Genetic tools for highly pathogenic *Francisella tularensis* subsp. *tularensis*

Eric D. LoVullo,[†] Lani A. Sherrill,[†] Lanyin L. Perez[‡] and Martin S. Pavelka, Jr

University of Rochester Medical Center, 601 Elmwood Ave, Box 672, Rochester, NY 14642, USA

Correspondence

Martin S. Pavelka, Jr
martin_pavelka@
urmc.rochester.edu

This paper is the first detailed description of the development and use of new genetic tools specifically for the safe manipulation of highly pathogenic *Francisella tularensis* subsp. *tularensis*. Most of these tools are also demonstrated to work with other *F. tularensis* subspecies. Kanamycin and hygromycin resistance determinants that function as genetic markers in *F. tularensis* subsp. *tularensis* strain Schu and sets of episomal shuttle vectors that are either unstable or stably maintained in the absence of selection were developed. In addition, the *hyg* gene, expressed from the *F. tularensis* *groESL* promoter, was successfully used as a marker for transposon mutagenesis. This work also includes the development of *sacB*-based suicide plasmids expressing kanamycin resistance that can be used for electroporation-mediated allelic exchange of unmarked mutations in Schu and the *F. tularensis* live vaccine strain (LVS). Using these plasmids, the two predicted β -lactamase genes, *blaA* and *blaB*, in Schu and LVS were deleted. Only the $\Delta blaB1$ mutants had increased susceptibility to ampicillin, and this phenotype was complemented by a plasmid expressing *blaB*⁺. The results suggest that the β -lactam antibiotic resistance phenotype of Schu and LVS is likely due to only one of the two β -lactamase genes present and that ampicillin resistance can be used as an additional selectable marker in β -lactamase deletion mutants. The collection of tools presented in this report will be helpful for the genetic analyses of *F. tularensis* subsp. *tularensis* pathogenesis.

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INTRODUCTION

Francisella tularensis is a fastidious Gram-negative bacterium responsible for the zoonotic disease tularemia (Sjostedt *et al.*, 1996). The disease has a variety of clinical presentations ranging from mild to more serious forms, such as pneumonic and typhoidal, which have a high mortality (Dennis *et al.*, 2001). There are four subspecies of *F. tularensis*, each differing in their relative degree of pathogenesis in the human host, ranging from the weakly pathogenic subsp. *novicida*, the moderately pathogenic subspecies *holarctica* and *mediasiatica*, to the highly pathogenic subspecies *tularensis* (Garcia Del Blanco *et al.*, 2002; Hollis *et al.*, 1989; Johansson *et al.*, 2001, 2004; Titball *et al.*, 2003). The high mortality of serious forms of tularemia, coupled with the very infectious nature of *F. tularensis* aerosols, prompted the past development of *F. tularensis* subsp. *tularensis* into a biological weapon (Dennis *et al.*, 2001).

The biology and molecular pathogenesis of this organism have been difficult to study, primarily due to the need for

biosafety level three conditions to manipulate the bacterium, its select agent status, and a lack of genetic tools. Most genetic studies have used the weakly pathogenic subspecies *novicida* or the live vaccine strain, LVS, derived from subsp. *holarctica* (Eigelsbach & Downs, 1961; Golovliov *et al.*, 2003; Lauriano *et al.*, 2003; Nano *et al.*, 2004; Sjostedt *et al.*, 1996). So far, three *Escherichia coli*–*Francisella* shuttle plasmids have been described for use in these two subspecies (Maier *et al.*, 2004; Norqvist *et al.*, 1996; Pavlov *et al.*, 1996); allelic exchange methods were developed for *novicida* and LVS (Golovliov *et al.*, 2003; Lauriano *et al.*, 2003), shuttle transposon mutagenesis has been used for *novicida* (Cowley *et al.*, 2000), while the EZ::TN transposome system (Kawula *et al.*, 2004) and a transposon based upon the *Himar1* element (Maier *et al.*, 2006) have been used for mutagenesis of LVS. However, few genetic tools have been tested with highly pathogenic, select agent strains of *F. tularensis* subsp. *tularensis*. A recent report was the first to show that site-directed mutagenesis of strain Schu S4 is possible, using a conjugative *sacB* plasmid encoding chloramphenicol resistance (Twine *et al.*, 2005).

A challenge in the development of genetic tools for *Francisella* is that only a few antibiotic resistance determinants can be used in highly pathogenic strains. All *F. tularensis* subspecies are intrinsically resistant to the

[†]These authors contributed equally to this work.

[‡]Present address: Biology Department, University of Maryland, Baltimore County, Baltimore, MD 21250, USA.

β -lactam classes of antibiotics (Baker *et al.*, 1985). Although most *F. tularensis* subspecies are sensitive to antibiotics such as streptomycin, gentamicin, tetracyclines, chloramphenicol, kanamycin and erythromycin, except LVS, which is resistant to erythromycin (Dennis *et al.*, 2001), the first four antibiotics can be used to treat tularaemia and thus the introduction of genes conferring resistance to these drugs into select agent strains of *F. tularensis* is restricted. The aminoglycosides kanamycin and nourseothricin are good antibiotics for genetic selection purposes in LVS but have not been tested in subsp. *tularensis* (Maier *et al.*, 2004, 2006). Some of the currently available genetic tools encode tetracycline and/or chloramphenicol resistance, which limits their use to non-select agent strains of *F. tularensis* (Golovliov *et al.*, 2003; Norqvist *et al.*, 1996). Thus, there is a need to test marker genes and develop genetic tools for highly pathogenic strains of subsp. *tularensis*.

In this paper, we report the development of new *E. coli*–*Francisella* shuttle vectors and genetic tools for use in *F. tularensis* subsp. *tularensis*. We demonstrate that the use of the kanamycin resistance gene *aphA-1* as a selectable marker in strain Schu requires that the gene be cloned downstream of a *F. tularensis* promoter. We chose *aphA-1* for this work, as it does not confer resistance to aminoglycosides used to treat *F. tularensis* infections (Dennis *et al.*, 2001; Shaw *et al.*, 1993). We also evaluated the hygromycin resistance gene, *hyg*, from *Streptomyces hygroscopicus* (Malpartida *et al.*, 1983), as a selectable marker. We chose this antibiotic because it is stable, it is not used to treat *F. tularensis* infections (Dennis *et al.*, 2001), and *hyg* does not confer resistance to other antibiotics (Leboul & Davies, 1982; Malpartida *et al.*, 1983). We also developed *sacB*-based suicide vectors and used them to delete the β -lactamase genes of strains Schu and LVS, and showed that efficient electroporation-mediated allelic exchange is possible in these organisms. Furthermore, we demonstrate that β -lactam resistance can be used as a selectable marker in these mutant strains.

METHODS

Bacterial strains, culture conditions and transformation. *E. coli* strain DH10B (Table 1) was used for routine cloning procedures and was grown in Luria–Bertani (LB) broth (BD Biosciences) or on LB agar. *F. tularensis* strains (Table 1) were grown at 37 °C in liquid modified Mueller–Hinton medium (MMH), which is Mueller–Hinton broth (BD Biosciences) supplemented with 1 mM CaCl₂, 1 mM MgCl₂, 0.1 % (w/v) glucose, 0.025 % (w/v) ferric pyrophosphate and 2.0 % (v/v) IsoVitalEx (BD Biosciences), or on MMH agar, which is the MMH medium above supplemented with 1.0 % (w/v) proteose peptone (BD Biosciences), 2.5 % (v/v) defibrinated sheep blood (Remel) and 1.5 % (w/v) bacto-agar (BD Biosciences) but lacking additional CaCl₂ and MgCl₂. Broth cultures of *F. tularensis* strains Utah 112 and LVS were grown in glass culture flasks with slow shaking (100 r.p.m.), while small (<20 ml) liquid cultures of *F. tularensis* strain Schu were grown in 30 ml plastic medium bottles with slow shaking and larger cultures of 50–100 ml were grown in 450 cm² plastic roller bottles (Corning) incubated on a roller apparatus. *F. tularensis* agar cultures were typically incubated

for 48 h. When necessary, ampicillin (Sigma-Aldrich Chemical) was added at 100 or 50 µg ml⁻¹, respectively, for *E. coli* or *F. tularensis*, while kanamycin (Sigma-Aldrich) was used at 50 µg ml⁻¹ for *E. coli*, 5 µg ml⁻¹ for LVS and Schu, or 15 µg ml⁻¹ for Utah 112. Kanamycin stock solutions were made by accounting for the concentration of active kanamycin reported for each lot by the supplier. Hygromycin B (Roche Applied Science) was used at 200 µg ml⁻¹ for all species and strains. Sucrose was used at a final concentration of 8 % (w/v). EZ::TN transposomes (Epicentre Technologies) were constructed as recommended by the manufacturer and stored at –20 °C for at least 3 days prior to use.

Electroporations were done with *F. tularensis* cells grown in MMH broth to an OD₆₀₀ of 0.2–0.5 for strain Schu, and 0.5–1.0 for Utah 112 and LVS. The cells were pelleted, washed twice in sterile, room temperature 0.5 M sucrose, and resuspended in sucrose at 1/100 of the original culture volume. Approximately 0.1–1.0 µg plasmid DNA was used for each electroporation mixed together with 100 µl prepared cells in a 0.2 cm gap cuvette. For allelic exchange experiments, we used at least 1 µg Qiagen column-prepared suicide plasmid per electroporation. The electroporations were done at room temperature with a Bio-Rad Gene Pulser II set for 2.5 kV, 25 µF and 600 Ω, followed by the addition of 1 ml room temperature MMH broth to the cuvette, transfer of the cell mixture to a 15 ml conical tube, and incubation with shaking at 37 °C for 3–4 h. Transformants were selected on MMH plates supplemented with the appropriate antibiotics.

DNA manipulation. DNA methods were performed essentially as described previously (Ausubel *et al.*, 1987). DNA fragments were isolated using agarose gel electrophoresis and absorption to a silica matrix (GeneClean; Bio 101), or by QIAquick spin columns (Qiagen). Oligonucleotides were synthesized by Invitrogen Life Technologies. All restriction endonucleases and DNA-modifying or polymerase enzymes were from New England Biolabs, Fermentas or Roche. PCR reactions with various thermostable polymerases were done according to the manufacturer's recommendations. All plasmids used in this study (Table 1) were from our laboratory's collection, except the cryptic *Francisella* plasmid pFNL10 (Pomerantsev *et al.*, 2001a), which was a generous gift from Fran Nano of the University of Victoria, BC, Canada, and the shuttle vector pFNLTP1 (Maier *et al.*, 2004), which was kindly provided by Tom Zahrt of the Medical College of Wisconsin. Plasmids were prepared from *E. coli* and *F. tularensis* strains using either a standard alkaline lysis protocol or with Qiagen columns. Genomic DNA from *F. tularensis* was prepared by a rapid guanidinium thiocyanate method previously described for mycobacteria (Pavelka & Jacobs, 1999).

Plasmid construction. Detailed descriptions of the construction of the plasmids used in this study can be obtained from the corresponding author. Information pertinent to the understanding of this work is described below for some of the plasmids.

Shuttle vector pMP393. Plasmid pMV261 (Table 1) was the source of a 2.1 kb *HincII*–*MluI* DNA fragment containing the ColE1 origin of replication and the *aphA-1* gene. This fragment was ligated to a 2.0 kb DNA fragment obtained from pFNL10 via PCR that encompasses the region from position 1 through 2000 of the pFNL10 GenBank sequence (accession no. NC_004952) to produce pMP393.

Shuttle vectors pMP527 and pMP529. The *aphA-1* gene was obtained from pMV261 using PCR to generate an *NdeI* site at the start codon of the gene. This fragment was cloned into pMP478, which is pMV261 bearing a PCR-generated *NotI*–*NdeI* DNA fragment from LVS (GenBank accession no. AM23362) corresponding to 290 bp upstream of the *groESL* operon, encompassing the promoter

Table 1. Bacterial strains and plasmids

Strains or plasmid	Description	Source or reference*
Bacteria		
<i>E. coli</i>		
DH10B	F ⁻ <i>mcrA</i> Δ (<i>mcrBC</i> - <i>hsdRMS</i> - <i>mrr</i>) [ϕ 80d Δ <i>lacZ</i> Δ M15] Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ (<i>ara</i> , <i>leu</i>)7697 <i>galU</i> <i>galK</i> λ^- <i>rpsL</i> <i>nupG</i>	
<i>F. tularensis</i>		
Utah 112	<i>F. tularensis</i> subsp. <i>novicida</i>	K. Elkins
LVS	<i>F. tularensis</i> subsp. <i>holarctica</i> live vaccine strain	J. Benach
Schu	<i>F. tularensis</i> subsp. <i>tularensis</i>	M. Schriefer
PM1521	LVS Δ <i>blaA1</i>	This work
PM1556	LVS Δ <i>blaB1</i>	This work
PM1560	LVS Δ <i>blaA1</i> Δ <i>blaB1</i>	This work
PM1516	Schu Δ <i>blaA1</i>	This work
PM1580	Schu Δ <i>blaB1</i>	This work
PM1571	Schu Δ <i>blaA1</i> Δ <i>blaB1</i>	This work
Plasmids		
pMOD-2	Ap ^R , EZ::TN cloning vector	Episentre
pFNL10	<i>Francisella</i> cryptic plasmid	Pomerantsev <i>et al.</i> (2001a)
pFNLTP1	Km ^R , <i>E. coli</i> - <i>F. tularensis</i> shuttle vector	Maier <i>et al.</i> (2004)
pMV261	Km ^R , <i>E. coli</i> -mycobacterial shuttle vector	Stover <i>et al.</i> (1991)
pMV261. <i>hyg</i>	Hyg ^R , <i>E. coli</i> -mycobacterial shuttle vector	Flores <i>et al.</i> (2005b)
pYUB631	Km ^R , pMV261 bearing the <i>sacR</i> <i>sacB</i> region of <i>Bacillus subtilis</i>	Pavelka & Jacobs (1999)
pMP393	Km ^R , <i>E. coli</i> - <i>F. tularensis</i> shuttle vector	This work
pMP478	Km ^R , pMV261 bearing a 290 bp <i>XbaI</i> - <i>NdeI</i> fragment containing the <i>groESL</i> promoter of LVS	This work
pMP492	Km ^R , pMV261 bearing a 1.8 kb <i>PstI</i> PCR fragment containing the <i>blaB</i> ⁺ region from LVS	This work
pMP521	Km ^R , pMV261 bearing a 3.2 kb <i>PstI</i> PCR fragment containing the <i>blaA</i> ⁺ region from LVS	This work
pMP523	Km ^R , pMP478 bearing the <i>aphA-1</i> gene from pMV261 cloned under control of <i>P_{groESL}</i>	This work
pMP524	Hyg ^R , pMP478 bearing the <i>hyg</i> gene from pMV261. <i>hyg</i> , cloned under control of <i>P_{groESL}</i>	This work
pMP527	Km ^R , <i>E. coli</i> - <i>F. tularensis</i> shuttle vector with the <i>P_{groESL}</i> - <i>aphA-1</i> cassette	This work
pMP528	Ap ^R , Hyg ^R , pMOD-2 bearing the <i>P_{groESL}</i> - <i>hyg</i> cassette	This work
pMP529	Hyg ^R , <i>E. coli</i> - <i>F. tularensis</i> shuttle vector with the <i>P_{groESL}</i> - <i>hyg</i> cassette	This work
pMP537	Km ^R , deletion derivative of pMP492 made by inverse PCR resulting in the Δ <i>blaB1</i> allele	This work
pMP552	Km ^R , pLLP18 bearing a 1.3 kb fragment that is the 3' partial Δ <i>blaA1</i> allele, forming the complete deletion allele	This work
pMP578	Km ^R , first-generation <i>F. tularensis</i> <i>sacB</i> suicide vector	This work
pMP582	Km ^R , pMP578 suicide vector bearing the 2.2 kb fragment containing the Δ <i>blaA1</i> allele from pMP522	This work
pMP590	Km ^R , 570 bp smaller, second-generation <i>F. tularensis</i> <i>sacB</i> suicide vector derived from pMP588	This work
pMP592	Km ^R , pMP590 suicide vector bearing the Δ <i>blaB1</i> allele from pMP537	This work
pMP607	Km ^R , <i>E. coli</i> - <i>F. tularensis</i> shuttle vector with the <i>P_{groESL}</i> - <i>aphA-1</i> cassette and ORF4-ORF5 of pFNL10	This work
pMP615	Hyg ^R , pMP529 with a 1.4 kb <i>MluI</i> PCR fragment bearing the <i>blaB</i> ⁺ gene of Schu	This work
pMP633	Hyg ^R , <i>E. coli</i> - <i>F. tularensis</i> shuttle vector with the <i>P_{groESL}</i> - <i>hyg</i> cassette and ORF4-ORF5 of pFNL10	This work

*K. Elkins, FDA, Rockville, MD, USA; J. Benach, SUNY, Stony Brook, NY, USA; M. Schriefer, CDC, Fort Collins, CO, USA.

and the *groES* start codon, engineered to have the *NdeI* site at the ATG. The resultant plasmid, pMP523 (Table 1), was used as the source of the *P_{groESL}-aphA-1* cassette for the construction of pMP527.

The *hyg* gene was amplified from pMV261.*hyg* using PCR to produce a 1012 bp *hyg* product with an engineered *NdeI* site at the start codon of the gene, which was cloned into pMP478 as described above for the *aphA-1* cassette, yielding pMP524. The *P_{groESL}-hyg* cassette was cloned into pMP393 to generate pMP529 in the same manner as the *P_{groESL}-aphA-1* cassette was cloned above in the construction of pMP527.

Shuttle vectors pMP607 and pMP633. pMP607 was derived from pMP527 and contains an ORF4–ORF5 DNA fragment (encompassing nucleotide positions 789–2596 of the pFNL10 sequence) obtained by PCR. pMP633 was made by swapping the *P_{groESL}-aphA-1* cassette of pMP607 with the *P_{groESL}-hyg* cassette from pMP529.

***sacB* suicide vectors.** Plasmid pMP578 was constructed by ligating a 2.3 kb *BglII*–*EcoRV* DNA fragment bearing the *sacB* region from pYUB631 (Table 1) with a 3.2 kb *BglII*–*HincII* DNA fragment from pMP527. pMP57 lacks ORF2 and part of *repA* of the *Francisella* origin of replication. Plasmid pMP590 is a modified version of pMP578 that includes a multiple cloning site and two deletions that move *sacB* closer to the *repA* promoter region.

Plasmid for Δ *blaA* allelic exchange, pMP582. A DNA fragment containing *blaA* was obtained from LVS genomic DNA using PCR and cloned into pMV261 to yield pMP521. An in-frame deletion of 771 bp within *blaA* was made using PCR. A 2.1 kb fragment con-

taining the Δ *blaA1* allele was cloned into pMP578 to yield pMP582. There is 1076 bp of DNA upstream and 1120 bp downstream of the Δ *blaA1* allele in this plasmid.

Plasmid for Δ *blaB* allelic exchange, pMP592. A DNA fragment containing *blaB* was obtained from LVS genomic DNA using the PCR and cloned into pMV261 to yield pMP492, which was used as a template for inverse PCR to generate an in-frame deletion of 735 bp within *blaB*. This allele was cloned into the *EcoRV* site within pMP590 to produce pMP592. There is 485 bp of DNA upstream and 399 bp downstream of the Δ *blaB1* allele in this plasmid.

Plasmid for *blaB* complementation, pMP615. The *blaB*⁺ gene with its putative promoter region was obtained from Schu genomic DNA (GenBank accession no. AJ749949) as a 1.4 kb DNA fragment with engineered *MluI* sites using PCR, and cloned into the *MluI* site of pMP529 to yield pMP615.

RESULTS

First-generation shuttle vectors

Our backbone *E. coli*–*Francisella* shuttle vector, pMP393 (Fig. 1), is derived from plasmid pMV261, an *E. coli*–mycobacterial shuttle vector (Stover *et al.*, 1991). We replaced the mycobacterial pAL5000 origin of replication in pMV261 with a PCR-generated, 2 kb DNA fragment

Fig. 1. Maps of *E. coli*–*F. tularensis* shuttle vectors. (A) The *F. tularensis* cryptic plasmid pFNL10 is shown for comparison. The plasmid pMP393 (B) contains the *Francisella* origin of replication region (*ori*, *repA*, ORF2) from pFNL10 and the *E. coli* ColE1 origin of replication, gene *aphA-1* (from Tn903) conferring resistance to kanamycin, and the *rrnABt1* transcriptional terminator. The plasmid pMP527 (C) is pMP393 with the wild-type *aphA-1* gene replaced with a *P_{groESL}-aphA-1* cassette, while pMP529 (D) is essentially the same as pMP527 but contains the *P_{groESL}-hyg* cassette. The ORF4–ORF5-containing plasmid, pMP607 (E), was derived from pMP527, and pMP633 (F) was derived from pMP607.

from the *Francisella* cryptic plasmid pFNL10 (Fig. 1) (Pomerantsev *et al.*, 2001a) that includes the origin of replication, the *repA* and ORF2 genes and their promoter region, but no other intact ORFs from pFNL10.

We routinely obtained $\sim 10^6$ kanamycin-resistant LVS transformants per electroporation with saturating amounts of pMP393 DNA (Fig. 2). Calculating our transformation efficiency on a per μg basis yields a value of $\sim 10^7$ kanamycin-resistant transformants $(\mu\text{g plasmid DNA})^{-1}$, which is the same as that previously reported by others for plasmid transformation of LVS following electroporation (Maier *et al.*, 2004).

We found that the transformation efficiencies of strains Utah 112 and Schu were three to four orders of magnitude lower than that of LVS (Fig. 2). The low transformation efficiency of Utah 112 we obtained has been reported by others and is likely due to a restriction–modification system previously described in this strain (Maier *et al.*, 2004). We hypothesized that the *aphA-1* gene in pMP393 might not be expressed well in Schu, and so we replaced the wild-type *aphA-1* in pMP393 with a PCR-generated cassette consisting of *aphA-1* expressed from the *F. tularensis* *groESL* promoter (Ericsson *et al.*, 1997) to produce pMP527 (Fig. 1). With this plasmid we obtained transformation efficiencies of at least 10^6 with both LVS and Schu, and a 10^3 transformation efficiency with Utah 112 (Fig. 2). Transformants of all three strains that received pMP393 or pMP527 grew at the same rate and had normal colony morphologies (data not shown).

Fig. 2. Transformation efficiencies of shuttle vectors with the three *F. tularensis* subspecies strains. The efficiencies are expressed as the number of antibiotic-resistant colony-forming units per electroporation with saturating amounts of plasmid DNA. Shown are the mean values and standard deviations of multiple experiments. Key: pMP393 (*aphA-1*, stippled bars), pMP527 (*P_{groESL}-aphA-1*, open bars) and pMP529 (*P_{groESL}-hyg*, hatched bars).

We compared transformation efficiencies of strains LVS and Schu with pMP393, pMP527 and the previously developed *E. coli*–*Francisella* shuttle vector pFNLTP1 capable of highly efficient transformation of LVS (Maier *et al.*, 2004) (Table 1). This vector consists of the *E. coli* pCR2.1-TOPO cloning vector fused to most of the pFNL10 cryptic plasmid. It differs from pMP393 and pMP527 in that it has ORF3, part of ORF5 and the *npt* gene for neomycin/kanamycin resistance, but it has *ori*, *repA* and ORF2 as they are present in our vectors. We found that the pFNLTP1 transformation efficiency was very high with strain LVS, as expected, but low with strain Schu. We obtained $\sim 10^6$ transformants with LVS and less than 50 transformants with strain Schu per electroporation (data not shown). The electroporations with pFNLTP1 were done in parallel with the cells and media used for the experiments with pMP393 and pMP527 shown in Fig. 2. The Schu/pFNLTP1 transformants took 2–3 days longer to grow than pMP393 or pMP527 transformants and had varying colony morphology and size, ranging from small pinpoints to an almost normal size. However, in agreement with a previous report (Maier *et al.*, 2004), we saw no growth or morphological changes for the LVS/pFNLTP1 transformants.

Hygromycin resistance as a selectable marker

We developed the *hyg* gene, encoding a hygromycin phosphotransferase, into a selectable marker by building a new derivative plasmid, pMP529, made by replacing *aphA-1* of pMP393 with a PCR-generated cassette bearing *hyg* cloned downstream of the *F. tularensis* *groESL* promoter as was done for the *aphA-1* cassette above (Fig. 1). We obtained essentially the same transformation efficiencies with pMP529 as we did with pMP527 and the three *F. tularensis* subspecies, obtaining $\sim 10^6$ transformants per electroporation with strains LVS and Schu, and $\sim 10^3$ hygromycin-resistant clones per electroporation with strain Utah 112 (Fig. 2). We used hygromycin at a concentration of $200 \mu\text{g ml}^{-1}$ for these experiments because this concentration is sufficient for selection against non-transformed *F. tularensis*. Furthermore, the frequency of spontaneous resistance of *F. tularensis* strains to the antibiotic was $< 10^{-8}$ when used at this concentration (data not shown).

Plasmid maintenance and second-generation shuttle vectors

We investigated the maintenance of the pMP527 shuttle vector in strains LVS and Schu in the absence of antibiotic selection and found that the plasmid was lost after subculture in media lacking kanamycin. For this determination, overnight cultures of pMP527 transformants grown in kanamycin medium were subcultured 1:10 into fresh medium with or without kanamycin and incubated for an additional 24 h, after which time we plated cells onto solid medium lacking antibiotic. Colonies were then picked and patched onto medium with or without kanamycin. The

percentage of kanamycin-sensitive clones in each population is shown in Table 2.

We hypothesized that introducing additional DNA from the cryptic plasmid pFNL10 into our first-generation shuttle vectors might allow us to develop a second-generation series of vectors that are stably maintained in a population grown without antibiotic selection. To this end, we cloned the ORF4–ORF5 region of pFNL10, encoding a putative ‘addiction system’ (Pomerantsev *et al.*, 2001a), into pMP527 to produce pMP607 (Fig. 1). The cloning was designed to reconstruct the relevant region in pMP607 as it organized in pFNL10 (Fig. 1).

Electroporation experiments with pMP607 and strains LVS and Schu revealed no defect in transformation efficiency as compared to the parental plasmid pMP527 (data not shown). When we repeated the stability tests, we found that pMP607 was effectively maintained in populations of transformed strains in the absence of selection. At most, only 2% of the population was kanamycin sensitive after 24 h growth in broth lacking the antibiotic (Table 2). Continued subculturing of LVS/pMP607 transformants for 2 days in the absence of antibiotic selection did not increase the percentage of kanamycin-sensitive cells (data not shown).

To improve the utility of the vector pMP607, we constructed a derivative, pMP633 (Fig. 1), which has the *P_{groESL}-aphA-1* cassette replaced with the *P_{groESL}-hyg* cassette. This plasmid has high transformation efficiency like its parent vector and is also maintained in a population of transformants without selection (Table 2).

Transposon mutagenesis

We tested the ability of the *P_{groESL}-hyg* cassette to function at single copy in the *F. tularensis* chromosome by incorporating the cassette into a transposon and using it to mutagenize *F. tularensis* strains LVS and Schu. We used the commercially available EZ::TN transposome system from Epicentre Technologies, which was previously shown to work well for mutagenesis of LVS (Kawula *et al.*, 2004). The transposome consists of a purified transposase enzyme bound to the EZ::TN transposon, a hyperactive version of

the transposon Tn5 characterized by two 19 bp mosaic ends (ME) flanking an antibiotic resistance marker (Goryshin *et al.*, 2000). A complex formed *in vitro* by the transposase and transposon DNA is stable in the absence of magnesium and can be electroporated into bacteria, where intracellular magnesium ions initiate transposition (Goryshin *et al.*, 2000).

We constructed a new transposon by cloning the *P_{groESL}-hyg* cassette into the pMOD-2 vector from Epicentre, which contains the mosaic ends of the EZ::TN transposon flanking a multiple cloning site, to produce the plasmid pMP528 (Fig. 3). Cleavage of the plasmid with the restriction enzyme *PvuII* releases transposon DNA fragments which are purified and complexed with the EZ::TN transposase.

We electroporated transposomes into strains LVS and Schu and selected for insertional mutants on MMH medium supplemented with 200 µg hygromycin ml⁻¹. For both strains we obtained hygromycin-resistant transformants at a frequency of ~10⁻⁶ per viable colony-forming unit. Three independent electroporations with LVS yielded 0.6 × 10³, 1.3 × 10³ and 1.6 × 10³ hygromycin-resistant mutants per electroporation while two electroporations of two independent cultures of Schu yielded 2.2 × 10³, 2.2 × 10³, 2.5 × 10³ and 2.6 × 10³ hygromycin-resistant mutants per electroporation. We did not obtain hygromycin-resistant clones in electroporations lacking transposomes. We performed Southern blots on genomic DNA prepared from ten arbitrarily chosen hygromycin-resistant transposon mutants of Schu using the *hyg* gene as the probe. Of the ten mutants, one had two insertions, while the other nine appeared to have only one transposon insertion each, which was located at a different site in each chromosome relative to the other mutants (Fig. 3). This frequency of multiple insertions was previously described by us, and others, who have used the EZ::TN system in mycobacteria, and is likely the result of a single cell receiving two or more transposomes during the electroporation (Derbyshire *et al.*, 2000; Flores *et al.*, 2005a).

Suicide plasmids and allelic exchange of *bla* genes

We used the first-generation shuttle vector pMP527 (Fig. 1) as the backbone plasmid to construct a *sacB*-based allelic exchange vector. Our first *sacB* vector, pMP578 (Fig. 4), was constructed by deleting part of *repA* and all of the ORF2 gene of the *Francisella* origin of replication of pMP527 (Fig. 1) and then inserting a fragment bearing the *sacB* region into the backbone of the plasmid. This was done to ensure that the vector would not replicate in *Francisella* and that *sacB* would be expressed from a strong *Francisella* promoter, presumably that which originally drove transcription of *repA* and ORF2.

We tested the ability of this plasmid to delete the β-lactamase genes in LVS and Schu using a two-step allelic

Table 2. Maintenance of shuttle vectors

Plasmid	Percentage loss of antibiotic resistance*	
	LVS	Schu
pMP527	74, 98 (100)	65, 59, 70 (100)
pMP607	0, 2 (50)	2, 0, 0 (50)
pMP633	0, 0 (50)	Not determined

*The percentage of screened clones that lost antibiotic resistance is shown with the number of clones screened in each experiment in parentheses. Two cultures were tested for each plasmid in LVS and three cultures tested for each plasmid in strain Schu.

Fig. 3. EZ::TN plasmid and Southern blot of Schu mutants. (A) Map of the pMP528 plasmid, a derivative of the EZ::TN construction vector pMOD-2, which contains the *PgroESL*-*hyg* cassette cloned between the mosaic ends of the EZ::TN transposon. The 1.6 kb transposon is removed from pMP528 by digestion with the restriction endonuclease *PvuII*. (B) Southern blot of *PvuII*-digested genomic DNA from Schu transposon mutants and wild-type, probed with *hyg*. Lanes: 1–10, transposon mutants; 11, wild-type Schu. The asterisk indicates the double insertion mutant (lane 2). Overexposure of the blot did not reveal any additional bands in lanes 7 and 8.

exchange. Previous work by another group showed that disruption of *bla* (designated *blaA* here), encoding a putative class A β -lactamase, does not affect the resistance phenotype of subsp. *novicida* strain Utah 112 and revealed that a second, putative class A β -lactamase gene (designated *blaB* here) may contribute to resistance (Lauriano *et al.*,

2003). We used the *sacB* vector pMP578 to construct pMP582, an allelic exchange plasmid for $\Delta blaA1$, and a second-generation *sacB* vector, pMP590 (Fig. 4), that is smaller than pMP578 and has multiple cloning sites, and used it to make the $\Delta blaB1$ allelic exchange plasmid pMP592.

Fig. 4. Maps of the *F. tularensis* *sacB* suicide vectors pMP578 and pMP590. The plasmid pMP578 (A) is derived from pMP527 and is missing ORF2 and part of *repA*. It also contains *sacB* and the regulatory region *sacR*. The plasmid pMP590 (B) is a 1.1 kb smaller version of pMP578 containing a multiple cloning site (MCS). The deletion in pMP590 removes the *sacR* region and moves the *sacB* gene closer to the *repA* promoter region. Both plasmids confer kanamycin resistance to Schu and LVS.

A typical allelic exchange procedure was done by electroporating the suicide vectors into LVS or Schu and plating on medium containing kanamycin at $5 \mu\text{g ml}^{-1}$. Each suicide plasmid yielded kanamycin-resistant recombinants at a frequency of 10^{-5} to 10^{-6} relative to the transformation efficiency ($\sim 10^6$ transformants per electroporation) with the replicating plasmid pMP527. Electroporations using suicide vectors lacking a *F. tularensis* DNA insert did not yield kanamycin-resistant recombinants. All the kanamycin-resistant primary recombinants arising from the $\Delta blaA1$ and $\Delta blaB2$ suicide plasmid transformations were sucrose sensitive, which was presumed to reflect integration of the suicide plasmid into the native locus of the chromosome by a single homologous recombination event.

We grew kanamycin-resistant, sucrose-sensitive, primary recombinants to saturation in medium lacking antibiotic and then plated the cells onto sucrose medium to select against clones that did not undergo a second recombination event. For all our experiments, sucrose-resistant clones arose from these cultures at frequencies of 10^{-3} to 10^{-4} relative to the viable counts. These putative secondary recombinants were then screened for loss of kanamycin resistance as an indication of successful recombination and loss of the integrated suicide plasmid. In the secondary recombinant pool, all the sucrose-resistant clones should be kanamycin sensitive, and in general, the population will consist of both wild-type and mutant clones. In practice, however, one always finds a few kanamycin-resistant clones in the sucrose-resistant population that represent clones that did not undergo a secondary recombination event but, instead, have a mutation (possibly an insertion element or deletion) within

the *sacB* gene or promoter region. In our experiments, we typically found kanamycin resistance in the sucrose-resistant population at frequencies ranging from 0 to 30 %.

We screened a set of sucrose-resistant, kanamycin-sensitive secondary recombinants by PCR to identify the $\Delta blaA1$ mutants. We picked two mutants, PM1521 (LVS $\Delta blaA1$) and PM1516 (Schu $\Delta blaA1$), for subsequent work. A Southern blot showing the deletion of *blaA* in these two strains is shown in Fig. 5(A). Consistent with the observation reported for the *blaA* knockout of *F. tularensis* subsp. *novicida*, we found that the $\Delta blaA1$ mutants grew as well as wild-type cells on media containing ampicillin (Fig. 6).

We then made mutants with only a deletion of *blaB*, strains PM1556 (LVS $\Delta blaB1$) and PM1580 (Schu $\Delta blaB1$). These mutants had increased susceptibility to ampicillin and could be distinguished from wild-type or $\Delta blaA$ mutants on medium containing ampicillin at $50 \mu\text{g ml}^{-1}$ (Fig. 6). In addition, we constructed double mutants, strains PM1560 (LVS $\Delta blaA1 \Delta blaB1$) and PM1571 (Schu $\Delta blaA1 \Delta blaB1$). These mutants were comparable to the single $\Delta blaB1$ mutants (Fig. 6). We confirmed allelic exchange in these mutants by Southern blotting as shown in Fig. 5.

We then demonstrated that *blaB*⁺ could be used as an additional selectable marker in the $\Delta blaA1 \Delta blaB1$ mutants. We cloned the wild-type *blaB* and its upstream region into the pMP529 shuttle vector to produce pMP615. This plasmid and the vector control were electroporated into the LVS and Schu double mutants, followed by dilution plating onto media containing either hygromycin at $200 \mu\text{g ml}^{-1}$ or

Fig. 5. Southern blots of genomic DNA from β -lactamase deletion mutants. (A) Genomic DNA of strains LVS and Schu digested with *HpaI* and probed with a 2.3 kb *PstI* fragment from plasmid pMP552 encompassing the $\Delta blaA1$ allele. Lanes: 1, 6, kb markers; 2, 7 and 4, 9, wild-type *blaA* (3.8 kb) LVS, Schu; 3, 5, $\Delta blaA1$ (3.0 kb) LVS, Schu mutants; 8, 10, $\Delta blaA1$ (3.0 kb) LVS, Schu double mutants. (B) Genomic DNA of strains LVS and Schu digested with *NdeI* and probed with a 1.0 kb *PstI* fragment from plasmid pMP537 encompassing the $\Delta blaB1$ allele. Lanes: 1, 6, kb markers; 2, 7 and 4, 9, wild-type *blaB* (1.9 kb) LVS, Schu; 3, 5, $\Delta blaB1$ (1.2 kb) LVS, Schu mutants; 8, 10, $\Delta blaB1$ (1.2 kb) LVS, Schu double mutants.

Fig. 6. Growth of LVS strains on MMH medium containing ampicillin at $50 \mu\text{g ml}^{-1}$. +, wild-type LVS; ΔA , $\Delta blaA1$ mutant PM1521; ΔB , $\Delta blaB1$ mutant PM1556; ΔAB , $\Delta blaA1 \Delta blaB1$ mutant PM1560; $\Delta AB/V$, the $\Delta blaA1 \Delta blaB1$ mutant vector control; $\Delta AB/B^+$, the double mutant complemented with gene *blaB*⁺ *in trans*. Similar results were obtained with the corresponding Schu strains.

ampicillin at $50 \mu\text{g ml}^{-1}$. We obtained $\sim 10^6$ transformants per electroporation with both plating methods for both strains regardless of antibiotic, with pMP615 generating equivalent numbers of hygromycin- and ampicillin-resistant clones (data not shown). As expected, electroporations with pMP529 generated only hygromycin-resistant clones. Thus, *blaB*⁺, carried on a plasmid *in trans*, complements the ampicillin phenotype and can be used for direct selection of ampicillin-resistant transformants in a $\Delta blaA1 \Delta blaB1$ background. The amount of BlaB enzyme expressed from the plasmid in the complemented strains appears to be higher than that produced by wild-type or $\Delta blaA1$ cells, as indicated by the relatively better growth of the complemented strains on ampicillin plates (Fig. 6).

DISCUSSION

The plasmids described here were derived from the cryptic plasmid pFNL10 originally found in ‘*F. novicida*-like strain F6168’, a strain isolated from human blood in 1984 (Hollis *et al.*, 1989; Pomerantsev *et al.*, 2001a). This plasmid is 3990 bp in size, carries six putative ORFs and is believed to replicate by the theta mode (Pomerantsev *et al.*, 2001a). Putative functions of the ORFs have been inferred from sequence comparisons to known proteins and the phenotypes of deletion derivatives of other pFNL10-based shuttle vectors (Maier *et al.*, 2004; Pavlov *et al.*, 1996; Pomerantsev *et al.*, 2001a, b). ORF1 is also known as *repA* and encodes the replication initiation protein, while ORF2 may be a helicase or endonuclease important for plasmid multimer resolution. The function of ORF3 is unknown but it has some similarity to recombinases and integrases, while ORF4 and ORF5 may constitute an addiction system important for

maintenance of pFNL10 (Pomerantsev *et al.*, 2001a, b). Addiction systems, or toxin–antitoxin systems, encode a long-lived toxic protein and a short-lived antitoxin protein. Loss of the two genes results in death or stasis of the cell by the toxic protein after the antitoxin is degraded by cellular proteases, as reviewed by Gerdes *et al.* (2005). The ORF5 gene product is similar to the Phd antitoxin encoded on bacteriophage P1 (part of the *phd-doc* addiction system of P1) and it is thought that the ORF4 gene product is the corresponding toxin, although its similarity to other toxic proteins, particularly Doc, is not very high (Pomerantsev *et al.*, 2001a). The remaining ORF of pFNL10, ORFm, may encode a protein with a role in regulating the expression or the functions of ORF4 and ORF5, but this hypothesis is untested (Pomerantsev *et al.*, 2001a).

Plasmid pFNL10 was previously used to create the first shuttle vector that could replicate in *E. coli* and *F. tularensis*. This vector, pFNL100, was made by fusing pFNL10 to the *E. coli* vector pBR328, and encodes resistance to tetracycline and chloramphenicol (Norqvist *et al.*, 1996). A deletion derivative of this vector, pFNL200, which lacks the *E. coli* origin, was used in the development of the shuttle vector pKK202, which bears the *E. coli* p15A origin from pACYC184 (Norqvist *et al.*, 1996). A more recent shuttle vector, pTOPO/FNL10, was made in much the same manner by fusing all of pFNL10 to an *E. coli* cloning vector bearing a kanamycin resistance marker (Maier *et al.*, 2004). Both pFNL200 and pTOPO/FNL10 contained a segment of pFNL10 DNA that was ultimately unstable in *F. tularensis* and was spontaneously deleted from the vectors when passed through the organism. In the case of pFNL200, the deletion derivative, pOM1 (Pomerantsev *et al.*, 2001a, b), lost part of ORF5, all of ORF4 and ORFm, while the deletion derivatives of pTOPO/FNL10 lost all of ORFm, all or most of ORF4, and in some cases (pFNLTP1), a part of ORF5 (Maier *et al.*, 2004). In the construction of pFNL200, the cloning vector was inserted into ORF5, the putative antitoxin gene, while in the construction of pTOPO/FNL10, the cloning vector was inserted between ORFm and ORF3, which might have affected expression of ORFm or ORF4–ORF5. These other researchers suggested that the deletion derivatives perhaps arose because the ORF4–ORF5 addiction system was probably dysregulated, with lethal consequences for *F. tularensis*.

In the construction of our first generation of shuttle vectors, we started with the minimal amount of DNA required for efficient replication in *F. tularensis*. However, these vectors were lost in the absence of selection, as demonstrated by the behaviour of pMP527. To rectify this, we inserted the ORF4–ORF5 region into our second generation of shuttle vectors. Our data from experiments with these vectors support the idea that these genes encode an addiction system because our second-generation plasmids are maintained in populations in the absence of selection.

The shuttle vector pFNLTP1, one of the deletion derivatives of pTOPO/FNL10 that contains *repA*, ORF2 and ORF3, is

stable in the absence of selective pressure (Maier *et al.*, 2004). Our stably maintained plasmids, pMP607 and pMP633, have *repA*, ORF2 and ORF4–ORF5 but lack ORF3. We think the maintenance of these two types of plasmids is the result of different mechanisms. Our original plasmids, pMP393, pMP527 and pMP529, have only *repA* and ORF2 and probably do not segregate as efficiently as pFNLTP1. We propose that the presence of the ORF4–ORF5 addiction system in our second-generation plasmids pMP607 and pMP633 does not increase the efficiency of plasmid segregation, but instead affects the maintenance of the plasmids in the population, as any daughter cell that lost the plasmid would be killed by the ORF4-encoded toxin. In contrast, the ORF3 gene present in pFNLTP1 might directly increase segregation efficiency by affecting plasmid partitioning, or it might play a role in copy number control. High-copy-number plasmids are less likely to be lost in daughter cells compared to low-copy-number plasmids.

It is possible that a plasmid bearing ORF3 is not well tolerated by strain Schu, as we did not obtain good transformation efficiencies with pFNLTP1 and Schu compared to our plasmids that lack ORF3. Furthermore, the Schu/pFNLTP1 transformants did not grow well and had altered colony morphologies. This could be explained by a low tolerance of high-copy-number plasmids by Schu, if ORF3 has a role in copy-number control. Alternatively, the effects of pFNLTP1 on strain Schu could be related to the plasmid's kanamycin resistance marker *npt*, which is different from the *aphA-1* marker present in our vectors.

The tools described here can be further developed for new applications. We are redesigning the shuttle vectors to include the *blaB* promoter region and a multiple cloning site for use as expression vectors. The unstable shuttle vectors can be used for transient expression of site-specific recombinases for the removal of antibiotic cassettes or for gene essentiality tests. We anticipate that these tools will be useful to others in the field meeting the challenge of *F. tularensis* genetics.

NOTE ADDED IN PROOF

While this paper was in review, another group (Bina, X. R., Wang, C., Miller, M. A. & Bina, J. E. 2006 *Arch Microbiol* **186**, 219–228) reported on the expression of the *F. tularensis* β -lactamase genes in *E. coli* and their data support our conclusions here that only the *blaB* gene contributes to β -lactamase resistance in *F. tularensis*. Another group (Qin, A. & Mann, B. J. 2006 *BMC Microbiol* **6**, 69) reported transposon mutagenesis of *F. tularensis* strain SchuS4 with the EZ::TN system using rifampicin resistance.

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