in MH+ broth by centrifuging at 8000 rpm for four minutes and finally combined in a suspension of MH+ broth containing 0.3% (wt/vol) DAP to support *E. coli* growth. Cells were plated onto chocolate agar plates, no antibiotics and maintained at 25ºC for about 20 hours. Bacteria were washed off plates with 1ml of

MH+ broth and diluted 1:10, 1:100, and 1:1000 before plating 100µl of each. Cells were spread onto chocolate agar media containing 100µg ml^{-1} Polymyxin-B (Sigma, St. Louis, MO) for *E. coli* counterselection and 5µg ml^{-1} kanamycin to promote the presence of pFT-mariner. Founder clones were maintained on chocolate agar plates containing 5µg ml^{-1} kanamycin.

Table 1. Strains and Plasmids used in this study.

Strain or Plasmid	Description of Use	Source
Escherichia coli Strains		
β2155	DAP^- used for conjugation	[60]
Top 10 Cells	Chemically competent cells used for cloning	Invitrogen
Francisella tularensis Strains		
LVS Strain 11	Transposon mutagenesis study	FDA [63]
Vibrio cholerae		
0395N1	Assess functionality of mutagenesis plasmid	[64, 65]
Plasmids		
pFT-mariner	*F. tularensis* LVS mutagenesis vector	[62]
pUC19	Cloning to detect the site of transposon integration	Invitrogen
Mammalian Cell Line		
RAW 264.7	Murine macrophages infected to determine *F. tularensis* LVS survival	ATCC

Detection of pFt-mariner in founder clones. Plasmid DNA was isolated according to the QIApre p Spin Miniprep Kit (Qiagen, Valencia, CA) and denoted pDNA. All pDNA of exconjugates were screened by PCR for the presence of the plasmid. There are three genes unique to the plasmid that primers were designed to detect: *sacB*, transposase, and a piece of the kanamycin-himar fragment. As a negative control genomic DNA (gDNA) isolated using the MasterPure™ DNA Purification Kit (Epicentre Biotechnoloies, Madison, WI) from LVS bacteria was used as a template attempting to detect the same three pieces of the plasmid. All preps of LVS bacteria were also run with a known unique *Francisella* gene, *iglC* to ensure that all samples were LVS and to verify the PCR reaction worked. The positive control for these PCR reactions was pFT-mariner, using primers to detect three unique plasmid features, *sacB*, transposase, and kanamycin fragments. Table 2. lists all primers used in this study.

PCR Protocol as follows:

Step 1: 94ºC – 2:00 min
Step 2: 94ºC – 0:30 min
Step 3: 58ºC – 0:30 min
Step 4: 72ºC – 1:10 min
Step 5: go to step 2 – 34 times

Step 6: 72ºc – 7:00 min
Step 7: 4ºC

Table 2. PCR primers used in this study.

Target	Primers	PCR product
iglC	F: 5' – TCATAATACCCCATGCTTCATCAG – 3' R: 5' – ACAAGACAACAGGTAACAAGTGGC – 3'	~ 500bp
sacB	F: 5' – GTCAAGTTCAGCCACATTTACATC – 3' R: 5' – TCTGCGTAGAATCCTCTGTTTGTC – 3'	~ 800bp
Kanamycin transposon	F: 5' – TAGTACCAACCTTCAAATGATTCCC – 3' R: 5' – CTGTAATGAAGGAGAAAACTCACCG – 3'	~ 900bp
Transposase	F: 5' – ATGGAAAAAAAGGAATTTCGTCTTTTG – 3' R: 5' – TTATTCAACATAGTTCCCTTCAAGAGC – 3'	~ 800bp
Transposon out primer	F: 5' – AAACAATCTGGCCCTGATAGTC – 3'	Varies in Size
20mer of T's	F: 5' – TTTTTTTTTTTTTTTTTTTN – 3'	Varies in Size
FT 1250	F: 5' – ATGAACGAAATAGTATAAAAAATCTAATCGCG- 3' R: 5' – TTATTCCTTATGGTCTACGCCTCTGAA – 3'	~ 900bp

N- variable nucleotide position

Induction and Selection Scheme. Induction of the transposition event is regulated through the addition of arabinose. *F. tularensis* LVS was grown in MH+ broth overnight and washed twice with MH- broth. Cultures were incubated in 1mL of MH- broth containing 0%, 0.5%, 1% or 2% (vol/vol) of L-Arabinose (Sigma, St. Louis, MO) and incubated rotating overnight at 37ºC, to find optimal induction conditions, Table 3. Following this 24-hour incubation period the cells were pelleted, to remove the arabinose and resuspended in 3mLs of MH+ broth, before being incubated overnight shaking at 37ºC.

To counterselect against pFT-mariner, bacteria were plated on chocolate agar plates containing 10% (wt/vol) sucrose. With sucrose present instead of glucose the *sacB* gene creates a levansucrase product that is toxic to cells [66]. Any colonies that survive the sucrose challenge were streaked onto chocolate agar plates with 5µg ml^{-1} kanamycin, and incubated at 37ºC with 5% CO_2.

Verification of Probe & Southern Blot Analysis. Individual clones were transferred to MH+ broth in preparation for genomic DNA harvesting. All clones and LVS samples were washed twice in 0.5M sucrose prior to DNA isolation. On each blot controls of 1µg of pFT-mariner prepared by a QIAmini-prep Kit (Qiagen, Valencia, CA), gDNA from LVS, and a biotinylated ladder (New England BioLabs, Ipswich, MA).

Southern Blot. Using freshly prepared gDNA only, 5µg of DNA was digested with *Nde*I (Invitrogen, Carlsbad, CA) overnight and resolved in 0.7%

(wt/vol) agarose gel made in Tris base, Acetic acid, EDTA (TAE). Gels were stained with ethidium bromide and visualized under UV light to verify both adequate digestion of the DNA and minimal degradation. Photographs were taken to provide documentation for all gels. Gels were then rinsed with distilled deionized water (ddH_20), washed twice for 20 minutes in denaturation solution (0.5M NaOH, 1.5M NaCl) followed by a second rinse in ddH_20 and finally two 20 minutes washes in neutralization buffer (0.5M Tris-HCl pH 7.0, 1.5M NaCl pH 7.5). The DNA was then transferred to a positively charged nylon membrane (Roche Diagnostics Corporation, Indianapolis, IN) through capillary action [67]. Using an electrophoresis chamber with 6x sodium chloride/sodium citrate solution (SSC) soaked whatman paper as a wick the gel was placed on it DNA side up. Soaking a piece of nylon membrane and two more pieces of whatman paper in 2x SSC, they were stacked on top of the gel. A 4-6" stack of paper towel the same size as the nylon membrane and a weight was placed on top the entire sandwich and left overnight to transfer.

Dot blot. Genomic DNA obtained fresh from LVS bacterial samples, at a concentration of 5µg and 1µg of pFT-mariner were denatured by boiling for 10 minutes and rapidly cooled on ice for 5 minutes to prevent re-annealing of the DNA prior to applying to the membrane. The DNA was then spotted by pipette onto a piece of dry nylon membrane.

DNA on membrane (via transfer or manual application). The membranes are baked at 80ºC for 2 hours, which cross-links the DNA to the membrane. The blots

were then soaked rotating at 68ºC in pre-hybridization buffer for 3 hours. Pre-hybridization buffer (6.25ml 20x SSC, 0.25ml 10% sarkosyl, 0.05ml 10% sodium dodecyl sulfate (SDS), 0.25 blocking reagent, 18.5ml H_20, 100µg mL^{-1} of Herring Sperm DNA (Promega)). After initial incubation the blots are exposed to fresh pre-hybridization solution and the appropriate biotinylated probe and left rotating at 68ºC overnight.

Probe. Blots were probed using biotinylated nucleotide PCR amplified sequences (New England Biolabs, Ipswich, MA). Using gDNA LVS as the template to detect FT.1250 a 726bp single copy gene and pFT-mariner as the template for an 900bp kanamycin-himar fragment that is specific to the transposon sequence [28][68]. Prior to addition the probe was column purified (Roche Diagnostics Corporation, Indianapolis, IN) then boiled for 10 minutes and put on ice for 5 minutes to denature the DNA probe.

Developing and Detection. After hybridization, the blots were washed twice for 15 minutes on an orbital shaker at room temperature in 0.1% (wt/vol) SDS and 2x SSC. Followed by a second set of washes rotating for 15 minutes each at 68ºC in 0.1% (wt/vol) SDS and 0.5X SSC. Blocking buffer (5% SDS, 125mM NaCl, 25mM sodium phosphate, pH7.2) was added to the blots and incubated for 45 minutes rocking at room temperature. Streptavidin and biotinylated alkaline phosphatase were diluted 1:1000, in blocking solution and added according to the NEB Phototope-Star Detection Kit (New England BioLabs, Ipswich, MA). Using the Phototope-Star Detection Kit, CDP-Star reagent at a dilution of 1:1000, blots washed for five minutes prior to developing. Autoradiography (AR) film (Kodak) was exposed to the blots

from 30 minutes to 2 hours and developed using an RP X-OMAT film processor (Kodak).

Identifying the site of insertion.

Cloning. Approximately 2µg of gDNA from a potential clone and 1µg of pUC19 pDNA were digested with *Nde*I at 37ºC overnight. Following digestion pUC19 was dephosphorylated using calf intestinal alkaline phosphatase (CIAP) at 37ºC for 1 hour. The CIAP was heat inactivated at 80ºC for 10 minutes, gDNA fragments and linear pUC19 were combined in a 2:1 ration, and ligated at 16ºC overnight with T4 ligase (New England BioLabs, Ipswich, MA). Following ligation DNA was transformed into One Shot TOP-10 Chemically Competent *E. coli* cells (Invitrogen, Carlsbad, CA). All cells were plated onto LB agar plates containing 50µg ml^{-1} kanamycin. Any colonies that result will be sequenced to determine the site of transposon insertion.

To verify the efficiency of cloning the kanamycin gene we artificially mimicked founder clone DNA with 1µg of pFT-mariner mixed with 2µg of gDNA from wild type (wt) LVS. The same digestion and ligation of the mixed LVS/ pFT-mariner DNA into pUC19 was followed. It is important to note that *Nde*I has restriction sites in both the wt LVS genome and multiple sites in pFT-mariner. To ensure pFT-mariner and pUC19 could not re-ligate a sample 10µl volume of each was used as a control during ligation and transformation. pFT-mariner was plated on LB agar plates with 50µg ml^{-1} kanamycin and pUC19 was plated on LB agar containing

100µg ml^{-1} ampicillin and 50µl of (conc.) 5-bromo-4chloro-3-indoyl-beta-D-galactopyranoside (X-gal) top spread.

Run-Off PCR. Approximately 5µg of genomic DNA was digested overnight at 37ºC with *Dra*I, which cuts gDNA from LVS about every 700bp but does not cut the kanamycin containing transposon element. The digested DNA was run on a 1% agarose gel in 1x TAE at 80V for 2 hours. Everything above 1kb was cut and gel purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) then used as a template for the first of two PCR reactions that uses a single primer that was designed specifically to read outward off the transposon fragment into the bacterial genome. The PCR reaction is as follows:

Step 1: 94ºC – 2:00 min
Step 2: 94ºC – 0:30 min
Step 3: 57ºC – 0:30 min
Step 4: 72ºC – 3:30 min
Step 5: go to step 2 – 34 times
Step 6: 72ºC – 7:00 min
Step 7: 4ºC

The resulting single strand DNA, ssDNA was spin column purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and eluted in a total volume of 30µl. Terminal deoxytransferase, TdT (New England BioLabs, Ipswich, MA) was used to add a series of dATPs were added to the 3' end of the ssDNA in the following reaction:

Step 1: 37ºC – 15:00 min
Step 2: 70ºC – 12:00 min
Step 3: 4ºC

The TdT reaction was used as a template for a second PCR, now with two distinct primers (Table 2).

Step 1: 94ºC – 2:00 min
Step 2: 94ºC – 0:30 min
Step 3: 58ºC – 0:30 min
Step 4: 72ºC – 5:00 min
Step 5: go to step 2 – 34 times
Step 6: 72ºC – 7:00 min
Step 7: 4ºC

The resulting reaction mixture was run on a 1% agarose gel in 1x TAE at 100V for 45 minutes. Any distinct bands were gel purified using QIAquick Gel Extraction Kit. The bands were then cloned into TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA) and sequenced using M13R, a TOPO vector (Figure 1).

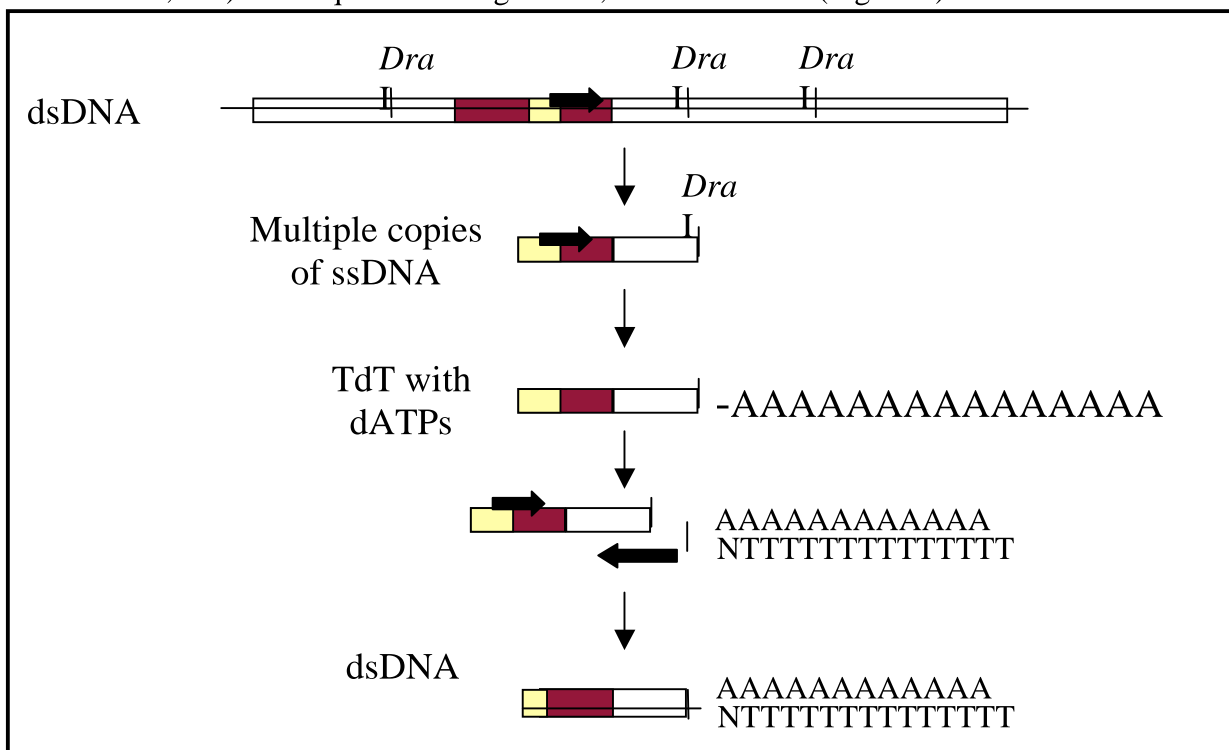

Figure 1. Schematic of Run-Off PCR amplification used to identify the site of the transposition event.

Macrophage Infection. RAW 264.7 murine macrophages were maintained in Dulbecco's Modified Eagle Medium with 10% Fetal Bovine Serum (FBS), complete DMEM (Gibco-Invitrogen, Carlsbad, CA) and plated at $2x10^5$ cells per well in a 24 well plate (Becton-Dickinson, Franklin Lakes, NJ) incubated at 37ºC with 5% CO_2 overnight. Bacterial cultures were adjusted to an OD of 1.00 at 560nm in phosphate buffered saline (PBS) for a concentration of approximately $1x10^9$ ml^{-1} [54]. Bacteria were added at a MOI of 1, 10, and 100 to the appropriate macrophage wells, the plates were then spun at 1000rpm for 5 minutes and incubated at 37ºC with 5% CO_2. Two hours post-infection, cells were washed a minimum of three times in serum free DMEM followed by the addition of complete DMEM, and then incubated overnight at 37ºC with 5% CO_2. Macrophage wells were washed several times with PBS and re-suspended in 300µl of ddH_20 to lyse the cells. After 20 minutes 100µl of cell lysates were added per well of the previously prepared ELISA plate and incubated at 4ºC overnight.

ELISA Screening. Nunc 96 well plates (Nunc, Rochester, NY) were coated with 1µg per well of anti-*Francisella*-LPS antibody (Fitzgerald, Concord, MA) overnight at 4ºC. Plates were washed with 0.05% PBS-Tween (EMD Biosciences, San Diego, CA) then blocked in PBS-10% FBS (Invitrogen, Carlsbad, CA) for two hours at room temperature.

To detect the LVS captured by the ELISA 1µl per well of a biotinylated 2º *F.t.*-anti LPS combined with a 1:250 streptavidin-HRP (New England BioLabs,

Ipswich, MA) made in blocking buffer was applied to each well and incubated at room temperature for a minimum of one hour. TMB substrate (PharMingen, San Diego, CA) was used for a colorimetric detection of LVS. TMB was mixed 1:1 and 100µl per well was added for about thirty minutes. To stop the reaction 50µl of 2N H_2SO_4 was applied prior to reading the plates at 450nm.

Chapter 3
RESULTS & DISCUSSION

pFT-mariner Construction

The *Francisella* mutagenesis vector, pFT-mariner constructed specifically for this study was designed to allow for regulation of transposition events through the addition of L-arabinose (Figure 2, Table 3). The plasmid contains a *F. tularensis* specific origin of replication (Ori) and can be introduced through conjugation using an origin of transfer (OriT) from a previously described RP4 plasmid [61]. The transposon insertion, Tn-mar, has a kanamycin resistance marker flanked by mariner family Himar-1 repeats. To allow us to regulate transposition the transposase enzyme is cloned separately under the control of a pBAD promoter. The transcription factor AraC will activate the pBAD promoter in response to the addition of arabinose. For counterselection of pFT-mariner following the transposition event we will utilize the *sacB* gene. In the presence of sucrose, SacB, a levansucrase enzyme is activated and leads to an accumulation of levan a toxic product that causes cell lysis [66]. This method of counterselection has been previously shown to be functional in *Francisella* [61].

Additional features of pFT-mariner that cannot be utilized in *F. tularensis* but are functional in other bacterial species are the ColE1 origin of replication and the ampicillin resistance marker. The ColE1 origin was added to this plasmid to maintain replication in *Escherichia coli*. Following the initial transformation of pFT-mariner into *E. coli* the ampicillin resistance gene was used for selection.

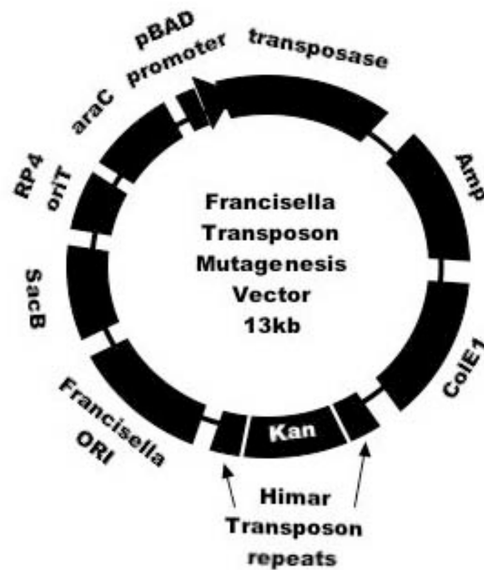

Figure 2. Diagram and features of the *Francisella* mutagenesis vector, pFT-mariner.

Table 3. Features of the *Francisella* mutagenesis vector, pFT-mariner [69].

Plasmid Element	Function	Source	Cloning Method
Himar Transposase	Transposase enzyme	pFD1 plasmid [70]	PCR
pBAD promoter	Regulated by AraC+ arabinose	pBAD-TOPO (Invitrogen)	Plasmid backbone
araC	Arabinose regulated transcription factor	pBAD-TOPO (Invitrogen)	Plasmid backbone
Himar Transposon +Kan	Transposon cassette carries Kan resistance	pFD1 plasmid [70]	Restriction digest
Francisella Ori	*Francisella* specific origin of replication	pKK214 plasmid [56]	PCR
sacB	Counter selection on sucrose	pPV plasmid [66]	Restriction digest
RP4 OriT	Conjugation	pPV plasmid [66]	Restriction digest
ColE1 Ori	*E. coli* origin of replication	pBAD –TOPO	Plasmid backbone
Amp^R	Selection in *E. coli*	pBAD – TOPO	Plasmid backbone

pFT-mariner & *Vibrio cholerae*

Functionality of pFT-mariner

To assess the functionality of the various plasmid genes we tested this system in *Vibrio cholerae*, a bacterium in which genetic tools have been well established. While the plasmid contains a *Francisella* origin of replication it also carries a ColE1 origin, which can be used in *Vibrio* species. Advantages of working with *V. cholerae* include rapid culture time, easy detection of certain phenotypes such as motility of the bacterium, and natural sensitivity to both ampicillin and kanamycin. Rapid overnight culture of *V. cholarae* allows for a quick generation and analysis of both founder and potential library clones. Since *V. cholerae* has a single flagella it is motile in soft agar medium, a trait that can be exploited in a rapid screening assay to detect a visual phenotype [71]. It is important to note that previous work has shown sucrose selection to be effective in *V. cholerae* [72]. The added advantage of the sensitivity of the bacteria to both ampicillin and kanamycin allows for efficient screening for loss of pFT-mariner. This is achieved by replicate plating colonies following sucrose counterselection.

Generating Founder Clones & Arabinose Induction

To verify that transposition of Tn-mar is possible we conjugated the donor *E. coli* strain carrying pFT-mariner with *V. cholerae* strain 0395N1, which yielded numerous potential library founder clones. Screening by PCR revealed the presence of three unique pFT-mariner genes encoding SacB, transposase, and kanamycin resistance (data not shown). Using four identified founder clones we carried out induction as described for *F. tularensis* LVS using varying concentrations of arabinose for 24 hours.

Efficiency of Counterselection

Following arabinose induction, bacteria were diluted, 1:10, 1:100, 1:1000 and plated on 10% sucrose LB agar plates to select for the loss of pFT-mariner. Resulting colonies could therefore have either the desired transposition event or have retained the plasmid and survived the sucrose counterselection. To test this, colonies from the sucrose plates were plated in duplicate onto both LB-kanamycin and LB-ampicillin plates. Colonies retaining the plasmid will be both ampicillin and kanamycin resistant. Those bacteria with the transposition event as well as loss of pFT-mariner will be ampicillin sensitive but kanamycin resistant. Several colonies were isolated and a few chosen at random to be screened by PCR for integration events. Chosen colonies seemed to differ in colony morphology, which suggests the presence of the Tn-mar. PCR analysis of these colonies revealed the transposon marker, kanamycin gene but no SacB gene indicating loss of pFT-mariner. Preliminary results indicated

that 1:6 retain pFT-mariner following counterselection on sucrose (Figure 3, Figure 4).

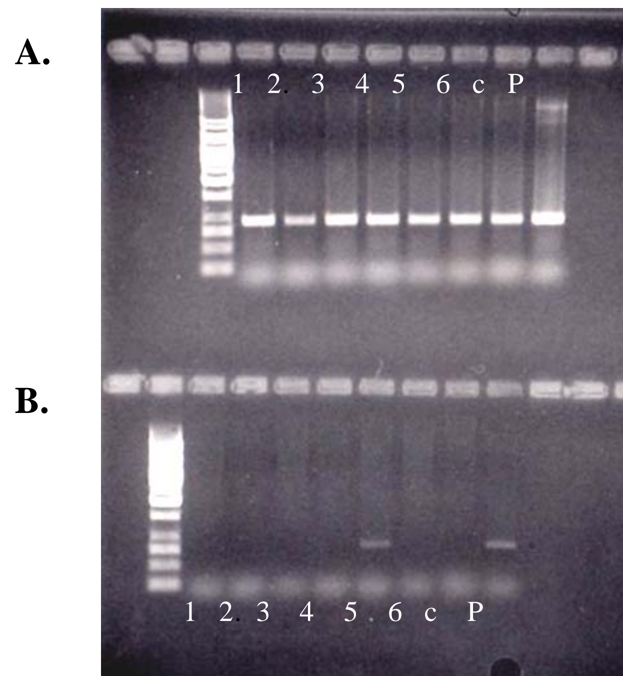

Figure 3. PCR screening for integration of Tn-mar from genomic DNA of potential *V. cholerae* library clones. 1kb standard DNA ladder, six potential clones are shown, c represents a colony PCR attempt, and P is the positive control lane where pFT-mariner was run as the DNA template. **A.** PCR detecting 800bp kanamycin transposon fragment. **B.** PCR to detect 800bp *sacB* fragment, a band indicates the presence of pFT-mariner.

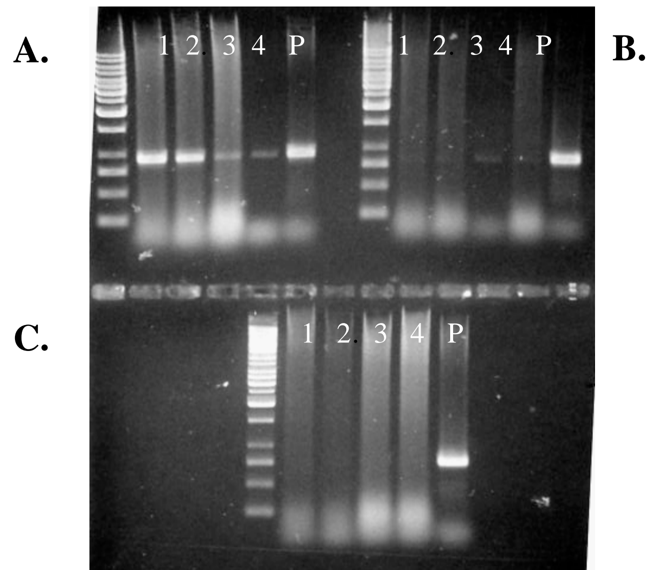

Figure 4. PCR screening the occurrence of transposition events of different potential library clones following arabinose induction and sucrose counterselection. Lanes contain genomic DNA from four potential *V. cholerae* library clones, lanes marked 'P' were run with pFT-mariner as the template.
A. Kanamycin primers **B.** *sacB* primers **C.** Transposase primers.

Positive evidence of transposition suggested that pFT-mariner is functional following conjugation and does integrate in *V. cholerae* using a concentration of 0.5%, 1%, and 2% arabinose. Table 4 shows the approximate number of colonies that resulted on kanamycin containing plates following arabinose induction of transposition at varying concentrations and sucrose counterselection.

Table 4. Concentrations of L-arabinose used during induction of pFT-mariner in *V. cholerae*.

% L-arabinose	# of colonies on Kan^R LB agar plate
0%	0
0.5%	> 10
1%	> 10
2%	> 10

Motility Screening Assay

V. cholerae has multiple genes associated with motility including those involved in creating the flagellum and those related to chemotaxis [73-77]. Thus, it is likely that if a random transposon library is created in *V. cholerae* numerous genes can be disrupted and effect motility. To rapidly screen for motility we stabbed several library clones into soft agar media and were able to detect a non-motile phenotype in 1 out of 30 colonies (Figure 5). DNA of the non-motile colony was subjected to PCR to ensure that a transposition event had occurred, although no follow-up was done to identify the site of insertion. As anticipated the presence of the kanamycin resistance marker, indicative of transposon integration was detected. However as expected, no *sacB* gene or transposase were identified (Figure 6).

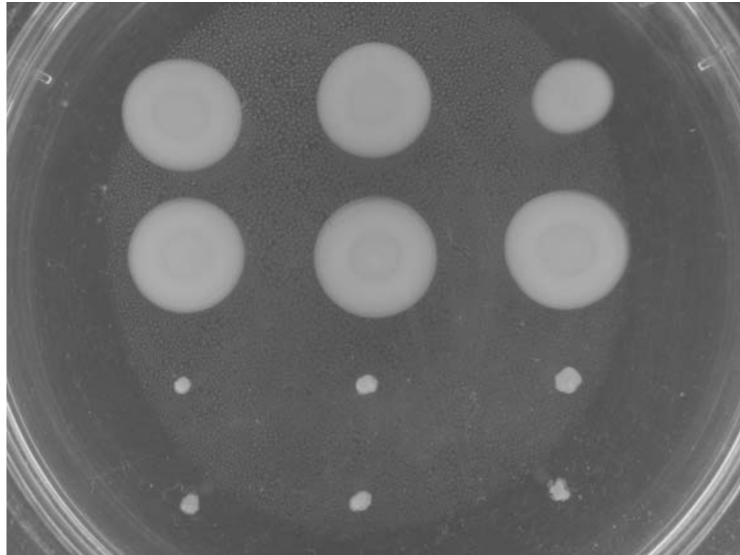

Figure 5. LB soft agar plate, showing multiple stabs of the non-motile phenotype of a *V. cholerae* library clone compared to stabs of other potential clones (representative data).

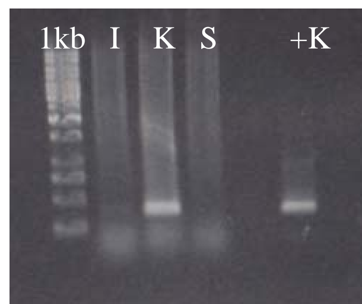

Figure 6. These reactions used genomic DNA from a previously identified non-motile library clone and pFT-mariner control. PCR screening for the presence of the *iglC* gene unique to *Francisella* (I), the kanamycin (K) and *sacB* (S) genes of pFT-mariner. +K lane is a control, which used kanamycin primers with pFT-mariner template. No SacB control is present.

From these results in *V. cholerae* we concluded that our mutagenesis plasmid can be passed through bacterial conjugation and that the transposase is functional

following arabinose induction. Furthermore using a rapid motility assay we were able to readily detect a mutant phenotype in a clone shown to contain Tn-mar, although no correlation was made between insertion site and phenotype.

Chapter 4

RESULTS & DISCUSSION

pFT-mariner & *Francisella tularensis* LVS

Generating Founder Clones & Induction Optimization

Conjugation into *F. tularensis* LVS was performed as previously described and several dilutions were plated onto kanamycin plates [54]. Although small colonies grew on kanamycin containing media many of these potential founder clones failed to grow substantially. When re-streaked onto kanamycin plates, twenty potential founder clones were successfully expanded to obtain enough biomass to allow isolation of plasmid DNA. PCR screening on these potential founder clones revealed the presence of pFT-mariner markers, the kanamycin resistance gene and the transposase gene (Figure 7). Observing positive PCR results for the kanamycin gene and transposase gene, four of these potential founder clones were also screened for the presences of the *sacB* gene. Our findings indicated that at least four clones had multiple markers of pFT-mariner present. Of the four clones screened for three pFT-mariner markers, founder clone 12 was used to optimize arabinose induction. Clone 12 was exposed to arabinose at concentrations of 0%, 0.5%, 1%, and 2% and recovered in complete media for 24 hours prior to selection plating on sucrose. Our preliminary findings suggested that 0.5% arabinose was sufficient for induction but yielded few colonies following sucrose counterselection. As both 1% and 2% arabinose induction resulted in numerous colonies (data not shown), further induction of integration used 1% arabinose exposure for 24 hours.

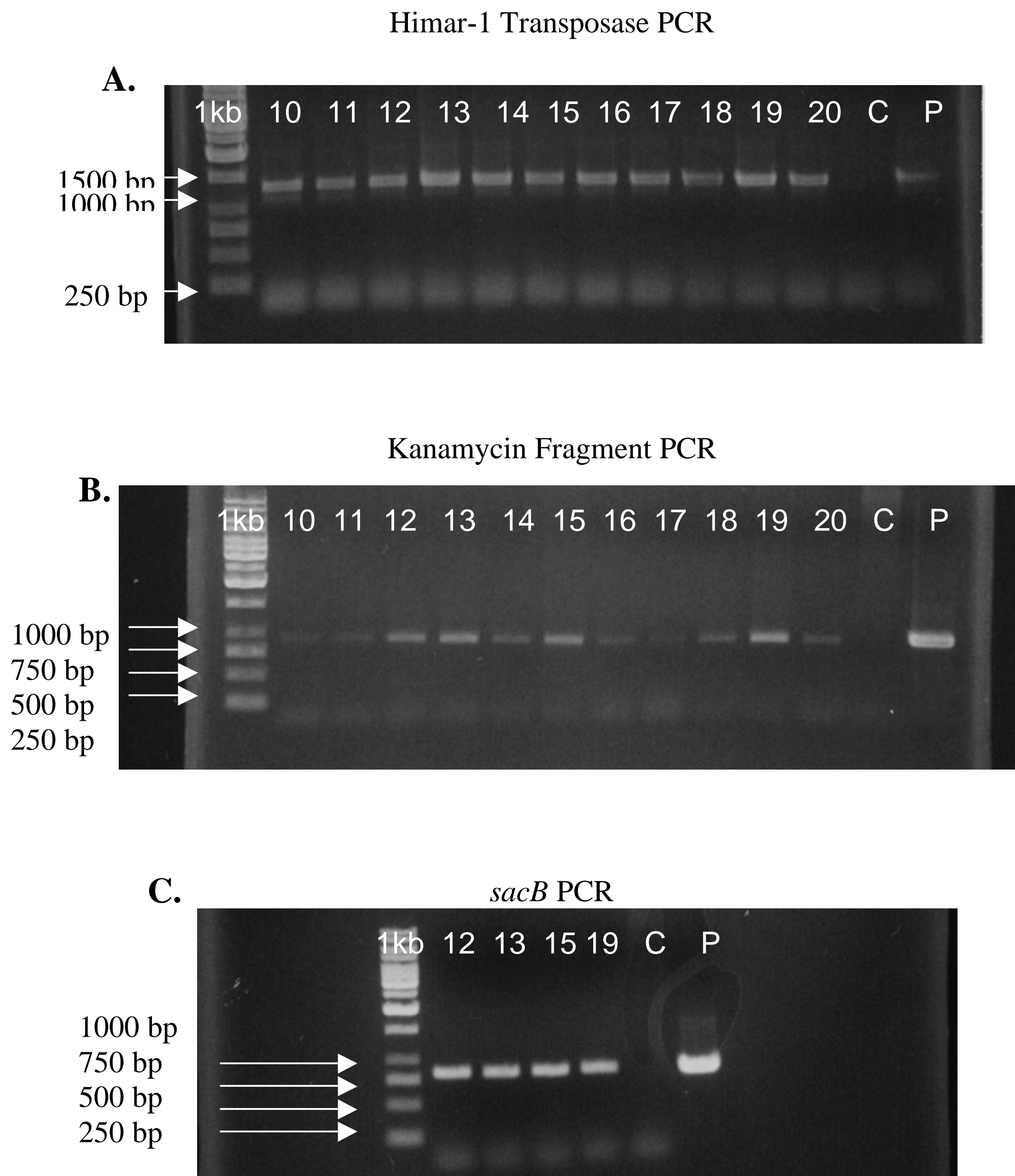

Figure 7. PCR results of potential founder clones, C is the negative control lane using gDNA of wt LVS in the PCR reaction, P lanes indicate use of pFT-mariner as the reactions template DNA. **A.** Eleven clones screened for a 1200bp fragment of the transposase gene. **B.** Eleven potential clones screened for an 800bp fragment of the kanamycin gene. **C.** Select clones were screened by PCR for an 800bp fragment of the *sacB* gene.

Library Generation

Founder clone #12 was chosen to create a comprehensive library and incubated in the presence of 1% arabinose for 24 hours to induce transposition of Tn-mar. Following induction, the culture was plated on chocolate agar containing 10% sucrose at dilutions of 1:100 and 1:1000 to avoid confluent growth, allowing for efficient sucrose counterselection. After approximately two days single colonies were transferred onto kanamycin containing agar plates and many colonies that grew were expanded to obtain enough biomass to isolate genomic DNA. These library clones were screened by PCR using primer specific for the kanamycin-himar fragment of Tn-mar and revealed the presence of this fragment and of the *F. tularensis* LVS specific *iglC* gene. Importantly, these same clones lacked other pFT-mariner identifying markers, such as *sacB* and transposase genes (Figure 8). Taken together, these data suggest that integration of Tn-mar into the chromosome of *F. tularensis* occurred and that sucrose counterselection was effective in eliminating pFT-mariner. Thus, for the first time a study has demonstrated that a mutagenesis library can be generated in *F. tularensis* LVS using a modified mariner-himar1 transposon system.

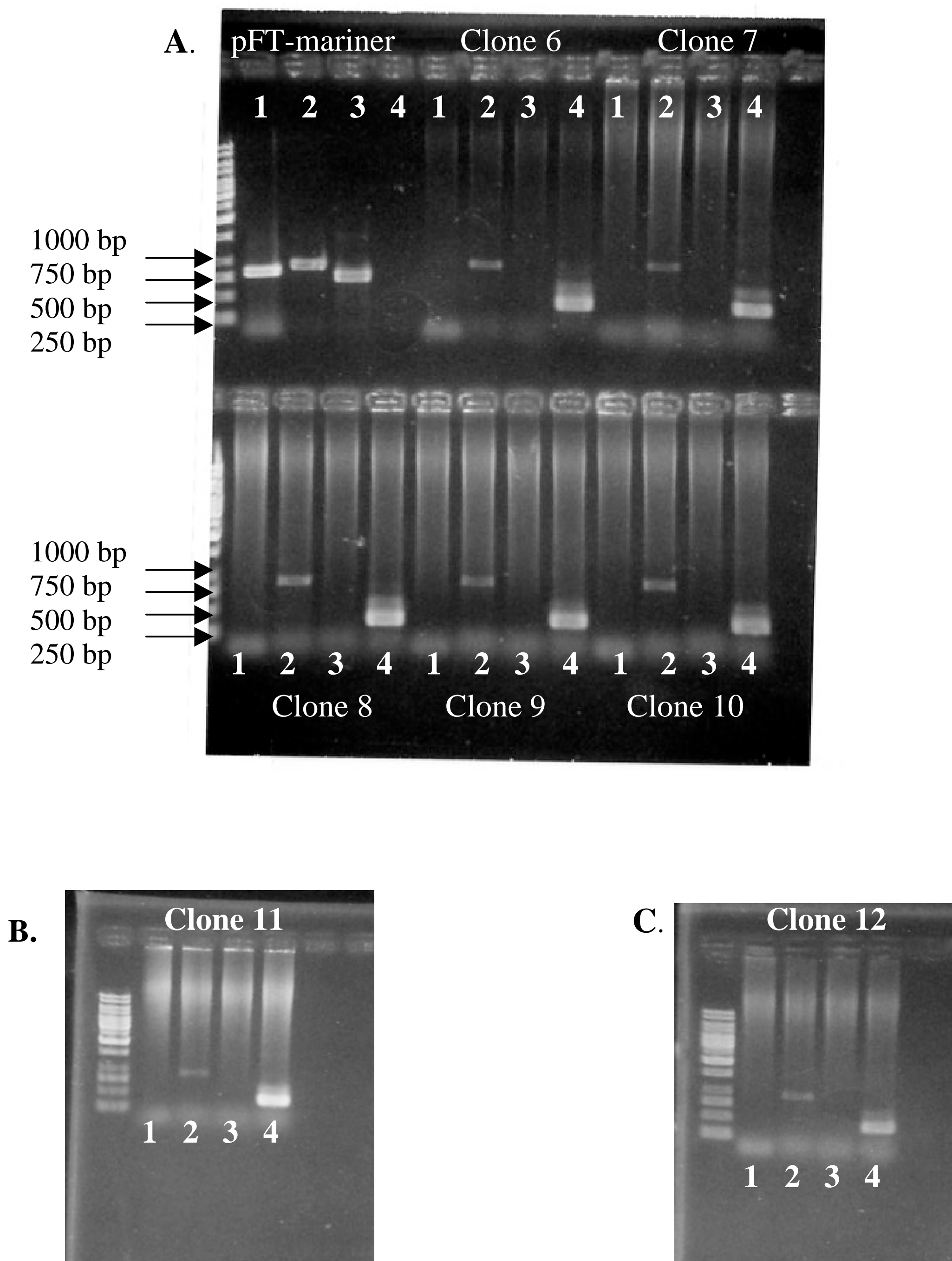

Figure 8. PCR screening for integration of pFT-mariner and several potential library clones. **1**. *sacB* **2.** Kanamycin gene **3.** Transposase gene **4.** *iglC*. **A**,**B**,**C** show results of PCR from different potential library clones.

Macrophage Survival Assay and ELISA Screen

Bacterial infection assays usually require plating serial dilutions of bacteria containing cell lysates post-infection. The difficulties of serial plating are the amount of resources needed and more importantly the incubation time required for this bacterium. To be able to examine the ability of a library mutant to invade and replicate within macrophages, an Enzyme-Linked ImmunoSorbent Assay (ELISA) screening assay was developed. By using an ELISA method we can quickly and accurately quantify bacterial presence from an infected macrophage cell suspension. Preliminary results investigating infection with wt LVS show this method to be accurate in detecting whole LVS, recovered from macrophages (Figure 9).

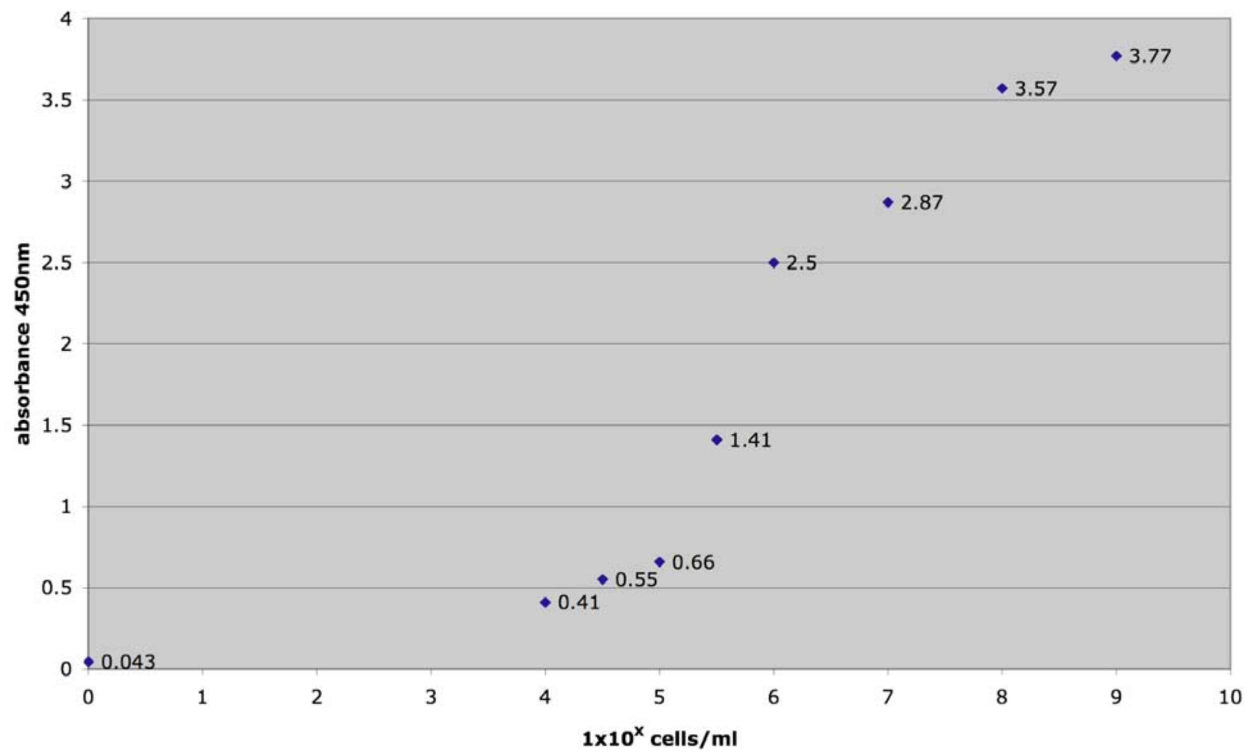

Figure 9. Saturation curve of whole LVS, standard dilutions from 10^3 to 10^9 bacteria/ml are represented.

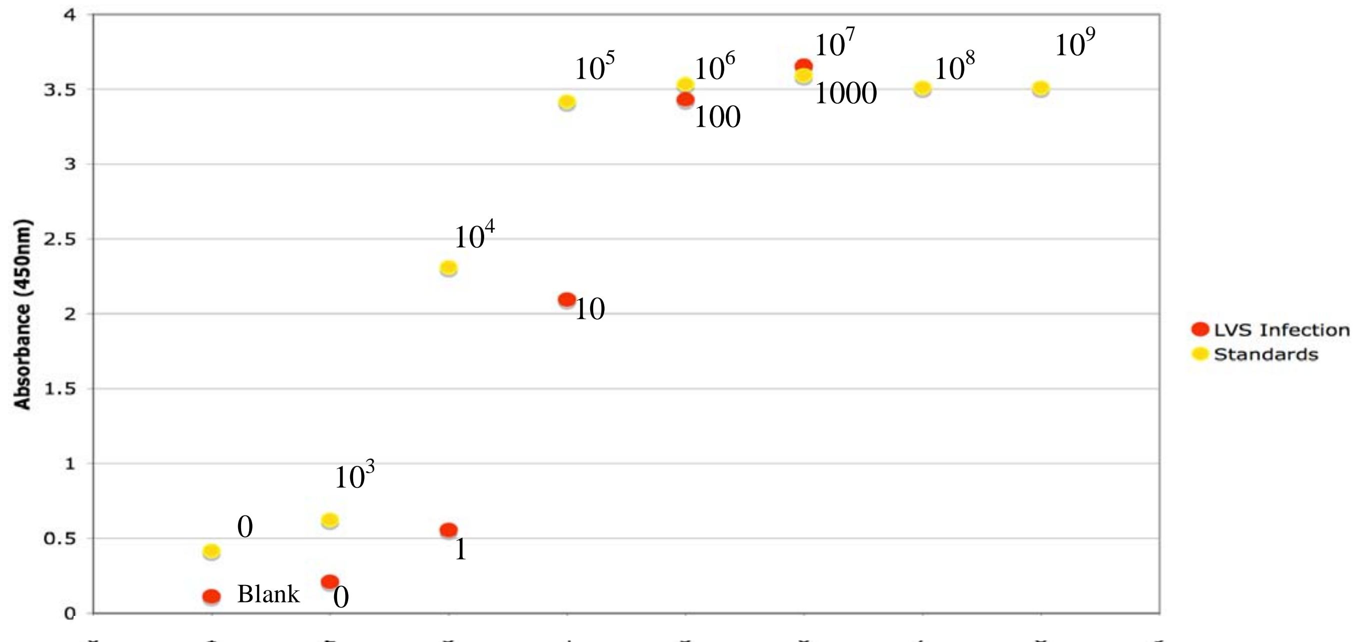

Figure 10. ELISA results of wt LVS recovered from a four-hour macrophage infection comparing varying multiplicities of infection (MOI). Red dots represent MOIs of 1, 10, 100, and 1000; yellow dots represent standard dilutions of LVS.

Identifying the Site of Transposon Insertion

Cloning into pUC19. One method to identify the site of transposon insertion is to clone the fragment of LVS DNA containing the kanamycin resistance gene into a cloning vector, such as pUC19. Should this technique work any colonies that grow on LB kanamycin plates following transformation would be expanded. Isolated plasmid DNA would then be sequenced using pUC19 vector primers to determine which gene the transposon integrated into.

Several attempts to clone the kanamycin gene following digestion with different restriction enzymes failed for unknown reasons (data not shown). Therefore an experiment was designed mixing *Nde*I digested pFT-mariner and digested gDNA from LVS to mimic a founder clone. The restriction enzyme *Nde*I was chosen because it cuts the plasmid in both the *sacB* gene and transposase region, but not within the Tn-mar sequence. A combination of pFT-mariner and LVS gDNA simulating the founder clone was digested along with pUC19. The pFT-mariner/LVS DNA mix was added to pUC19 DNA in a 3:1 ratio to favor ligation into the vector. Controls for this experiment included the digestion and ligation of each fragment individually as well as the digestion of pUC19 alone, followed by plating on ampicillin plates with X-gal to determine cloning efficiency. Figure 11 shows the results of this simulated cloning and the efficiency suggests that if the kanamycin resistance gene were present in the sample it could be identified. Selected colonies that grow on kanamycin plates would be subjected to further investigation to identify the site of insertion.

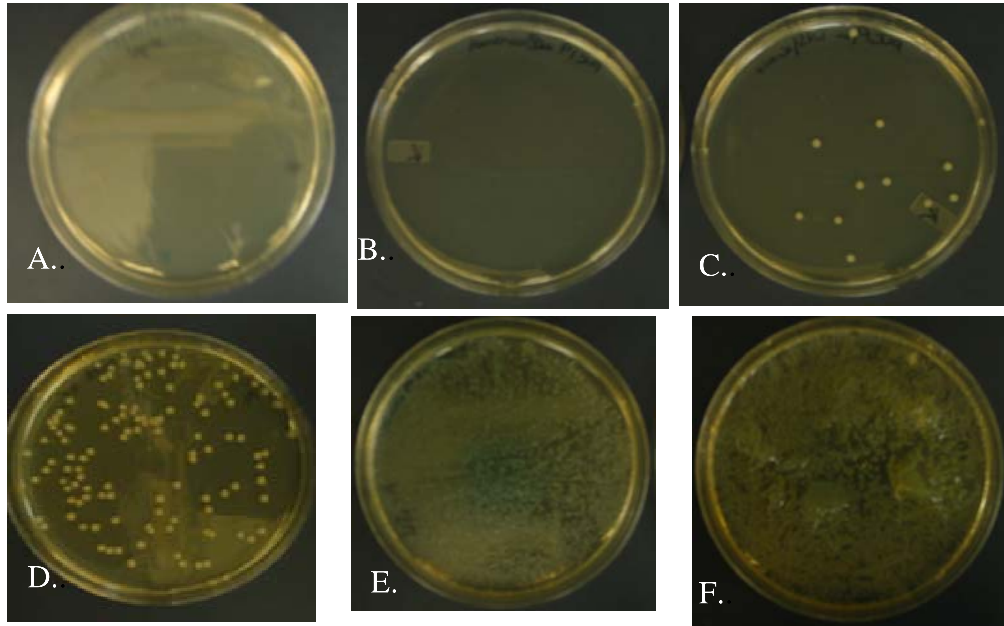

Figure 11. Cloning the kanamycin gene from a 2:1 combination of pFT-mariner and gDNA from *F. tularensis* LVS into pUC19. **A.** Control for *Nde*I digestion of LVS gDNA, ligated to itself and plated on kanamycin. **B.** Control for pUC19 digested with *Nde*I ligated to itself, plated on kanamycin. **C.** pFT-mariner/LVS control digested with *Nde*I and ligated. **D.** pFT-mariner/LVS ligated to pUC19 and plated on kanamycin. **E.** Ligation of pUC19 with pFT-mariner/LVS plated on ampicillin plates supplemented with Xgal. **F.** *Nde*I digested gDNA and pUC19, ligated and plated on LB ampicillin containing Xgal.

Run-Off PCR. Another method to identify the site of Tn-mar insertion is a modified 5' RACE, denoted run-off PCR. Genomic DNA from a potential library clone was digested with *Dra*I, which cuts LVS approximately every 700bp but does not cut the insertion fragment. This strategy takes advantage of the known transposon sequence, using a transposon specific primer a single stranded DNA fragment was created from digested DNA of potential library clones. The terminal deoxytransferase

addition of dATPs to the 3' end of each DNA fragment generates a second unique primer site. The final PCR reaction uses the original transposon specific primer and an oligomer of 'T's anchored by a variable nucleotide position at the 3' end. Any resulting DNA bands can then be cloned into an *E. coli* vector or sequenced directly.

Run-Off PCR was used to determine the insertion site of library clone 6 (C6) and revealed the presence of Tn-mar in the FT0538 gene, a ubiquinone biosynthesis protein [68]. Figure 12 shows the results of C6 following the second PCR reaction of the run-off system. The two bands circled represent the same site of transposon insertion, the larger one is likely the result of an incomplete digestion with *Dra*I. The lower band was then cut and cloned into the TOPO-TA vector prior to sequencing with the vector primer, M13 reverse. Preliminary data using C6 show this modified technique to be a valid method for identifying the site of transposon insertion (Figure 13).

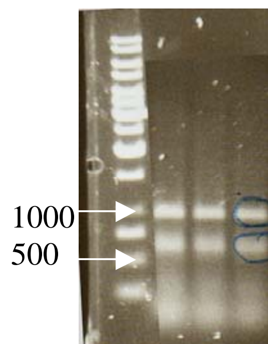

Figure 12. The two bands used to identify the site of integration following Run-Off PCR amplification of *F. tularensis* LVS mutagenesis library clone 6 (C6).

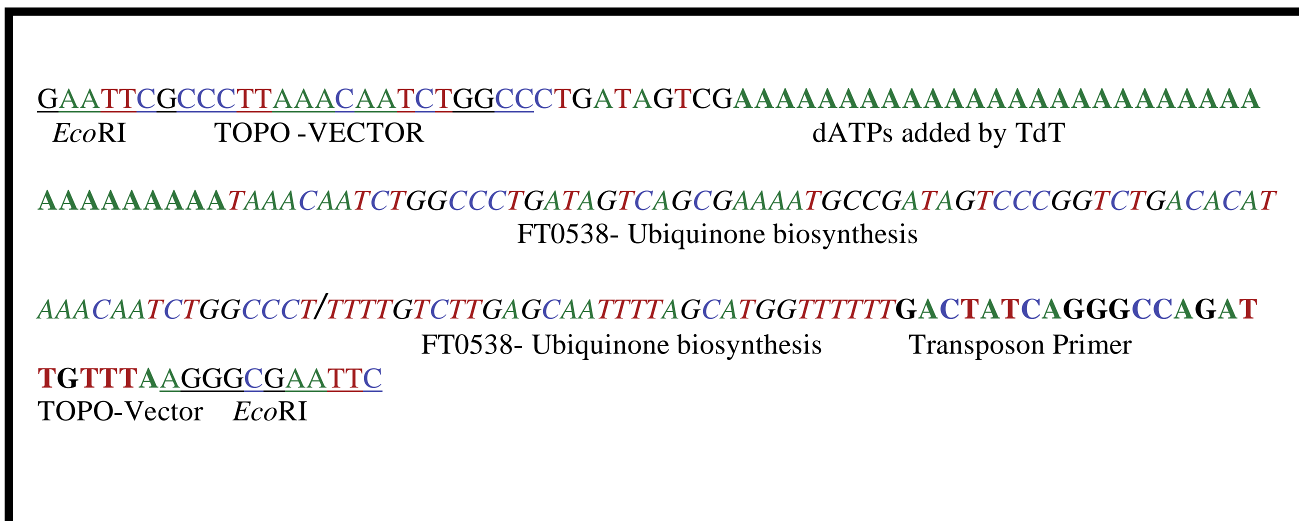

Figure 13. Run-off PCR sequence showing the site of Tn-mar insertion in C6.

Run-off PCR attempts to duplicate this result were unsuccessful, failing to produce distinct DNA bands but rather yielding smears of DNA (data not shown). These data indicate a potential non-specific priming event during the initial Run-off reaction, creating multiple single stranded DNA fragments. These results suggest that either, Tn-mar is no longer present in the C6 genome or that the initial run-off PCR was due to non-specific priming.

To independently verify the presence of Tn-mar in C6, primers were designed to amplify the previously identified disrupted gene, FT0538 and gDNA from LVS compared to gDNA from C6. The expectation was to observe the wild-type gene (636bp) in the reaction containing LVS DNA but a band 1432bp larger in the reaction containing C6. However, analysis of C6 revealed no difference between the library clone and LVS, strongly suggesting the transposon is no longer present in FT0538 (Figure 14). It is important to note that the isolated DNA used in these PCR reactions

were from different growth passages of bacteria than the original run-off PCR. These results could be indicative of transposon instability among other possibilities.

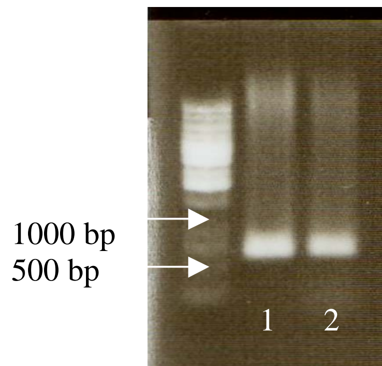

Figure 14. PCR amplification to verify the previously identified transposon integration site using gDNA from LVS (**Lane 1**) and C6 (**Lane 2**) as template.

Southern Blot Analysis

Although, the copy number of pFT-mariner is unknown in *Francisella*, it is not expected to be very high. Since the mariner transposon uses the "cut and paste" method of transposition, every copy of the plasmid could induce a single Tn-mar integration event potentially leading to multiple transposition events per genome. While the total number of integration events is important to know it is also crucial to this investigation to verify random insertion of Tn-mar.

However, several problems were encountered during southern blot analysis, including low yields of genomic DNA, poor DNA quality, poor transfer to the nylon membrane, and varying probe sensitivity. Changing to a different genomic DNA isolation kit proved more effective for this particular bacterial species and using only freshly prepared DNA helped to significantly increase the DNA yield and quality.

Samples were stored at -80°C rather than -20°C, which seemed to substantially reduce degradation. To overcome difficulties with probe sensitivity various detection methods were used including biotinylated nucleotide, DIG and ^{32}P-labeled probes.

Controls for the technique included performing simultaneous duplicate blots to be probed for a known *F. tularensis* LVS gene and for the transposon specfic kanamycin marker. In addition, all probes were made to be of similar length, approximately 800bp of PCR amplified DNA fragments that were generated with biotinylated nucleotides. To verify amplification of the desired length probe, a sample of each PCR reaction was visualized on an agarose gel (Figure 15).

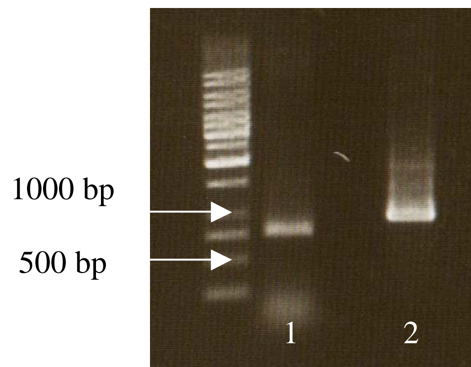

Figure 15. PCR amplified biotinylated probes; **Lane 1**- 800bp kanamycin-himar fragment from pFT-mariner, **Lane 2**- 900bp fragment of FT1250 from LVS.

The restriction enzyme, *Nde*I was chosen specifically to digest all southern blot samples as it cuts gDNA from LVS to generate a 4kb fragment that carries the FT1250 gene sequence. In addition, the Tn-mar kanamycin marker remains intact, both the transposase and *sacB* genes of pFT-mariner contain *Nde*I restriction sites. A

test *Nde*I digest of LVS gDNA and pFT-mariner was run on an agarose gel to ensure presence of the desired products (Figure 16).

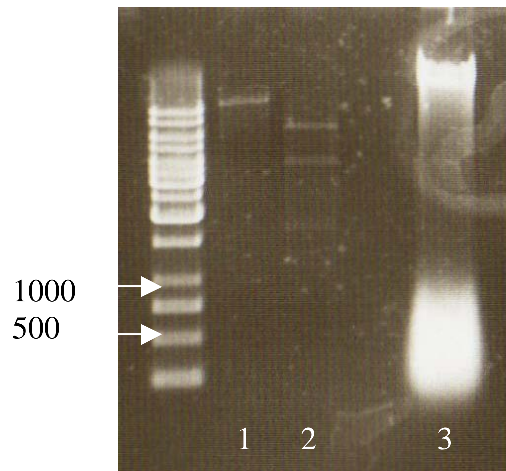

Figure 16. Approximately 1µg of pFT-mariner DNA and 5µg of *F. tularensis* LVS were digested with *Nde*I for three hours. **Lane 1** is undigested pFT-mariner DNA. **Lane 2** is digested pFT-mariner **Lane 3** is mostly undigested gDNA from *F. tularensis* LVS.

Agarose gels loaded with DNA from pFT-mariner, original founder clones, and potential library mutants were electrophoresed for approximately 2.5 hours at 70V prior to visualizing the DNA fragments with ethidium bromide (Figure 17). It is important to note that gels were prepared in the same manner before transfer. To ensure acceptable DNA transfer had occurred gels subjected to transfer were re-stained with ethidium bromide (Figure 18). Any remaining DNA was found to be located in regions of highest DNA concentration and likely represents uncut samples. After adequate DNA transfer, the blots were hybridized with the previously tested probes.

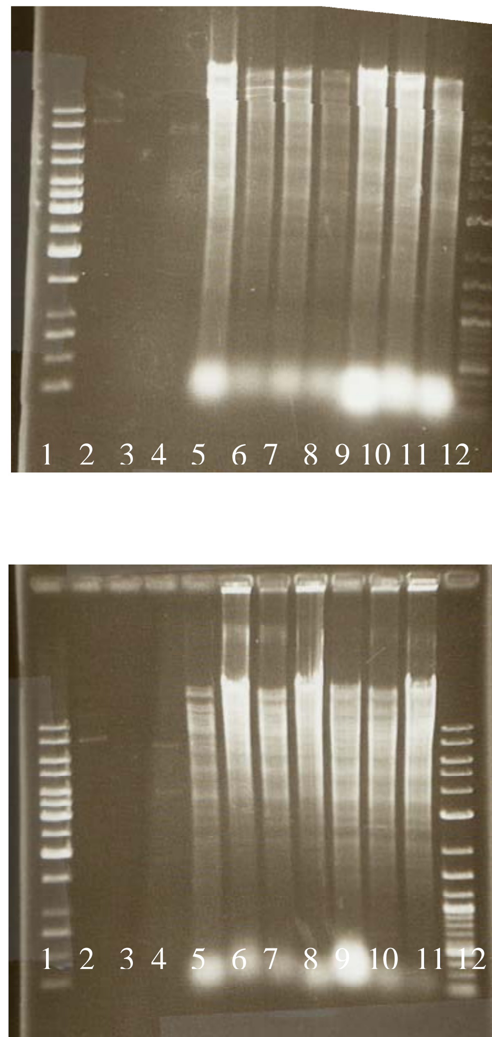

Figure 17. Southern blot agarose gels following overnight digestion with *Nde*I, stained with ethidium bromide. Each gel has samples loaded in the same order beginning with the control lanes, 1kb DNA ladder, uncut pFT-mariner, 1:10 dilution of uncut pFT-mariner. The next lanes contain approximately 5µg of *Nde*I digested samples; LVS, founder clone 15, founder clone 13, and library clone C8, library clone C58, library clone 66, library clone 80 followed by a biotinylated ladder (representative data).

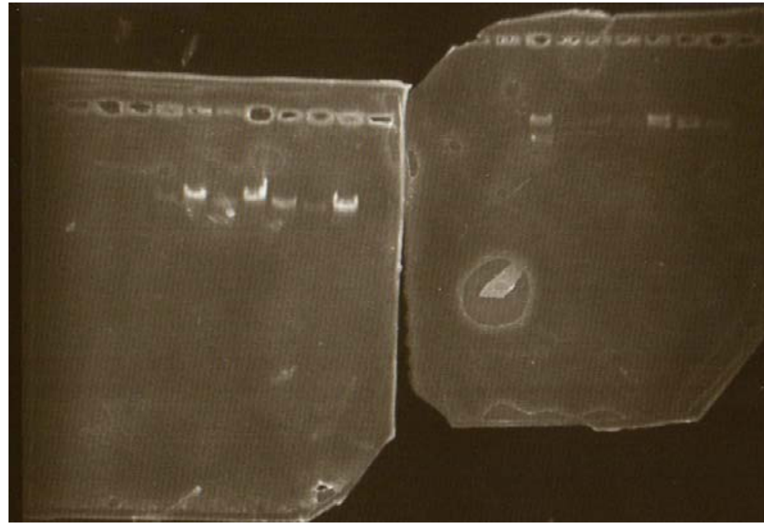

Figure 18. Southern blot gels re-stained with ethidium bromide to verify adequate DNA transfer (representative data).

Blot Probed with FT1250. *Francisella* gene, FT1250 is a unique single copy gene it should yield a single distinct band in all lanes containing LVS gDNA. The only samples that should not hybridize with the FT1250 probe are the three pFT-mariner control lanes. Experimental results showed that FT1250 yielded one intense band and a pattern of several smaller bands in lanes containing gDNA from LVS as well as the tested founder and library clones (Figure 19). After a shorter exposure time, the same series of bands was observed suggesting that, while FT1250 is a unique gene, this blot was not performed under high enough stringency to detect the FT1250 gene.

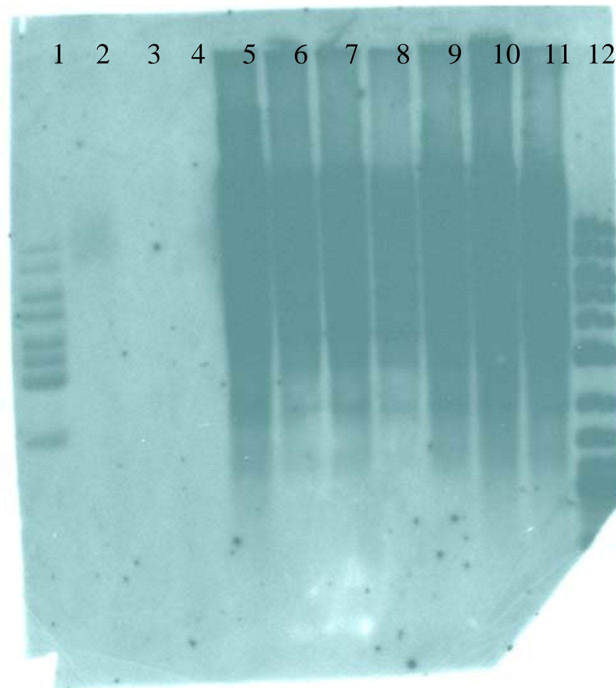

Figure 19. Southern blot probed with a 900bp biotinylated gene from *F. tularensis* LVS, FT1250 PCR amplified from LVS gDNA. Lanes: **1**- 10kb ladder, **2**- 1µg undigested pFT-mariner, **3**- 100ng undigested pFT-mariner, **4**- 1µg pFT-mariner digested with *Nde*I. Lanes 5-11 all have 5µg of gDNA digested with *Nde*I; **5**- LVS, **6**- founder clone 15, **7**- founder clone 13, **8**- clone 8, **9**- clone 58, **10**- clone 66, **11**- clone 80. Lane **12**- biotinylated ladder.

Blot Probed for Transposon Marker, the Kanamycin Gene. Controls for this blot include lanes loaded with various concentrations of the pFT-mariner plasmid, which should each result in distinct bands. Hybridization of this probe is also expected in lanes loaded with founder clone gDNA, and a minimum of one but potentially several bands in library clone gDNA lanes. The only anticipated negative sample for this blot is the lane containing gDNA from wt LVS.

Figure 20 shows the results of probing for the kanamycin resistance marker. As expected the blot demonstrates the presence of the kanamycin gene in the detected pFT-mariner lanes. However, in all other sample lanes hybridization is not observed including DNA from some of the original founder clones. The duplicate blot (Figure 19) probed for FT1250 suggests that this is not a technical problem but rather due to the lack of kanamycin gene present in these DNA samples. Together, these results strongly suggest the plasmid, as well as the transposon are unstable in this system.

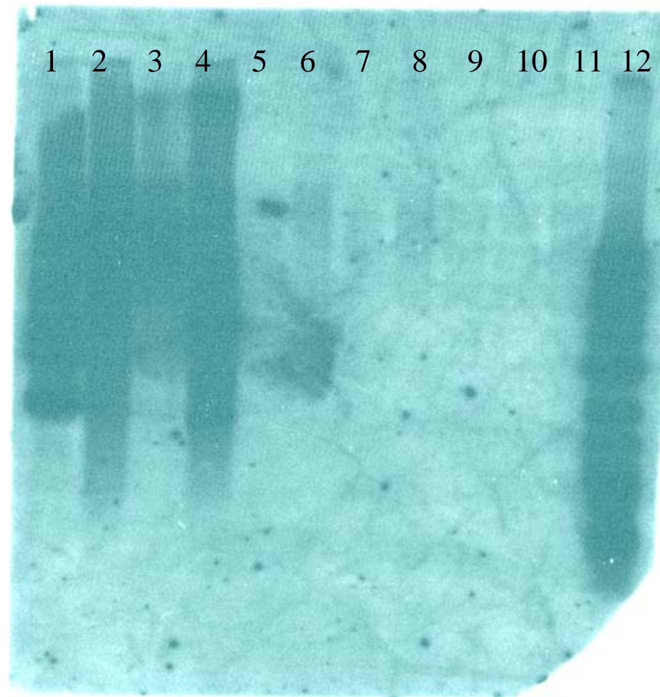

Figure 20. Southern blot probed with an 800bp biotinylated kanamycin-himar PCR fragment amplified from pFT-mariner. Lanes: **1**- 1kb ladder, **2**- 1µg undigested pFT-mariner, **3**- 100ng undigested pFT-mariner, **4**- 1µg pFT-mariner digested with *Nde*I. Lanes 5-11 all have 5µg of gDNA digested with *Nde*I; **5**- LVS, **6**- founder clone 15, **7**- founder clone 13, **8**- clone 8, **9**- clone 58, **10**- clone 66, **11**- clone 80. Lane **12**- biotinylated ladder.

Dot Blot Analysis

Concerned by our findings that none of the potential library clones which were previously positive by PCR did not contained a detectable kanamycin gene we attempted a second blot to explain our results. Even more disconcerting is that the two founder clones tested that were also positive by PCR did not show a kanamycin resistance gene by southern blot. Interested in ascertaining simply presence or absence of our target genes we used another type of blot, the dot blot. The advantages of this technique are that DNA samples are not digested, no agarose gel separation is required, and transfer via capillary action is avoided. Instead denatured DNA is spotted directly onto the nylon membrane, which allows for maximum DNA concentration in a small area. Since we eliminated many of the steps where DNA can be lost or degraded, this technique allows for a more accurate determination if a certain gene is present.

Since the dot blots have the same DNA samples as the southern blots and are probed for both the FT1250 and kanamycin genes, the expectations for this type of blot are similar to those previously discussed for the southern blot. These blots were spotted with denatured DNA from two founder clones, five different potential library clones, and several pFT-mariner controls. Spots of pFT-mariner should show hybridization to the kanamycin probe, while those that contain LVS gDNA should bind the FT1250 probe. The importance of this screen is determining the presence of the kanamycin marker in the potential founder and library clones.

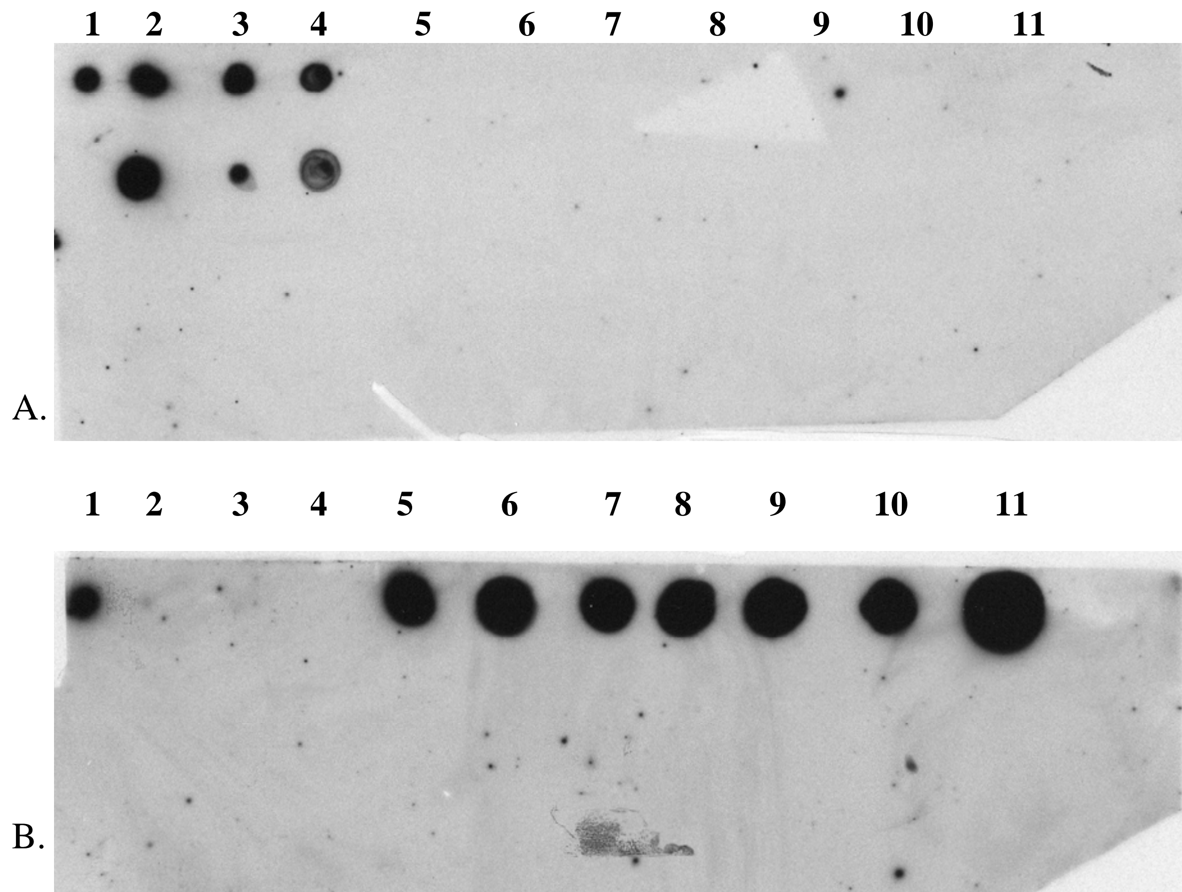

Figure 21. Duplicate dot blots spotted with identical DNA samples **Lanes**: **1**- biotinylated ladder, **2**- 1µg undigested pFT-mariner in duplicate **3**- 100ng undigested pFT-mariner in duplicate, **4**- 1µg pFT-mariner digested with *Nde*I in duplicate. Lanes 5-11 all have 5µg of gDNA digested with *Nde*I; **5**- LVS, **6**- founder clone 15, **7**- founder clone 13, **8**- clone 8, **9**- clone 58, **10**- clone 66, **11**- clone 80; exposed for 35 minutes.
A. probed for kanamycin fragment. **B.** probed for FT1250.

The results confirm those from the previous southern blot, FT1250 can be detected in all lanes containing LVS bacteria but not in the pFT-mariner controls (Figure 21A). Notably the most significant outcome of the dot blot analysis is that no

detectable kanamycin gene is present in any of our library clones or in the founder clones tested (Figure 21B).

Stability of pFT-mariner

The next step was to determine when the original founder clones lost pFT-mariner. Once conjugation had occurred we expected pFT-mariner to be stable in *F. tularensis* LVS. During the course of this investigation it was discovered that six months following conjugation all the desired plasmid genes are still detected (Figure 22) in founder clones. Almost nine months after conjugation another original founder clone was screened for the presence of plasmid genes. Figure 23 indicates that this founder clone still contains pFT-mariner. However pDNA isolated from a recent culture of the same founder clone was also screened for multiple plasmid markers and was found to no longer contain detectable pFT-mariner genes (Figure 24).

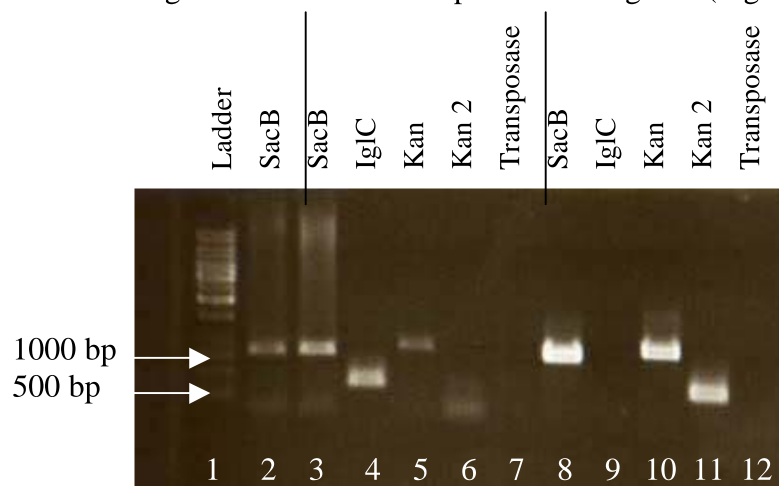

Figure 22. PCR amplified fragments of pFT-mariner and *F. tularensis* LVS founder clone DNA six months post-conjugation. **Lanes: 1**- ladder, **2**- founder clone 19 as template **Lanes 3-7** founder clone 13 pDNA as template **Lanes 8-11** pFT-mariner as template.

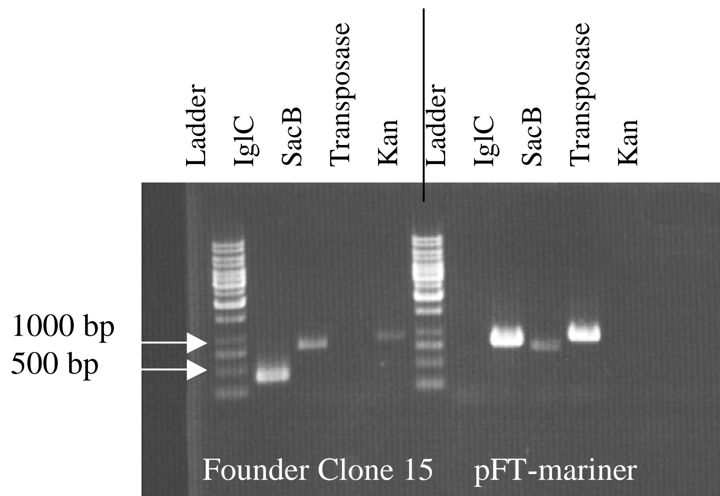

Figure 23. PCR analysis to identify marker genes of pFT-mariner on pDNA of founder clone 15 compared to a pFT-mariner control nine months following conjugation.

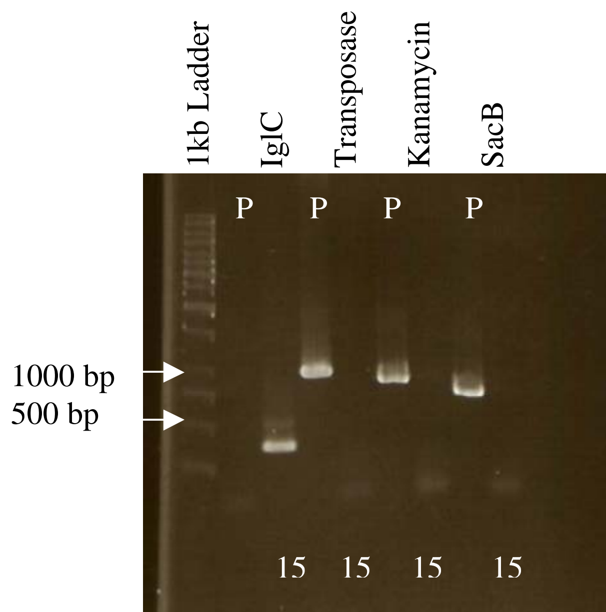

Figure 24. PCR amplification using P, pFT-mariner control and 15, pDNA from freshly cultured founder clone.

These observations address the previously considered question of pFT-mariner stability in *F. tularensis* LVS. This data suggests that pFT-mariner is stable in LVS for several months at -80ºC but for an unknown reason can be lost over time, despite maintaining kanamycin selection in the growth media. Loss of the mutagenesis plasmid would explain the majority of our failures to isolate and detect Tn-mar in founder and library clones. The most confounding observation in this study is, if there is no kanamycin gene present how does this bacteria continuously grow in the presence of kanamycin while LVS is consistently sensitive.

Chapter 5

CONCLUSIONS

The genetic approaches used to investigate the pathogenicity of *Francisella* species has grown significantly but still remain limited for a number of reasons. Traditional bacterial genetic techniques are notoriously inefficient, potentially requiring repeated introduction of foreign DNA to create a library. Although transformation of plasmids has been reported in *F. tularensis*, these tools have been severely hindered due to the few useful selective markers and a lack of stably replicating plasmids.

Prior to this study several generations of vectors have been constructed for use in *Francisella* species including, pFNL10, pKK214 and pKK202 [54, 56]. While pFNL10 contains a *Francisella* origin of replication, it fails to replicate in *E. coli* and adversely affects bacterial survival *in vivo*, which again limits the plasmids usefulness [56]. A derivative of pFNL10, pFNLTP1 was described for use as a shuttle vector [54]. Among the advantages to using this plasmid are increased electroporation efficiency over the standard *E. coli* vectors and the ability to stably replicate [54]. However, pFNLTP1 has been observed to alter in sequence once transformed into bacteria [54]. Second generation plasmids, pKK214 and pKK202 are both reported to replicate in *E. coli* but have low transformation efficiencies, and carry antibiotic markers inappropriate for use in *Francisella* [56]. A recent study has described the construction of yet another vector, pPV that has been used for targeted gene deletion [61]. pPV contains the pKK214 backbone with an additional *sacB* gene for

counterselection on sucrose and a chloramphenicol antibiotic marker for selection pressure [61]. Therefore, a plasmid that can be maintained in both *Francisella* and *E. coli*, carries an effective antibiotic marker, and can be efficiently introduced would aid in investigating the virulence of *Francisella* species.

An additional problem that has been demonstrated in efforts to generate a mutant library of *Francisella* species, is the instability of bacterial transposon systems. Among the previously tested transposons are TnMax2, Tn1721 and Tn10, all of which were ultimately found to be unstable in this bacterium for unknown reasons. It seems that most prokaryotic-based transposon systems can be reactivated by resident *Francisella* transposases.

Thus, designing a mutagenesis system requiring a single founder clone to generate an entire library of mutants circumvents the problem of repeated DNA introduction. Furthermore, using the Tc-1 mariner family transposase and the Himar-1 transposon should avoid reactivation by a native *Francisella* bacterial transposase. Our vector uses the *sacB* and RP4 origin of transfer region from pPV, which have been proven to function in *F. tularensis* [61]. To regulate transposition events, the transposase gene used in this study is under the control of an arabinose inducible promoter. Therefore, once a founder clone is generated through conjugation, an entire library can be created through the addition of arabinose, which activates the himar1 specific transposase and counterselection of pFT-mariner on sucrose plates.

Our initial results indicate that the constructed plasmid can be efficiently introduced and maintained in *F. tularensis* LVS for several generations. Findings also

suggest that the himar1 transposon can integrate into the *F. tularensis* LVS genome to create library clones from a single parent clone. This eliminates the need for repeated attempts at traditional techniques to introduce foreign DNA. Methods to determine the transposon insertion site were utilized and found to be effective. Furthermore, a rapid ELISA screening assay was developed to assess intra-macrophage survival of any mutant library clones. However, our attempts to generate a *F. tularensis* LVS transposon library were unsuccessful, due to instability of the transposon for unknown reasons. Following up on our results we also determined that the original founder clones lose the plasmid over time. Thus, the design of our mutagenesis vector and system are not yet optimized.

A recent study has demonstrated the ability of a mariner family himar1 transposon to generate a *F. tularensis* LVS library [78]. This investigation does not describe any stability issues as a result of the eukaryotic-based transposon and thus provides evidence to support our overall design. Interestingly, the sequencing of the *Francisella* genome revealed several copies of ISFtu1, one of two types of IS elements associated with IS630 Tc-1 mariner family transposons [79] [28]. However, this transposon requires a double frameshift event to be translated [28]. However unlikely, the occurrence of these frameshifts might explain the observed instability of our transposon system. The loss of pFT-mariner in the original founder clones can be explained through an emerging idea in pathogen evolution. Genome biology has begun to suggest a new mechanism to increase virulence through which bacteria lose rather than gain DNA [28]. This evolution is particularly advantageous for

intracellular pathogens, as the host cell provides much of the required nutrients and growth factors. Limiting the bacterial genome allows for faster replication of the genome, more rapid infection and thus increased pathogenicity.

While it remains unclear why our plasmid and transposon were unable to generate a library, in an effort to continue our investigation of identifying virulence factors an alternative strategy of targeted gene deletions was employed. Using the recent genome sequence three genes were selected through homology for deletion, *oxyR*, *mviN*, and *mce* [67]. Two approaches are currently in progress to disrupt these genes including a nonpolar and a polar method, which utilize the pPV and TOPO TA plasmids. The creation of the deletion mutants will be verified using several techniques optimized in this study. Screening for intra-macrophage survival will be done as described above using the ELISA technique developed. Furthermore, to assess that any observed changes in virulence are a direct result of the removed gene, the pKK214 plasmid will be used for complementation. Thus, this study was able to develop and optimize techniques useful for future *F. tularensis* investigation.

Chapter 6

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