Genetic tools for highly pathogenic Francisella tularensis subsp. tularensis

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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 ΔblaB1 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.

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 mediiasiatica, 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

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$\beta$-lactam classes of antibiotics (Baker et al., 1985). Although most \textit{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 tularemia and thus the introduction of genes conferring resistance to these drugs into select agent strains of \textit{F. tularensis} is restricted. The aminoglycosides kanamycin and nourseothricin are good antibiotics for genetic selection purposes in LVS but have not been tested in subs. \textit{tularensis} (Maior 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 \textit{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 subs. \textit{tularensis}.

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

**METHODS**

**Bacterial strains, culture conditions and transformation.** \textit{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. \textit{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 CaCl$_2$, 1 mM MgCl$_2$, 0.1% (w/v) glucose, 0.025% (w/v) ferric pyrophosphate and 2.0% (w/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% (w/v) defibrinated sheep blood (Remel) and 1.5% (w/v) bacitracin (BD Biosciences) but lacking additional CaCl$_2$ and MgCl$_2$. Broth cultures of \textit{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 \textit{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 cm$^2$ plastic roller bottles (Corning) incubated on a roller apparatus. \textit{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 \textit{E. coli} or \textit{F. tularensis}, while kanamycin (Sigma-Aldrich) was used at 50 µg ml$^{-1}$ for \textit{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 \textit{F. tularensis} cells grown in MMH broth to an OD$_{600}$ 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 \textit{Francisella} plasmid pFN1.0L10 (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 \textit{E. coli} and \textit{F. tularensis} strains using either a standard alkaline lysis protocol or with Qiagen columns. Genomic DNA from \textit{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 \textit{HinClI–MluI} DNA fragment containing the ColE1 origin of replication and the \textit{aphA-1} gene. This fragment was ligated to a 2.0 kb DNA fragment obtained from pFN1.0L10 via PCR that encompasses the region from position 1 through 2000 of the pFN1.0L10 GenBank sequence (accession no. NC_004952) to produce pMP393.

**Shuttle vectors pMP527 and pMP529.** The \textit{aphA-1} gene was obtained from pMV261 using PCR to generate an \textit{NdeI} site at the start codon of the gene. This fragment was cloned into pMP478, which is pMV261 bearing a PCR-generated Not–NdeI DNA fragment from LVS (GenBank accession no. AM23362) corresponding to 290 bp upstream of the \textit{groEL} operon, encompassing the promoter.
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Description</th>
<th>Source or reference*</th>
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<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
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<tr>
<td>E. coli DH10B</td>
<td>F- mcrA Δ(mcrBC-hsdRMS-mrr) [Δ80dΔlacZΔM15] ΔlacX74 deoR recA1 endA1 araD139 Δ(ara,leu)7697 galU galK i- rpsL nupG</td>
<td></td>
</tr>
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<td>F. tularensis Utah 112</td>
<td>F. tularensis subsp. novicida</td>
<td>K. Elkins</td>
</tr>
<tr>
<td>LVS</td>
<td>F. tularensis subsp. holarctica live vaccine strain</td>
<td>J. Benach</td>
</tr>
<tr>
<td>Schu</td>
<td>F. tularensis subsp. tularensis</td>
<td>M. Schriefer</td>
</tr>
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<td>PM1521</td>
<td>LVS ΔblaA1</td>
<td>This work</td>
</tr>
<tr>
<td>PM1556</td>
<td>LVS ΔblaB1</td>
<td>This work</td>
</tr>
<tr>
<td>PM1560</td>
<td>LVS ΔblaA1 ΔblaB1</td>
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</tr>
<tr>
<td>PM1516</td>
<td>Schu ΔblaA1</td>
<td>This work</td>
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<td>PM1580</td>
<td>Schu ΔblaB1</td>
<td>This work</td>
</tr>
<tr>
<td>PM1571</td>
<td>Schu ΔblaA1 ΔblaB1</td>
<td>This work</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pMOD-2</td>
<td>ApR, EZ-::TN cloning vector</td>
<td>Epicentre</td>
</tr>
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<td>pFNLT1</td>
<td>Francisella cryptic plasmid</td>
<td>Pomerantsev et al. (2001a)</td>
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<td>pFNLT1</td>
<td>KmR, E. coli–F. tularensis shuttle vector</td>
<td>Mai et al. (2004)</td>
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<td>pMV261</td>
<td>KmR, E. coli–mycobacterial shuttle vector</td>
<td>Stover et al. (1991)</td>
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<td>pMV261.hyg</td>
<td>HygR, E. coli–mycobacterial shuttle vector</td>
<td>Flores et al. (2005b)</td>
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<td>pYUB631</td>
<td>KmR, pMV261 bearing the sacR sacB region of Bacillus subtilis</td>
<td>Pavelka &amp; Jacobs (1999)</td>
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<td>pMP393</td>
<td>KmR, E. coli–F. tularensis shuttle vector</td>
<td>This work</td>
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<td>pMP478</td>
<td>KmR, pMV261 bearing a 290 bp XbaI–NdeI fragment containing the groESL promoter of LVS</td>
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<td>pMP492</td>
<td>KmR, pMV261 bearing a 1.8 kb PstI PCR fragment containing the blaB+ region from LVS</td>
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<td>pMP521</td>
<td>KmR, pMV261 bearing a 3.2 kb PstI PCR fragment containing the blaA+ region from LVS</td>
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<td>pMP523</td>
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<td>KmR, first-generation F. tularensis sacB suicide vector</td>
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<td>KmR, pMP578 suicide vector bearing the 2.2 kb fragment containing the ΔblaA1 allele from pMP522</td>
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<td>KmR, 570 bp smaller, second-generation F. tularensis sacB suicide vector derived from pMP588</td>
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<td>pMP592</td>
<td>KmR, pMP590 suicide vector bearing the ΔblaB1 allele from pMP537</td>
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<td>pMP607</td>
<td>KmR, E. coli–F. tularensis shuttle vector with the PgroESL-aphA-1 cassette and ORF4–ORF5 of pFNLT1</td>
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<td>pMP615</td>
<td>HygR, pMP529 with a 1.4 kb MluI PCR fragment bearing the blaB+ gene of Schu</td>
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<td>pMP633</td>
<td>HygR, E. coli–F. tularensis shuttle vector with the PgroESL-hyg cassette and ORF4–ORF5 of pFNLT1</td>
<td>This work</td>
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*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 Nde 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 Nde site at the start codon of the gene, which was cloned into pMP78 as described above for the aphA-1 cassette, yielding pMP74. 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 2.5 kb Bgl–EcoRV DNA fragment bearing the sacB region from pYUB631 (Table 1) with a 3.5 kb Bgl–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 ΔblaA 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 containing the ΔblaA allele was cloned into pMP578 to yield pMP582. There is 1076 bp of DNA upstream and 1120 bp downstream of the ΔblaA allele in this plasmid.

**Plasmid for ΔblaB 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 ΔblaB 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

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**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 CoIE1 origin of replication, gene aphA-1 (from Tn903) conferring resistance to kanamycin, and the mmAB1 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 \(~10^6\) kanamycin-resistant LVS transformants per electroporation with saturating amounts of pMP393 DNA (Fig. 2). Calculating our transformation efficiency on a per \(\mu\)g basis yields a value of \(~10^7\) kanamycin-resistant transformants (\(\mu\)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).

We compared transformation efficiencies of strains LVS and Schu with pMP393, pMP527 and the previously developed \(E.\) coli–Francisella shuttle vector pNLTP1 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 pNLTP1 transformation efficiency was very high with strain LVS, as expected, but low with strain Schu. We obtained \(~10^6\) transformants with LVS and less than 50 transformants with strain Schu per electroporation (data not shown). The electroporations with pNLTP1 were done in parallel with the cells and media used for the experiments with pMP393 and pMP527 shown in Fig. 2. The Schu/pNLTP1 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/pNLTP1 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 \(~10^6\) transformants per electroporation with strains LVS and Schu, and \(~10^5\) hygromycin-resistant clones per electroporation with strain Utah 112 (Fig. 2). We used hygromycin at a concentration of 200 \(\mu\)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
We hypothesized that introducing additional DNA from the cryptic plasmid pFN1L0 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 pFN1L0, encoding a putative ‘addiction system’ (Pomerantshev 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 pFN1L0 (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 PgroESL-aphA-1 cassette replaced with the PgroESL-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 PgroESL-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 PgroESL-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 *Pvu*II 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 × 10^3, 1·3 × 10^3 and 1·6 × 10^3 hygromycin-resistant mutants per electroporation while two electroporations of two independent cultures of Schu yielded 2·2 × 10^3, 2·2 × 10^3, 2·5 × 10^3 and 2·6 × 10^3 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

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**Table 2. Maintenance of shuttle vectors**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Percentage loss of antibiotic resistance*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LVS</td>
</tr>
<tr>
<td>pMP527</td>
<td>74, 98 (100)</td>
</tr>
<tr>
<td>pMP607</td>
<td>0, 2 (50)</td>
</tr>
<tr>
<td>pMP633</td>
<td>0, 0 (50)</td>
</tr>
</tbody>
</table>

*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.*
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 _ΔblaA1_, 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 _ΔblaB1_ allelic exchange plasmid pMP592.

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**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 _P_{groEL}·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.

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**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 electroporting the suicide vectors into LVS or Schu and plating on medium containing kanamycin at 5 μg ml⁻¹. Each suicide plasmid yielded kanamycin-resistant recombinants at a frequency of 10⁻⁵ to 10⁻⁶ relative to the transformation efficiency (~10⁶ 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 ΔblaA1 and ΔblaB2 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 against clones that did not undergo a second recombination and then plated the cells onto sucrose medium to select recombinants to saturation in medium lacking antibiotic. We then made mutants with only a deletion of ΔblaA1, strains PM1556 (LVS ΔblaB1) and PM1580 (Schu ΔblaB1). These mutants had increased susceptibility to ampicillin and could be distinguished from wild-type or ΔblaA1 mutants on medium containing ampicillin at 50 μg ml⁻¹ (Fig. 6). In addition, we constructed double mutants, strains PM1560 (LVS ΔblaA1 ΔblaB1) and PM1571 (Schu ΔblaA1 ΔblaB1). These mutants were comparable to the single ΔblaB1 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 ΔblaA1 ΔblaB1 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 μg ml⁻¹ or 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 ΔblaA1 mutants. We picked two mutants, PM1521 (LVS ΔblaA1) and PM1516 (Schu ΔblaA1), for subsequent work. A Southern blot showing the deletion of blaA in these two strains is shown in Fig. 5A. Consistent with the observation reported for the blaA knockout of F. tularensis subsp. novicida, we found that the ΔblaA1 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 ΔblaB1) and PM1580 (Schu ΔblaB1). We confirmed allelic exchange in these mutants by Southern blotting as shown in Fig. 5B. We then demonstrated that blaB⁺ could be used as an additional selectable marker in the ΔblaA1 ΔblaB1 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 μg 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 ΔblaA1 allele. Lanes: 1, 6, kb markers; 2, 7 and 4, 9, wild-type blaA (3-8 kb) LVS, Schu; 3, 5, ΔblaA1 (3-0 kb) LVS, Schu mutants; 8, 10, ΔblaA1 (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 ΔblaB1 allele. Lanes: 1, 6, kb markers; 2, 7 and 4, 9, wild-type blaB (1-9 kb) LVS, Schu; 3, 5, ΔblaB1 (1-2 kb) LVS, Schu mutants; 8, 10, ΔblaB1 (1-2 kb) LVS, Schu double mutants.](image-url)
ampicillin at 50 μg ml⁻¹. We obtained ~10⁶ 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 ΔblaA1 ΔblaB1 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 ΔblaA1 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 pFNLT1. 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 pFNLT1 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 pFNLT1 and Schu compared to our plasmids that lack ORF3. Furthermore, the Schu/pFNLT1 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 pFNLT1 on strain Schu could be related to the plasmid’s kanamycin resistance marker 

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.

ACKNOWLEDGEMENTS

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