

## ABSTRACT

DIANA MURRO

Creation of a Transposon Mutant Library in Live Vaccine Strain *Francisella tularensis*  
Under the direction of DR. RUSSELL KARLS

Tularemia is a disease of small animals and humans caused by the bacterium *Francisella tularensis*. The most virulent subspecies to humans is *F. tularensis* subspecies *tularensis*. Due to its highly-infectious nature, this pathogen was classified as a category A agent by the Center for Disease Control and Prevention. The Live Vaccine Strain (LVS) is derived from *F. tularensis* subspecies *holarctica*. LVS is avirulent in humans, but still replicates in macrophages and causes a lethal infection in mice. Therefore, identifying LVS genes that are required for intra-macrophage survival should also aid in efforts to attenuate the category A strain.

The transposon Tn5 is a mobile genetic element that inserts into the target DNA with very little site-specificity, resulting in virtually-random transposition. To generate a transposon system that functions in *Francisella* species, a Tn5 transposome was constructed containing the gene encoding green fluorescent protein (GFP) and the gene encoding hygromycin resistance under the control of *Francisella* promoters. Transformation into LVS should result in GFP-expressing transformants resistant to hygromycin. As transposition is a rare event, experiments are underway to optimize transformation into LVS. The optimized conditions should enable the isolation of a large set of transposon mutants which can then be screened for failure to replicate in cultured macrophages. The transposon is engineered to facilitate the identification of its chromosomal location. Identifying the disrupted genes is a first step in understanding the mechanisms by which *F. tularensis* bacteria escape killing by the host.

INDEX WORDS: Live Vaccine Strain *Francisella tularensis*, transposon mutant library, transposition

CREATION OF A TRANSPOSON MUTANT LIBRARY IN LIVE VACCINE STRAIN

*FRANCISELLA TULARENSIS*

by

DIANA MURRO

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## DEDICATION

I would like to dedicate this work to my family for their love, support, and encouragement.

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I would like to acknowledge Dr. Karls, Dr. Quinn, and the members of their labs for their guidance and support.

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## CHAPTER 1 INTRODUCTION

*Francisella tularensis* is a fastidious Gram-negative coccobacillus and the causative agent of tularemia (Oyston 2008). There are four subspecies of *F. tularensis*. Subspecies *tularensis* constitutes Type A strains while *holarctica*, *mediasiatica*, and *novicida* constitute Type B strains (Conlan and Oyston 2007). The North American subspecies *tularensis* is highly virulent; as few as 10 organisms transmitted subcutaneously can lead to severe infection (Lee et al. 2006). Type B strains are less virulent but still highly infectious (Nano et al. 2004). Strains of subspecies *novicida* rarely cause disease in humans (Broekhuijsen et al. 2003). Due to its high infectivity and lethality, *F. tularensis* has been classified as a biosafety level III category A agent by Centers for Disease Control and Prevention (Nano et al. 2004).

Tularemia is a zoonotic infection found in rodent populations (Oyston 2008). Whether these animals are the true reservoir is debatable, as arthropod vectors have been implicated in transmission of the pathogen (Oyston 2008). Humans can acquire infection from bites by infected arthropods, contact with infected animal fluids or tissues, contact with contaminated soil, food or water, or inhalation of infectious aerosols. There is no documented transmission from person-to-person. Ulceroglandular tularemia results from infection through the skin. A lesion develops, and lymphadenopathy can later occur. Respiratory or pneumonic tularemia is caused by inhalation of the pathogen. Symptoms vary and depend on the virulence of the strain. Infection with the most virulent strains has a fatality rate of up to 30% without treatment (Oyston 2008).

*F. tularensis* is able to proliferate in large numbers within the host and grow within macrophages. The host inflammatory response induced by this pathogen appears to contribute to disease, though the bacterium's lipopolysaccharide layer (LPS) does not seem to be a major inflammation-inducing factor (Oyston 2008). The LPS of *Francisella* is distinct because lipid A of subspecies *novicida* and *tularensis* possesses unusual modifications. The low toxicity of LPS from this pathogen may enable the bacteria to evade host innate immune responses (Cole et al. 2006).

Despite the virulence of tularemia there is no commercially-available vaccine approved by the Food and Drug Administration of the United States. To combat an intracellular pathogen, a vaccine should elicit a cell-mediated immune response. The only vaccines in widespread clinical use against intracellular pathogens are the BCG vaccine against tuberculosis and the Ty21a vaccine to combat typhoid fever (Conlan et. al.).

A vaccine was developed from an attenuated *F. tularensis* subsp. *holarctica* strain known as live vaccine strain (LVS). This strain was developed in the 1940s by repeatedly passing the Type B strain on agar plates and then through mice. The molecular basis of attenuation is unclear. LVS can still replicate in macrophages, and although it is attenuated in humans, it is lethal to mice (Cole). LVS administered by scarification provides protection against 10-100 infectious doses of virulent *F. tularensis* subsp. *tularensis*, but illness develops at a challenge of 1000 infectious doses (Oyston). Aerosol delivery of LVS provides better protection against aerosol challenge than administration of LVS via scarification. Both routes of administration provide protection against subcutaneous challenge. However, in humans, LVS is more virulent when administered as an aerosol; thus LVS was only approved when administered by scarification (Conlan et. al.). The virulence of the vaccine raises safety concerns about

vaccination of children and immunocompromised individuals. Additionally, the immunoprotective moieties in the vaccine have not been determined (Lee et. al.). Safety issues, variable immunogenicity, inability to protect against aerosol challenge, and other issues all stall the licensing of the LVS vaccine (Conlan and Oyston 2007).

Other approaches could lead to the development of a more effective vaccine against *F. tularensis*. One approach to creating a vaccine is to engineer live vaccines with precisely-defined genetic defects. Live vaccines are advantageous because they should elicit a cell-mediated immune response and an antibody response to multiple bacterial proteins and LPS (Conlan and Oyston 2007). Molecular techniques to create mutations in LVS or more virulent strains could lead to the generation of a defined attenuated live vaccine. Thus, identification of virulence genes could be an important step in vaccine development.

Development of a transposon library can lead to identification of virulence genes. The transposon Tn5 is a mobile genetic element consisting of an antibiotic-resistance gene and the Tn5 transposase gene flanked by mosaic ends (MEs). The transposase binds to the mosaic ends and catalyzes the movement of the DNA. This transposon inserts into the target DNA with very little site-specificity, resulting in virtually-random transposition. Transposon mutants of LVS can be collected and screened for interesting phenotypes. Inability to replicate in murine macrophages indicates that the transposon likely inserted into a gene necessary for virulence, resulting in disrupted gene function. Locating the transposon insertion aids in identification of the virulence gene.

## CHAPTER 2 MATERIALS AND METHODS

The methods described were used to produce a *F. tularensis*-specific transposon vector. Plasmid construction steps are illustrated in Figures 1 and 2. Sequences of PCR primers used in these experiments are shown in Table 1. Relevant features of plasmids used in this project are indicated in Table 2.

### Construction of pKEK1041hyg

The pKEK1041 plasmid was obtained by the Klose laboratory (Rodriguez et al. 2008). The pKEK1041hyg plasmid was created by replacing the *Tn903aph* region (which confers resistance to the antibiotic kanamycin) with the *hygR* gene encoding resistance to the antibiotic hygromycin gene obtained from plasmid p16R1 (Garbe et al. 1994). The pKEK1041 plasmid was digested with the restriction enzyme *NdeI*; digestion was confirmed by agarose gel electrophoresis. The digested DNA was purified using a Zymoclean DNA Clean and Concentrator kit and eluted twice with 8 µl TE buffer. The product was then digested with the restriction enzyme *NotI*, purified with the Zymoclean DNA Clean and Concentrator kit, and eluted twice with 8 µl TE buffer. The recovered DNA was quantified by agarose gel electrophoresis relative to known quantities of DNA standards. The 3000-base pair vector fragment was extracted from the gel and purified with a Zymoclean Gel DNA recovery kit. The product was eluted twice with 8 µl of TE buffer. A plasmid concentration of 12 ng/µl was determined by comparison of band intensities to known concentrations of λBstEII DNA standards.



### Construction of pKEKhygEGFP

Plasmid pEGFP (BD Biosciences Clontech) was digested with *Hind*III to linearize the plasmid for use as a PCR template. Primers P1047 and P1048 (Table 1) were used to obtain the *egfp* gene flanked by the *Spe*I restriction sites. The PCR reactions included 85  $\mu$ l dH<sub>2</sub>O, 10  $\mu$ l 10x Native Plus Pfu buffer, 1  $\mu$ l P1047 [10  $\mu$ M], 1  $\mu$ l P1048 [10  $\mu$ M], 1  $\mu$ l pEGFP/*Hind*III DNA, 1  $\mu$ l 25 mM dNTPs, and 1  $\mu$ l Native *Pfu* DNA polymerase. PCR was performed using the following parameters.

97 °C ;	97 °C	51 °C	72 °C ;	97 °C	65 °C	72 °C ;	72 °C	;	4 °C
4 min ;	<u>30s</u>	<u>30s</u>	<u>2min</u> ;	<u>30s</u>	<u>30s</u>	<u>2min</u>	;	10min ;	$\infty$
	2 cycles			25 cycles					

Successful PCR was confirmed by agarose gel electrophoresis. The PCR product was then digested with *Spe*I and purified using a Zymoclean DNA Clean and Concentrator kit. The DNA product was eluted twice in 8  $\mu$ l of TE buffer. A final concentration of 6 ng/ $\mu$ l was determined by comparison to  $\lambda$ BstEII DNA standards.

Plasmid pKEK1041hyg was digested with *Spe*I and purified using the Zymoclean DNA Clean and Concentrator kit. The plasmid DNA was eluted twice in 8  $\mu$ l of TE buffer. The ends were dephosphorylated using Calf Intestinal Alkaline Phosphatase. The dephosphorylated DNA product was purified again using the Zymoclean DNA Clean and Concentrator kit and eluted twice with 8  $\mu$ l of TE buffer. The DNA was quantified by agarose gel electrophoresis. To remove the undigested portion, the 4609-bp band was extracted and purified using a Zymoclean

Gel DNA Recovery kit. A concentration of 4.6 ng/ $\mu$ l was determined by comparison band intensities to known concentrations of  $\lambda$ BstEII DNA standards.

Ligation of *egfp* into pKEK1041hyg was performed using T4 DNA ligase. The ligated DNA was transformed into chemically-competent *E. coli* TAM1 cells. The cells were plated onto LB agar supplemented with 200  $\mu$ g/ml of hygromycin and incubated overnight at 37 °C. Six colonies resistant to hygromycin were selected and streaked for isolation. The candidates were viewed under a microscope using the FITC filter for cells that fluoresced green (Figure 4a). Two candidates expressed EGFP. A QIAgen Maxiprep kit was used to purify plasmid DNA from one of these isolates; the plasmid was named pKEKhygEGFP.

#### Construction of plasmid pMOD5hygEGFP

The pMOD5 Transposon Construction Vector (Epicentre) was digested with *Sph*I. The digested DNA was concentrated using a MicroCon spin column (Amicon, Inc.) and DNA bands separated by agarose gel electrophoresis. Gel extraction of the approximately 2000-bp band was performed using the Zymoclean Gel DNA Recovery kit. The DNA was eluted twice with 8  $\mu$ l of TE buffer. A concentration of the recovered DNA was determined to be 15 ng/ $\mu$ l by comparison to bands of  $\lambda$ BstEII DNA standards.

The pKEKhygEGFP plasmid was digested with *Sph*I and *Hind*III to linearize the DNA in preparation for use as a PCR template. The 100- $\mu$ l reaction consisted of 85  $\mu$ l dH<sub>2</sub>O, 10  $\mu$ l 10x Cloned *Pfu* buffer, 1  $\mu$ l 10 mM primer P1110, 1  $\mu$ l 10 mM primer P1111, 1  $\mu$ l of a 1:100 dilution of pKEKhygEGFP digest with *Hind*III and *Sph*I, 1  $\mu$ l 25 mM dNTPs, and 1  $\mu$ l Cloned *Pfu* DNA polymerase. Successful PCR was performed using the following parameters:

Temperature (°C): 97 ; 97 49.8 72 ; 72 ; 4

Time: 4 min. ; 30s 30s 4min ; 7min ; ∞  
27 cycles

The PCR products were purified using a Zymoclean DNA Clean and Concentrator kit and eluted twice in 8 µl TE buffer. The PCR product was digested with *HindIII* and successful digestion was confirmed by agarose gel electrophoresis. The recovered product was then digested with *SphI*. Successful digestion was again confirmed by agarose gel electrophoresis. The digestion product was purified using a Zymoclean Clean and Concentrator Kit and eluted twice with 8 µl TE buffer.

The pMOD5 plasmid obtained from Epicentre Biotechnologies was digested with *HindIII*. Successful digestion was confirmed by agarose gel electrophoresis. A gel extraction of the 2050-bp band was performed, and the DNA, and the band was purified using a Zymoclean DNA Gel Recovery kit. The final product was eluted twice with 8 µl TE buffer. The pMOD5/*HindIII* fragment was then digested with *SphI*. The product was purified using a Zymoclean DNA Clean and Concentrator kit and eluted in 6 µl TE buffer. A concentration of 1.4 ng/µl was determined using  $\lambda$ BstEII DNA standards.

The ligation reaction was prepared by mixing 6 µl of pMOD5 DNA previously-digested with *HindIII* and *SphI*, 1.5 µl of hygEGFP previously-digested with the same enzymes, 2 µl of 5x NEB ligase buffer, and 0.5 µl of T4 DNA ligase. The ligated products were electroporated into competent cells of *E. coli* strain EC100D pir+ cells according to the Epicentre TransforMax EC100D pir+ Electrocompetent *E. coli* Electroporation protocol. Colonies were selected on plates containing 100µg/ml of carbenicillin and 200µg/ml of hygromycin. Plasmid DNA from candidates was digested with *KpnI* and *PacI* restriction enzymes. Correct banding pattern was confirmed by the presence of bands at approximately 2600-bp and 1200-bp on a 1% agarose/1X

TBE gel (Figure 5). Cells harboring this plasmid were then viewed under a microscope for green fluorescence using the FITC filter. Isolates A and B expressed EGFP. A QIAGEN Maxiprep kit was used to purify plasmid DNA from Isolate A. The final plasmid concentration was 90 ng/μl.

#### Transformation of Live Vaccine Strain *Francisella tularensis*

A 50-mL culture of Live Vaccine Strain *Francisella tularensis* was grown in Mueller-Hinton (MH) broth containing 1.23 mM CaCl<sub>2</sub>, 1.03 mM MgCl<sub>2</sub>, 2% (vol/vol) IsoVitaleX (BD), 1% glucose, and 0.024% (wt/vol) iron(III) pyrophosphate. The culture was grown in a T-75 ventilated canted-neck flask in a 34 °C incubator containing 5% carbon dioxide. Cells were cultured until an optical density at 600 nm of 0.6 – 0.9 was reached. The culture was then centrifuged at 3400 rpm in a clinical centrifuge for 10 minutes at 4 °C. The cells were washed twice with an equal volume of 0.5 M sucrose and 1 mM EDTA buffer (centrifugation at 3400 rpm for 8 minutes was used to collect the cells after each wash). Cells were then washed twice with an equal volume of 0.5 M sucrose. All reagents were at room temperature. After centrifugation, the final pellet was suspended in 0.3 ml of 0.5 M sucrose. Next, 100 μl of cells were aliquoted into tubes and mixed with 1 μl of plasmid DNA. The reactions were incubated for 10 minutes at room temperature. The reactions were then transferred to 2-mm electroporation cuvettes. Electroporation was performed using a BioRad GenePulser Xcell machine set at the following parameters: 2500 V, 50 μF, and 100Ω. Immediately after electroporation, 0.9 ml of supplemented MH broth was added to the cuvette and the contents were transferred to a 15-ml Falcon tube. The cells were incubated for 3-5 hours in a 34 °C incubator set at 5% carbon dioxide. Cells were plated on MH agar containing 100 μg/ml hygromycin, 2.5% heat-inactivated bovine serum albumin, 2 % IsoVitaleX, and 0.024% iron(III)

pyrophosphate. Colonies were selected from the plates and streaked onto MH agar plates containing the same supplements. LVS *Francisella tularensis* was successfully transformed with pKEK1041hyg and pKEKhygEGFP. Cells expressing pKEKhygEGFP are shown in Figure 4b.

#### Creation of the MOD5hygEGFP Transposome

The pMOD5hygEGFP plasmid was digested with the *ScaI* restriction enzyme. PCR was then performed to amplify the region containing the *Francisella tularensis* promoters and the *hygR* and *egfp* genes. MCS Forward and Reverse PCR primers provided by Epicentre were used. The reaction contained 81.5  $\mu$ l dH<sub>2</sub>O, 10  $\mu$ l 10x *Pfu* buffer, 2  $\mu$ l 100 mM MgSO<sub>4</sub>, 1  $\mu$ l 10  $\mu$ g/ml pMOD5 MCS Forward PCR primer, 1  $\mu$ l 10  $\mu$ g/ml pMOD5 MCS Reverse PCR primer, 2.5  $\mu$ l 10 mM dNTPs, 1  $\mu$ l Cloned *Pfu* DNA polymerase, and 1  $\mu$ l pMOD5hygEGFP digested with *ScaI*. The following PCR parameters were utilized:

Temperature (°C): 97 ; 97 59.2 72 ; 72 ; 4

Time: 4 min ; 30s 30s 6min ; 10min ;  $\infty$

30 cycles

Successful PCR amplification was confirmed by agarose gel electrophoresis. The product was then purified using a Zymoclean DNA Clean and Concentrator kit. The transposome reaction contained 2  $\mu$ l MOD5hygEGFP DNA (100  $\mu$ g/ml), 4  $\mu$ l EZ-Tn5 transposase (Epicentre), and 2  $\mu$ l of 100% glycerol. The reaction was mixed by vortexing and incubated at room temperature for 30 minutes.

### Subsequent Transformations

Subsequent transformations of LVS *Francisella tularensis* were unsuccessful; probably because the 5% CO<sub>2</sub> incubator was no longer available. LVS *F. tularensis* cultures were grown in a 34 °C incubator without 5% CO<sub>2</sub>.

The first attempt utilized the same procedure described previously. LVS was transformed with the EZ-Tn5 transposome MOD5hygEGFP, but the cultures were grown in the incubator that did not have 5% CO<sub>2</sub>. A transformation efficiency based on colony counts of  $1.08 \times 10^4$  colonies/μg MOD5hygEGFP DNA was obtained. However, no colonies that fluoresced green were obtained, indicating that the transposon did not insert into the bacterial chromosome or that the gene does not produce enough EGFP when expressed in single copy from the chromosome.

The next experiment utilized pKEKhygEGFP as a positive control. Transformation in tandem of LVS with pKEKhygEGFP and the MOD5hygEGFP transposome was performed utilizing the same conditions in the first attempt. A lawn of growth was observed on several plates but no green fluorescent colonies were found.

The plates were then tested for contamination. One colony from the transformation was mixed in 100 μl dH<sub>2</sub>O in a PCR tube. In separate PCR tubes, 1 μl and 10 μl aliquots of LVS *Francisella tularensis* freezer stock were mixed in 100 μl of dH<sub>2</sub>O. The last PCR tube contained 100 μl of freezer stock. The cells were lysed and heat-killed at 99°C for 10 minutes. Each PCR reaction included 12.5 μl Promega PCR Mastermix, 0.5 μl 10 μM RD1A, 0.5 μl 10 μM RD1B, 2 μl heat-killed cells, and 9.5 μl dH<sub>2</sub>O. PCR was performed using the following parameters:

Temperature (°C): 94 ; 94 57 72 ; 72 ; 4

Time: 5min ; 30s 30s 1 min ; 10 min ; ∞

40 cycles

Agarose gel electrophoresis was performed on the PCR products. The presence of a band of approximately 1000 bp indicated the colony was LVS *F. tularensis*.

Transformation was again attempted using the same procedure as in the second attempt. However, the transformants were plated onto modified Mueller-Hinton media (MMH) containing 1% protease peptone, 2.5% defibrinated sheep blood, 1.5% bactoagar, 0.1% glucose, 2% IsoVitaleX, and 100 µg/ml hygromycin. However, neither the pKEKhygEGFP nor the MOD5hygEGFP transposome transformation plates contained colonies.

The fourth transformation attempt was attempted using only the pKEKhygEGFP plasmid. After transformation using the same procedure, 20 µl of cells were plated onto Mueller-Hinton agar (MHA) plates containing 2% IsoVitaleX, 2.5% Heat-inactivated bovine serum, and 0.024% iron(III) pyrophosphate. The plates contained hygromycin at concentrations of 50 µg/ml and 100 µg/ml. No growth was observed on the plates. To determine if a longer outgrowth time would affect results, 100 µl of transformants were plated onto the supplemented MHA plates and the remaining transformants were allowed to grow in the 34 °C incubator overnight without shaking. The overnight cultures were then plated onto the same media. However, no colonies were observed on either plate.

The fifth transformation attempt utilized different media. A 50-ml inoculum of LVS was cultured in Brain Heart Infusion (BHI) broth with 1x Casamino Acids (CAA) at pH 6.8. The same transformation procedure as before was utilized, except 0.9 ml of BHI broth+ 1x CAA pH 6.8 was added to the cells after electroporation instead of MHB. Transformants were plated onto chocolate MH agar plates supplemented with 100 µg/ml hygromycin. No colonies were observed.

The sixth transformation attempt utilized reduced iron pyrophosphate. A 0.48% stock of iron pyrophosphate was reduced in an anaerobic environment and used to supplement the media. Two 5-ml LVS starter cultures were made in MHB supplemented with 2% IsoVitaleX, 1.23 mM CaCl<sub>2</sub>, 1.03 mM MgCl<sub>2</sub>, and 0.1% glucose. One culture contained 0.024% iron(III) pyrophosphate while the other contained 0.024% iron(II) pyrophosphate. Each culture was subcultured into 50 ml of the same media in sealed 250-ml flasks and incubated at 34°C without shaking. The culture containing iron(III) pyrophosphate only reached an optical density at 600 nm reading of 0.242. An unidentified black precipitate formed in the 50-ml iron(II)-containing culture. Transformation of the iron(II) culture was performed, and 1, 10, and 100 µl of transformants were plated onto MH plates supplemented with 2.5% heat-inactivated bovine serum, 2% IsoVitaleX, 0.024% iron(II) pyrophosphate, and 100 µg/ml of hygromycin. A lawn on growth was observed on all plates, including the negative control. Due to the possibility that the hygromycin concentration was too low to inhibit growth of non-candidates, the transformants were then plated onto MH plates supplemented with 2.5% heat-inactivated bovine serum, 2% IsoVitaleX, 0.024% iron(II) pyrophosphate, and 200 µg/ml of hygromycin. However, a lawn of growth was observed on all plates with the higher hygromycin concentration, including the negative control.

## CHAPTER 3 RESULTS AND DISCUSSION

The objective of this project was to develop an EZ-Tn5 transposome for LVS *F. tularensis* in order to develop a transposon library of the strain. A transposon construction vector was created using several steps. The first derived plasmid, pKEK1041hyg, was created by removing the *Tn903aph* region of pKEK1041 (Rodriguez) by digestion enzymes *NdeI* and *NotI* and replacing it with the hygromycin-resistance gene, *hygR*, engineered to contain *NdeI* and *NotI* ends by PCR from plasmid p16R1 (Figure 1). Proper insertion was verified by restriction analysis after digestion with *EcoR1* (Figure 3). Important features of pKEKhyg include an *E. coli* origin replication (pACYC184*ori*), a *F. tularensis* origin of replication (pFNL10*ori*), the *hyg* gene under control of *F. tularensis* promoter *omp26* and the *F. tularensis* *groEL* promoter located upstream of a multiple cloning site for insertion of new genes.

Plasmid pKEKhygEGFP was created by inserting *egfp*, obtained as a *SpeI*-encoded PCR product from plasmid pEGFP, into the *SpeI* site located downstream of the *F. tularensis* *groEL* promoter (Figure 1). *E. coli* TAM1 transformants with the correct plasmid produced hygromycin-resistant colonies consisting of bacteria that fluoresced green by fluorescence microscopy (Figure 4a). The plasmid was transformed into the LVS strain. Transformants also produced hygromycin-resistant colonies and individual bacteria fluoresced green under the fluorescence microscope (Figure 4b).

The *hygegfp* region was amplified from plasmid pKEKhygEGFP as a PCR product with *SphI* and *HindIII* ends (Figure 1). This DNA element was ligated into the pMOD5 Transposon

Construction Vector (Epicentre) at the *SphI HindIII* sites to create plasmid pMOD5hygEGFP. Proper insertion was confirmed by restriction analysis following digestion with *HindIII* (Figure 5). The *hygegfp* region of pMODhygEGFP is flanked by Mosaic Ends for binding the EZ-Tn5 transposase (Epicentre Technologies). The plasmid also contains the R6*Kori* for replication in *E.coli* strains expressing the pi protein. The region of pMOD5hygEGFP containing R6*Kori*, *hygegfp*, and the Mosaic Ends was amplified by PCR with MCS Forward and Reverse primers supplied by Epicentre Technologies. Mixing this DNA with EZ-Tn5 Transposase yielded an EZ-Tn5 transposome that should result in random transposition into the *F. tularensis* genome conferring resistance to hygromycin.

Before an LVS transposon library can be made, the transformation efficiency of this bacterium must be optimized using supercoiled plasmids. Electroporation of plasmid pKEK1041hyg into LVS *F. tularensis* yielded hygromycin-resistant colonies with a transformation efficiency of  $1 \times 10^5$  transformants/ $\mu\text{g}$  pKEK1041hyg DNA. Electroporation of the slightly-larger plasmid pKEKhygEGFP into LVS *F. tularensis* yielded hygromycin-resistant colonies at a transformation efficiency of  $5 \times 10^4$  transformants/ $\mu\text{g}$  pKEKhygEGFP DNA. The transposome vector pMOD5hygEGFP plasmid replicates in *E. coli* but not in LVS *F. tularensis*, so it was not tested. Transformation with the MOD5hygEGFP transposome was attempted several times; however, it does not appear to have been successful. One possible explanation is that incubator set at 34°C and 5% carbon dioxide used previously to culture LVS was put into storage because one of the labs was being renovated. The more recent cultures were grown in an incubator set at 34°C that was unable to supply carbon dioxide. Variations of growth media and transformation protocol were made and numerous attempts were made to improve the transformation efficiency using both with the MOD5hygEGFP transposome and pKEKhygEGFP

as described in the Materials and Methods section. Even supercoiled plasmids that were previously shown able to transform LVS could no longer be taken up by the bacteria cultured under conditions without carbon dioxide. Recently, a new incubator that has the ability to supply carbon dioxide has been set up in the lab. With the addition of an incubator providing 5% carbon dioxide, successful transformations should now be possible. Experiments to transform the LVS strain using cultures grown in an incubator containing 5% carbon dioxide are currently in progress.

### Future Studies

Once the MOD5hygEGFP transposome is successfully electroporated into electrocompetent LVS cells, the transposase will catalyze transposition at random sites into the bacteria's chromosome. This procedure will be used to generate a library of LVS *F. tularensis* transposon mutants. In future studies, the library can be screened for mutants that cannot survive in murine macrophages. Screening for these mutants can be done by infecting cultured macrophages in microplate dishes. These dishes can be examined under the microscope to identify any wells that no longer fluoresce green. These mutants will grow on media supplemented with hygromycin and fluoresce green *in vitro* but not *in vivo*. These characteristics demonstrate that the mutant contains insertion of MOD5hygEGFP but the lack of fluorescence *in vivo* indicates inability to survive in mice macrophages. It can be concluded that these mutants contain a transposon insertion into a gene essential for virulence. Location of the insertion site will be possible by digesting chromosomal DNA with a restriction enzyme that does not cut within the transposome, followed by ligation, and transformation into *E. coli* selecting for resistance to hygromycin. As linear foreign DNA is digested rapidly upon bacterial entry, only circular plasmids that contain

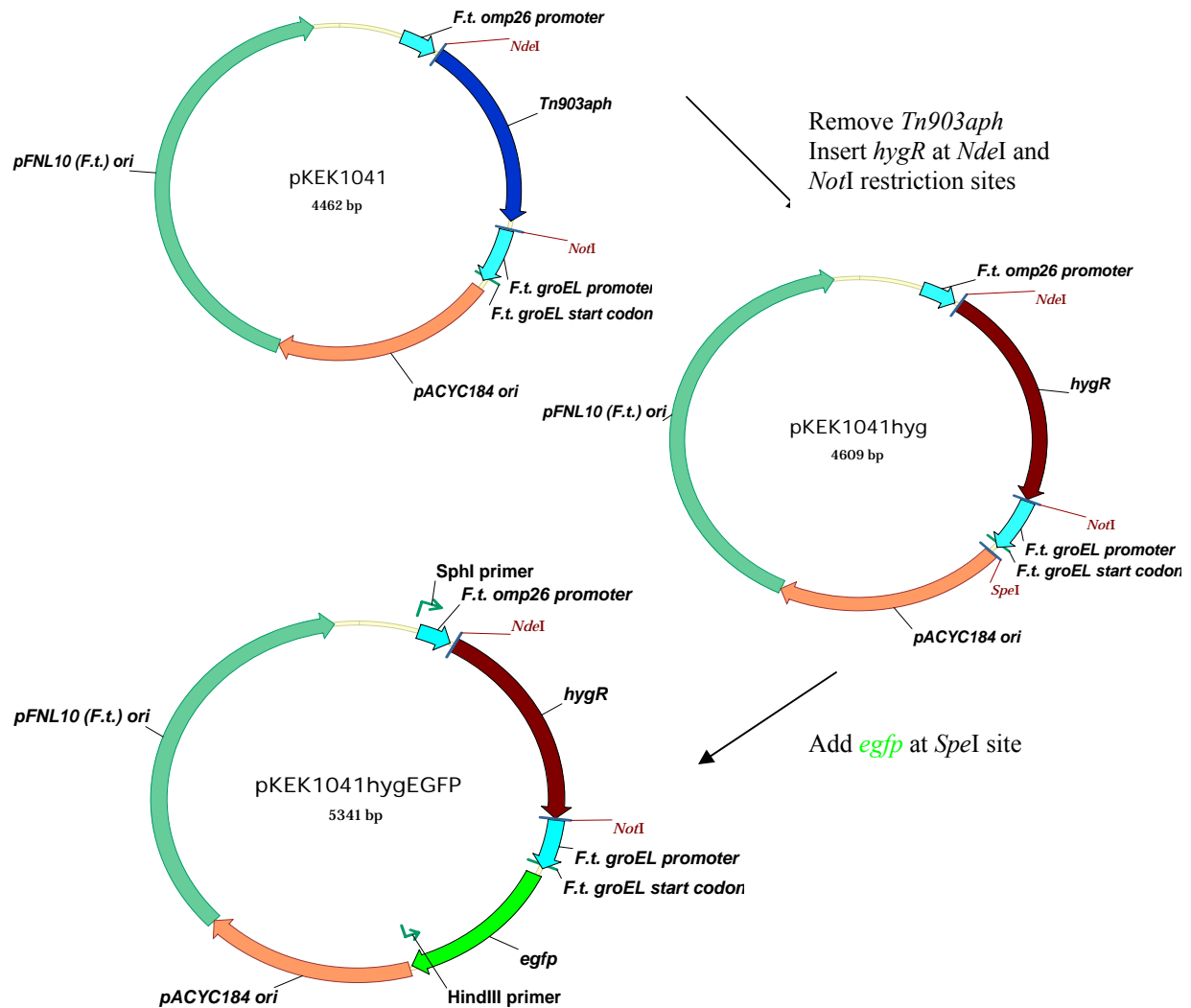
the R6K origin should be able to confer resistance to hygromycin. The plasmid DNA can then be isolated and primers facing the mosaic ends can be used to sequence the sequence into which the transposome was inserted. Identification of the disrupted gene or genes (if the gene is part of a multi-gene operon) should enable future researchers to study how this region is involved in survival of the bacteria in host macrophages. It may be possible to develop a live, attenuated vaccine from mutants that fail to replicate in macrophages.

Table 1: PCR Primers Used

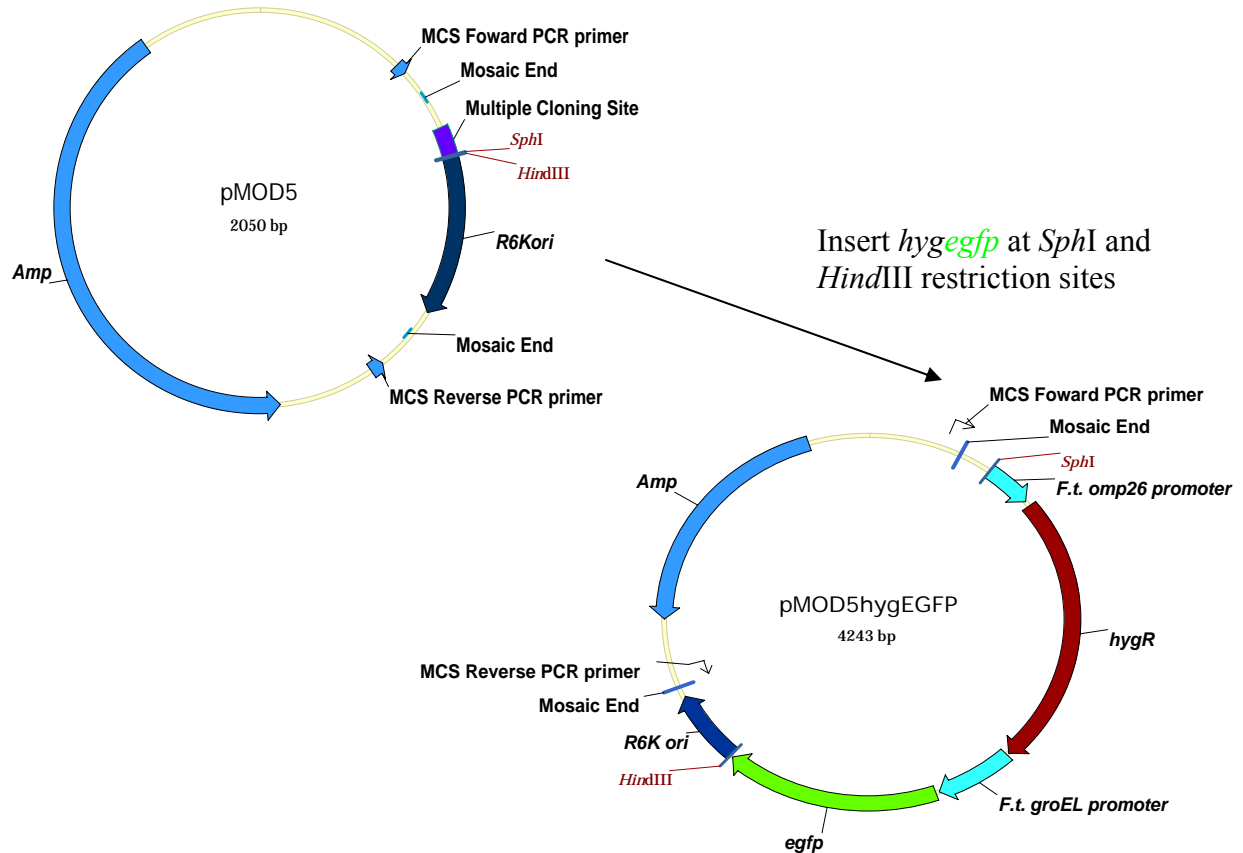
Primer Name	Primer Sequence
P1049	5'-ACT GTG ACA TAT GAC ACA AGA ATC CCT GTT AC-3'
P1050	5'-AC TAC TAG CGG CCG CTC AAG CAC CTG GAG CAG TAT CTG GTG GAC CCC AGA GGA ACT GCG C-3'
P1047	5'-GGACTAGT-3'
P1048	5'-TGATCAGGG-3'
P1110S	5'-GTATGCATG-3'
P1111	5'-TTCGAAGTG-3'
MCS Forward PCR primer	5'-ATTCAGGCTGCGCAACTGT-3'
MCS Reverse PCR primer	5'-GTCAGTGAGCGAGGAAGCGGAAG-3'
RD1A	5'-TTTATATAGGTAAATGTTTTACCTGTACCA-3'
RD1B	5'-GCCGAGTTTGATGCTGAAAA-3'

Table 2: Plasmids

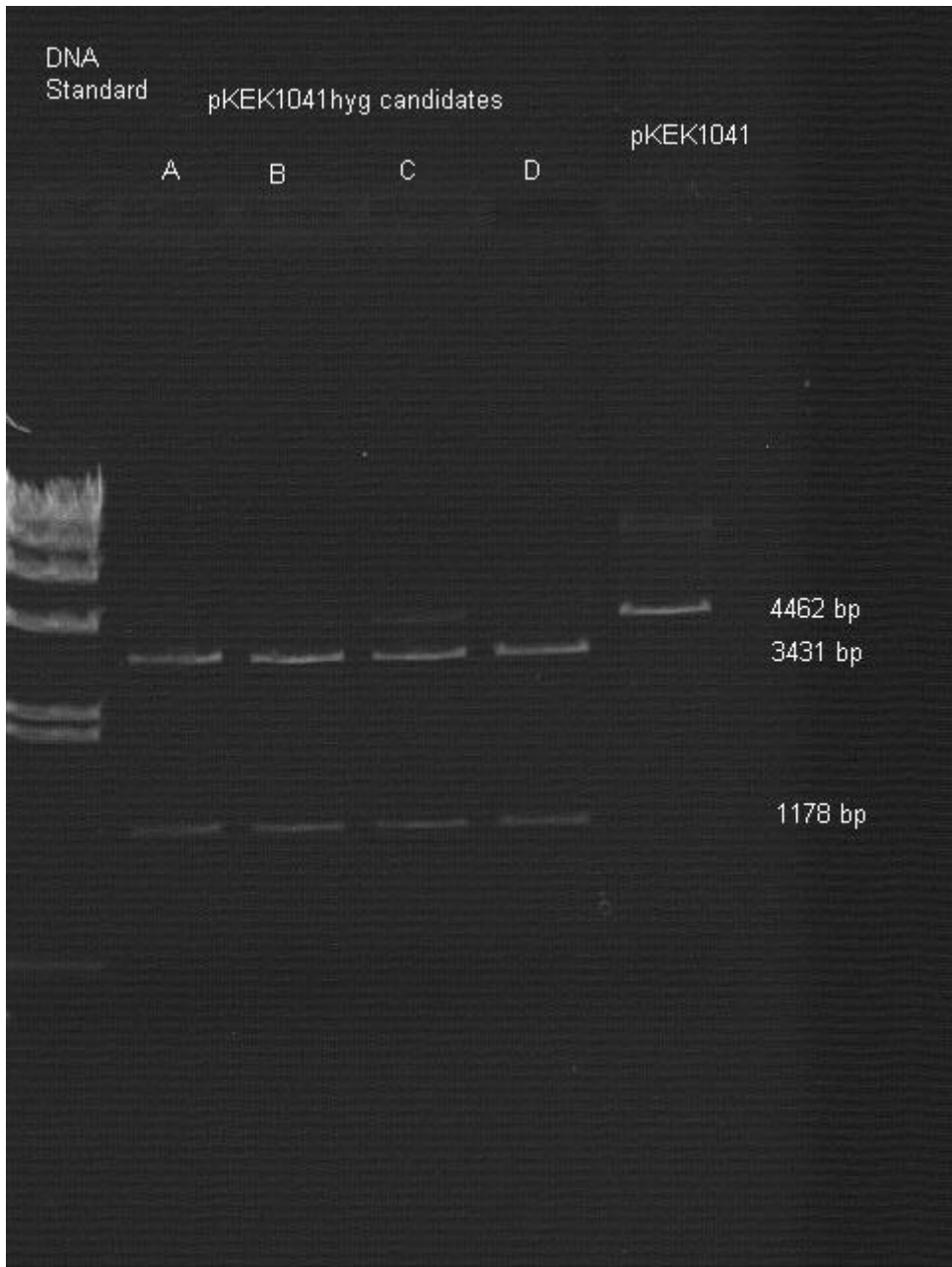
Plasmid Name	Relevant Features
pKEK1041	pFNL10ori, pACYC184ori, <i>Tn903aph</i> , <i>F.t. omp26</i> and <i>groEl</i> promoters
pKEK1041hyg	pKEK1041 with <i>Tn903aph</i> replaced with <i>hyg</i>
pKEKhygEGFP	pKEK1041hyg + <i>egfp</i>
pMOD5	MCS, <i>R6Kori</i> , <i>Tn5</i> mosaic ends, <i>amp</i>
pMOD5hygEGFP	pMOD5 + <i>hygegfp</i>
p16R1	Source of <i>hygR</i>
pEGFP	Source of <i>egfp</i>



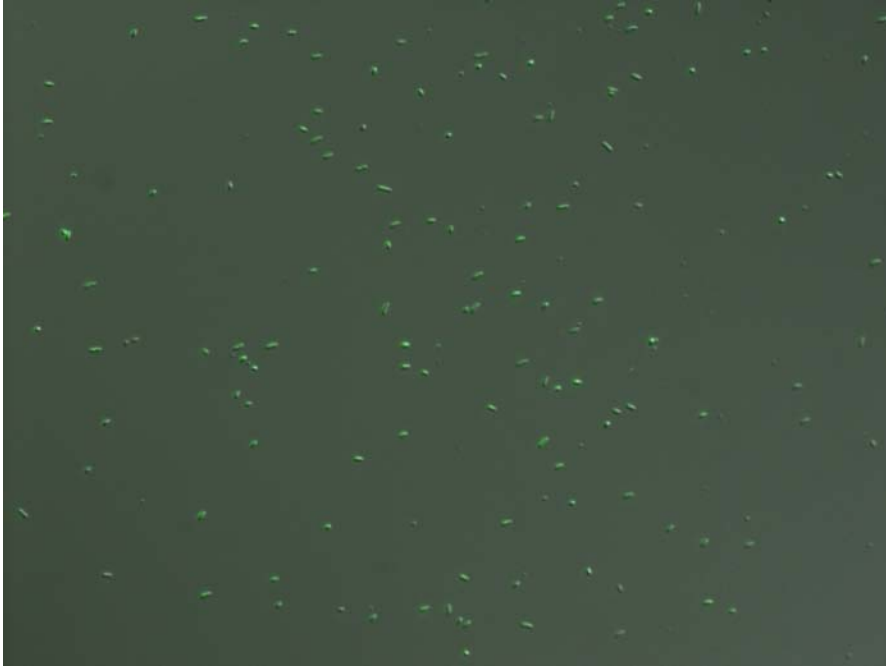
**Figure 1. Plasmid Construction Diagram.** The pKEK1041 plasmid (Rodriguez et. al.) can replicate in *E. coli* via the *E. coli* origin of replication *pACYC184ori* and in *F. tularensis* via the *F.t* origin of replication from plasmid pFNL10. The *Tn903aph* gene encodes resistance to kanamycin and is expressed from a *F. tularensis* promoter (*F.t. omp26*). The *F.t. groEL* promoter is located upstream of cloning sites to allow insertion of a foreign gene to be expressed in *F. tularensis*. *F. tularensis* contains an adenine+thymidine-rich genome; thus, the addition of *F. t* promoters facilitates transcription of foreign genes with a lower A+T DNA content. Plasmid pKEK1041 was digested with enzymes *NdeI* and *NotI* to remove *Tn903aph*. The vector band lacking the *Tn903aph* gene was ligated with a *hygR* gene encoding resistance to the antibiotic hygromycin. To create pKEKhygEGFP, a promoterless *egfp* gene encoding the protein Enhanced Green Fluorescent Protein (EGFP) was inserted into the *SpeI* site of pKEK1041hyg downstream of the *F.t. groEL* transcription and translation signals. The region containing *hygR*, *egfp*, and the *F.t.* promoters can be amplified by PCR with the *SphI* and *HindIII* primers.



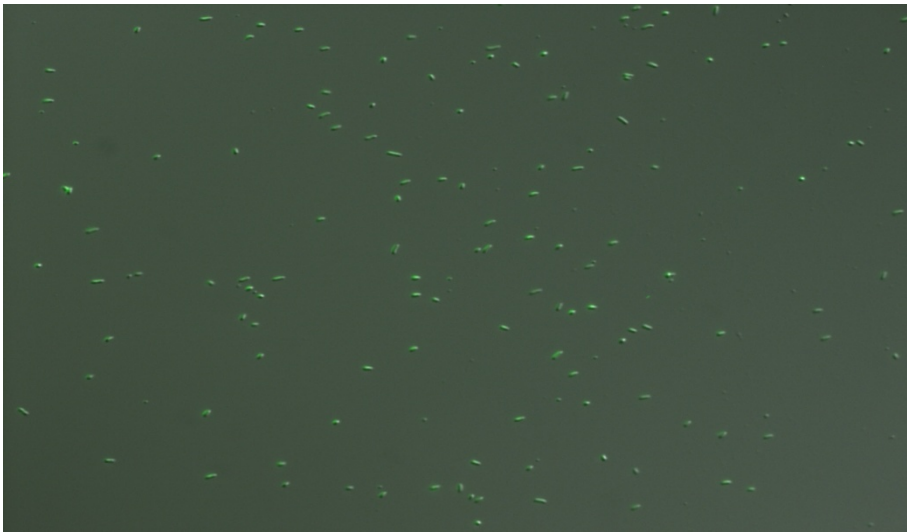
**Figure 2: Creation of a Transposon Construction Vector for *F. tularensis*.** Plasmid pMOD5 (Epicentre) contains a multiple cloning site (MCS) for gene insertion and an *R6Kori* for replication in *E. coli*. These genes are flanked by Mosaic Ends (ME) for binding EZ-Tn5 transposase (Epicentre). This entire region can be amplified by PCR using the MCS Forward and Reverse PCR primers. The plasmid also harbors the gene for ampicillin resistance (*amp*). This plasmid cannot replicate in *F. tularensis*. Insertion of the *hygegfp* fragment from pKEK1041hygEGFP results in the formation of plasmid pMOD5hygEGFP. The entire region containing *hygR*, *egfp*, and *R6Kori* was amplified with the MCS Forward and Reverse PCR primers to create an EZ-Tn5 transposon specific for *F. tularensis*.



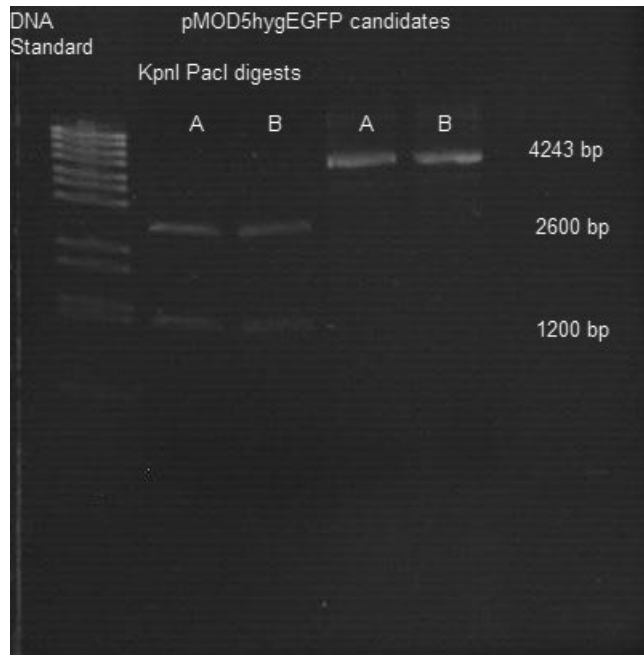
**Figure 3: Screening Candidates for pKEK1041hyg.** Plasmid DNA isolated from *E. coli* TAM1 pKEK1041hyg candidates was digested with *EcoR*I in parallel with pKEK1041 and examined by agarose gel electrophoresis. Lambda DNA digested with *Hind*III was used as the DNA standard; sizes from top to bottom (in kb) are: 8.4, 7.2, 6.4, 5.6, 4.8, 4.3, 3.7, 2.3, 1.9, 1.4, 1.9, 1.4, 1.3, 0.7. The expected bands for pKEK1041hyg digested with *EcoR*I are 3431 bp and 1178 bp. All candidates (A through D) have this pattern.



**Figure 4a: EGFP Expression in *E. coli* TAM1 cells.** Fluorescence microscopy of *E. coli* TAM1 cells harboring the pKEK1041hygEGFP plasmid reveals expression of the enhanced green fluorescent protein (EGFP).



**Figure 4b: EGFP Expression in LVS *Francisella tularensis*.** Fluorescence microscopy of LVS *F. tularensis* cells harboring the pKEK1041hygEGFP plasmid reveals EGFP expression. *Image provided by Dr. Tuhina Gupta*



**Figure 5: Screening Candidates for Plasmid pMOD5hygEGFP.** Plasmid DNA isolated from *E. coli* EC100D pir<sup>+</sup> cells was digested with *Kpn*I and *Pac*I and examined by agarose gel electrophoresis with plasmid DNA from the candidates. Lambda DNA digested with *Hind*III was used as the DNA standard; sizes from top to bottom (in kb) are: 8.4, 7.2, 6.4, 5.6, 4.8, 4.3, 3.7, 2.3, 1.9, 1.4, 1.9, 1.4, 1.3, 0.7. The expected band sizes for pMOD5hygEGFP digested with *Kpn*I and *Pac*I are approximately 2600 bp and 1200 bp. Candidates A and B have this pattern.

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