Microbiology (2009), **155**, 1152–1163 **DOI** 10.1099/mic.0.022491-0

Single-copy chromosomal integration systems for *Francisella tularensis*

Eric D. LoVullo,[1] Claudia R. Molins-Schneekloth,[2]† Herbert P. Schweizer[2] and Martin S. Pavelka, Jr[1]

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

[1]Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY 14642, USA

[2]Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO 80523, USA

Received 21 July 2008
Revised 8 December 2008
Accepted 29 December 2008

Francisella tularensis is a fastidious Gram-negative bacterium responsible for the zoonotic disease tularemia. Investigation of the biology and molecular pathogenesis of *F. tularensis* has been limited by the difficulties in manipulating such a highly pathogenic organism and by a lack of genetic tools. However, recent advances have substantially improved the ability of researchers to genetically manipulate this organism. To expand the molecular toolbox we have developed two systems to stably integrate genetic elements in single-copy into the *F. tularensis* genome. The first system is based upon the ability of transposon Tn*7* to insert in both a site- and orientation-specific manner at high frequency into the *att*Tn*7* site located downstream of the highly conserved *glmS* gene. The second system consists of a *sacB*-based suicide plasmid used for allelic exchange of unmarked elements with the *blaB* gene, encoding a β-lactamase, resulting in the replacement of *blaB* with the element and the loss of ampicillin resistance. To test these new tools we used them to complement a novel D-glutamate auxotroph of *F. tularensis* LVS, created using an improved *sacB*-based allelic exchange plasmid. These new systems will be helpful for the genetic manipulation of *F. tularensis* in studies of tularemia biology, especially where the use of multi-copy plasmids or antibiotic markers may not be suitable.

INTRODUCTION

Tularemia has a variety of clinical manifestations depending on route of entry, subspecies of bacteria and inoculum size, while the disease state can range from mild to fatal (Ellis *et al.*, 2002). There are three official subspecies of the causative agent *Francisella tularensis*: the highly pathogenic *F. tularensis* subsp. *tularensis*, and the less pathogenic *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *mediaasiatica*. The high mortality of *F. tularensis* subsp. *tularensis* pneumonic tularemia and its high infectivity has raised concerns about its potential use as a biological weapon (Dennis *et al.*, 2001). These concerns have prompted the US Centers for Disease Control (CDC) to classify *F. tularensis* as a Select Agent.

A wide variety of genetic tools have recently been developed for the manipulation of *Francisella*. These include *Escherichia coli–Francisella* shuttle vectors (Bina *et al.*, 2006; LoVullo *et al.*, 2006; Ludu *et al.*, 2008; Maier *et al.*, 2004; Rasko *et al.*, 2007), random transposon mutagenesis systems based on EZ-Tn5, *Himar1* and Tn*5* (Buchan *et al.*, 2008; Kawula *et al.*, 2004; LoVullo *et al.*, 2006; Maier *et al.*, 2006; Qin & Mann, 2006), as well as methods for allelic exchange (Golovliov *et al.*, 2003; LoVullo *et al.*, 2006; Ludu *et al.*, 2008; Rodriguez *et al.*, 2008; Twine *et al.*, 2005). One methodology that has not been fully explored is the integration of genetic elements into the *F. tularensis* chromosome. In other bacteria this has been accomplished using non-replicative vectors containing an attachment site and integrase gene from a lysogenic bacteriophage (Hoang *et al.*, 2000; Stover *et al.*, 1991). This approach is not possible for *F. tularensis*, since phages that are infective for this organism have yet to be discovered.

In this paper, we report the development of two single-copy integration systems for incorporating genetic elements into the *Francisella* genome. The first system is based on the transposon Tn*7* and takes advantage of its ability to insert in both a site- and orientation-specific manner at high frequency into the *att*Tn*7* site, located downstream of the highly conserved *glmS* gene, which encodes the essential glucosamine-6-phosphate synthetase (Peters & Craig, 2001). This system has been used in a number of

†Present Address: Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases, Bacterial Diseases Branch, Fort Collins, CO 80521, USA.

Abbreviations: D-AAT, D-amino acid transferase; PGA, poly-γ-D-glutamic acid.

pathogens, including *Pseudomonas aeruginosa*, *E. coli*, *Salmonella typhimurium* and the Select Agents *Burkholderia mallei*, *Burkholderia pseudomallei* and *Yersinia pestis* (Choi *et al.*, 2005, 2006, 2008; McKenzie & Craig, 2006). As the insertion occurs in an intergenic region the fitness of the modified organisms appears to be unchanged (Choi *et al.*, 2005; Peters & Craig, 2001).

We constructed a mini-Tn*7* vector, which has a kanamycin-resistance marker flanked by γδ-*res* sites, and a helper plasmid, which encodes the site-specific Tn*7* transposase complex TnsABCD, expressed from an *F. tularensis* promoter. We confirmed the ability of the mini-Tn*7* to stably insert at the *att*Tn*7* site in the *F. tularensis* chromosome. We also showed that the kanamycin marker can be efficiently excised by the γδ-resolvase. The ability to remove the kanamycin marker is important, because *F. tularensis* genetics has a limited repertoire of Select Agent approved markers (Titball *et al.*, 2007).

The second system uses a *sacB*-based suicide plasmid expressing kanamycin resistance that is used for allelic exchange of unmarked elements with the *blaB* gene, which encodes the only functional β-lactamase in *F. tularensis* (Bina *et al.*, 2006; LoVullo *et al.*, 2006). The deletion of the *blaB* gene allows for convenient screening of desired recombinants based on their sensitivity to ampicillin.

METHODS

Bacterial strains, culture conditions, and transformation. *E. coli* DH10B (Table 1) was used for routine cloning procedures and was grown in Luria–Bertani (LB) broth (BD Biosciences) or on LB agar. *E. coli* HB101 was used to maintain all plasmids containing the γδ-*res* cassettes and was grown as described above. *F. tularensis* strains (Table 1) were grown as previously reported (LoVullo *et al.*, 2006). Specifically, strains were grown at 37 °C in liquid modified Mueller–Hinton medium (MMH), which is Mueller–Hinton broth (BD Biosciences) supplemented with 1.0 % (w/v) glucose, 0.025 % (w/v) ferric pyrophosphate (Sigma-Aldrich) and 0.05 % (w/v) L-cysteine free base (Calbiochem), or on MMH agar, which is the MMH medium described 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). When necessary, ampicillin (Ap; Sigma-Aldrich) was added at 100 or 50 μg ml^{-1}, respectively, for *E. coli* or *F. tularensis*, while kanamycin (Km; Sigma-Aldrich) was used at 50 μg ml^{-1} for *E. coli* and 5 μg ml^{-1} for *F. tularensis* strains LVS and Schu. Kanamycin stock solutions were made by accounting for the concentration of active kanamycin in each lot. Hygromycin B (Hyg; Roche Applied Science) was used at 200 μg ml^{-1} for all species and strains. Sucrose was used at a final concentration of 8 or 5 % (w/v) depending on the *sacB* vector. The β-galactosidase substrate X-Gal (Invitrogen) was used at 50 μg ml^{-1} in MMH agar lacking sheep blood. D-Glutamic acid (Sigma-Aldrich) was used at a final concentration of 200 μg ml^{-1} in MMH broth and in MMH agar lacking proteose peptone and sheep blood.

Electroporations and allelic exchange experiments were done as described previously (LoVullo *et al.*, 2006).

DNA manipulation. DNA methods were performed essentially as described by Ausubel *et al.* (1987). DNA fragments were isolated using agarose gel electrophoresis and QIAquick spin columns (Qiagen). Oligonucleotides were synthesized by Invitrogen Life Technologies. Oligonucleotides flanking the Tn*7* attachment site were attF, 5′-ATGCAGGACATGATTTTAGTG (forward 5′ to *att*Tn*7*), and attR, 5′-TTATGTTGAGTCCATATTTCAG (reverse 3′ to *att*Tn*7*). All restriction endonucleases and DNA modifying or polymerase enzymes were from New England Biolabs or Fermentas. PCRs were performed with Iproof High-Fidelity DNA Polymerase (Bio-Rad) according to the manufacturer's recommendations. All plasmids used in this study (Table 1) were from the authors' collections. Preparation of plasmid and genomic DNA from *E. coli* and *F. tularensis* was done as previously reported (LoVullo *et al.*, 2006).

***β*-Galactosidase assay.** The assays were performed on whole cell suspensions according to a standard protocol (Miller, 1972).

Plasmid construction. Plasmids used in this study are described in Table 1. Detailed descriptions of the construction of the plasmids used in this study can be obtained from the corresponding author. Information about plasmid construction that is pertinent to the understanding of this work is described below.

Unstable replicating helper plasmid, pMP720 (Fig. 1a). The R6K origin from plasmid pTNS2 (accession no. AY884833) was replaced with the pUC *ori* from pBluescript II KS(+) to produce pMP650. The *tnsABCD* operon from pMP650 was cloned into the multiple cloning site of pMP658 to produce pMP685. Inverse PCR was performed on pMP685, eliminating 380 bp between the *tnsABCD* operon and the *blaB* promoter, producing pMP720.

Non-replicative mini-Tn*7* vector, pMP749 (Fig. 1b). The R6K origin from plasmid pUC18R6KT mini-Tn*7*T (GenBank accession no. AY712953) was replaced with the pUC *ori* from pBluescript II KS(+) to produce the empty mini-Tn*7* pMP651. The P_{groESL}–*aphA-1* from pMP527 was flanked with γδ-*res* sites and ligated into pMP651 to produce pMP749.

Non-replicative mini-Tn*7* GFP vector, pMP793 (Fig. 1c). The R6K origin from plasmid pUC18R6KT mini-Tn*7*T gnELp-Kan-GFP was replaced with the pUC *ori* from pBluescript II KS(+) to produce pMP661. The *F. tularensis rpsL* promoter region was amplified from Schu genomic DNA by PCR and placed upstream of *gfp*, encoding GFPmut3 (Cormack *et al.*, 1996), which produced pMP761. This P_{rpsL}–*gfp* fragment was then inserted into the multiple cloning site of pMP749, generating pMP793.

Unstable γδ-resolvase plasmid, pMP672 (Fig. 1d). The γδ-resolvase gene, *tnpR*, was obtained from pGH542 and inserted into the multiple cloning site of pMP658 to generate pMP672.

***blaB* integration vectors, pMP719, pMP790 and pMP815 (Fig. 4).** Inverse PCR was performed on the suicide vector pMP590 to eliminate the pFNL10 *ori* yielding pMP671. The flanking regions of *blaB* were amplified from Schu genomic DNA and cloned upstream (5′ flanking) and downstream (3′ flanking) of the multiple cloning site of suicide vector pMP671 to produce the *blaB* integration vector pMP719. The *lacZ* gene was cloned into the multiple cloning site of pMP719 to form pMP741, and then the *rpsL* promoter region was amplified from Schu genomic DNA and placed upstream of the *lacZ* gene to form pMP790. This construct was created to test for heterologous gene expression in the *blaB* locus.

To improve the counterselectable marker, the *repA* promoter region upstream of *sacB* of pMP719 was removed by restriction digestion. This region was replaced with the *dnaK* promoter region amplified

Table 1. Bacterial strains and plasmids

Sources: J. Benach, State University of New York (SUNY), Stony Brook, NY, USA; M. Schriefer, CDC, Fort Collins, CO, USA.

Strain or plasmid	Description	Reference or source
Strains		
E. coli strains		
DH10B	F$^-$ *mcrA* Δ(*mcrBC-hsdRMS-mrr*) [ϕ80dΔ*lacZ*ΔM15] Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara,leu*)*7697 galU galK* λ$^-$ *rpsL nupG*	Invitrogen
HB101	F$^-$ Δ(*gpt-proA*)*62 leuB1 glnV44 ara-14 galK2 lacY1 hsdS20 rpsL20 xyl-5 mtl-1 recA13*	Boyer & Roulland-Dussoix (1969)
F. tularensis strains		
LVS	*F. tularensis* subsp. *holarctica* live vaccine strain	J. Benach
Schu	*F. tularensis* subsp. *tularensis*	M. Schriefer
PM2181	LVS Δ*murI1*	This work
PM2194	LVS Δ*murI1 att*Tn*7*::P$_{rpsL}$–*murI*$^+$ *res-aphA-1-res*	This work
PM2209	LVS Δ*murI1 att*Tn*7*::P$_{rpsL}$–*murI*$^+$ *res*	This work
PM2210	LVS Δ*murI1* Δ*blaB*::P$_{rpsL}$–*murI*$^+$	This work
Plasmids		
pBluescript II KS (+)	Ap^R, cloning vector	Stratagene
pGH542	Tc^R, source of *tnpR*	Piuri & Hatfull (2006)
pUC18R6KT mini-Tn*7*T	Ap^R, R6K replicon, mini-Tn*7* base vector	Choi *et al.* (2005)
pUC18R6KT mini-Tn*7*T gnELp-Kan-GFP	Ap^R, Km^R, R6K replicon, mini-Tn*7 gfp*	This work
pTNS2	Ap^R, R6K replicon, plasmid expressing *tnsABCD* from P$_{lac}$	Choi *et al.* (2005)
pMP527	Km^R, *E. coli–F. tularensis* shuttle vector with the P$_{groESL}$–*aphA-1* cassette	LoVullo *et al.* (2006)
pMP529	Hyg^R, *E. coli–F. tularensis* shuttle vector with the P$_{groESL}$–*hyg* cassette	LoVullo *et al.* (2006)
pMP590	Km^R, *F. tularensis sacB* suicide vector	LoVullo *et al.* (2006)
pMP650	Ap^R, plasmid expressing *tnsABCD* from P$_{lac}$	This work
pMP651	Ap^R, mini-Tn*7* base vector	This work
pMP658	Hyg^R, pMP529 with P$_{blaB}$ upstream of a multiple cloning site	LoVullo *et al.* (2008)
pMP661	Ap^R, Km^R, mini-Tn*7 gfp*	This work
pMP671	Km^R, *sacB* suicide vector lacking pFLN10 *ori* derived from pMP590	This work
pMP672	Hyg^R, pMP658 with P$_{blaB}$–*tnpR*	This work
pMP685	Hyg^R, pMP658 with P$_{blaB}$–*tnsABCD*	This work
pMP719	Km^R, *sacB*-based *blaB* integration vector	This work
pMP720	Hyg^R, pMP685 with P$_{blaB}$ moved 380 bp closer to *tnsABCD*	This work
pMP741	Km^R, *sacB*-based *blaB* integration vector based on pMP719 with *lacZ* in the multiple cloning site	This work
pMP749	Ap^R, Km^R, mini-Tn*7* vector with *res-aphA-1-res*	This work
pMP761	Ap^R, Km^R, mini-Tn*7* with P$_{rpsL}$–*gfp*	This work
pMP767	Km^R, *E. coli–F. tularensis* shuttle vector with P$_{rpsL}$–*gfp*	This work
pMP780	Km^R, *sacB* suicide vector derived from pMP590 with P$_{dnaK}$–*sacB*	This work
pMP790	Km^R, *blaB* integration vector based on pMP741 with P$_{rpsL}$–*lacZ*	This work
pMP793	Ap^R, Km^R, pMP749 with P$_{rpsL}$–*gfp* in the multiple cloning site	This work
pMP799	Km^R, *blaB* integration vector derived from pMP719 with P$_{dnaK}$–*sacB*	This work
pMP812	Km^R, 192 bp smaller, improved *sacB* suicide vector derived from pMP780	This work
pMP815	Km^R, 192 bp smaller, improved *blaB* integration vector derived from pMP719 with P$_{dnaK}$–*sacB*	This work
pMP880	Km^R, pMP812 suicide vector bearing the *murI* region of LVS	This work
pMP884	Km^R, pMP812 suicide vector containing Δ*murI* allele derived from pMP880	This work
pMP889	Km^R, P$_{rpsL}$–*murI*$^+$	This work
pMP890	Ap^R, Km^R, pMP749 with P$_{rpsL}$–*murI*$^+$ from pMP889 in the multiple cloning site	This work
pMP895	Km^R, pMP815 with P$_{rpsL}$–*murI*$^+$ from pMP889 in the multiple cloning site	This work

from *F. tularensis* Schu genomic DNA using PCR and cloned upstream of *sacB* to form pMP799. Inverse PCR was then performed with pMP799 to eliminate 192 bp of unnecessary DNA upstream of P$_{dnaK}$ to produce pMP815.

Modified *sacB* suicide vector, pMP812 (Fig. 6a). The *Francisella ori* region and *repA* promoter region upstream of *sacB* were removed from suicide vector pMP590 by restriction digestion. This region was replaced with the *dnaK* promoter and cloned upstream of *sacB* to

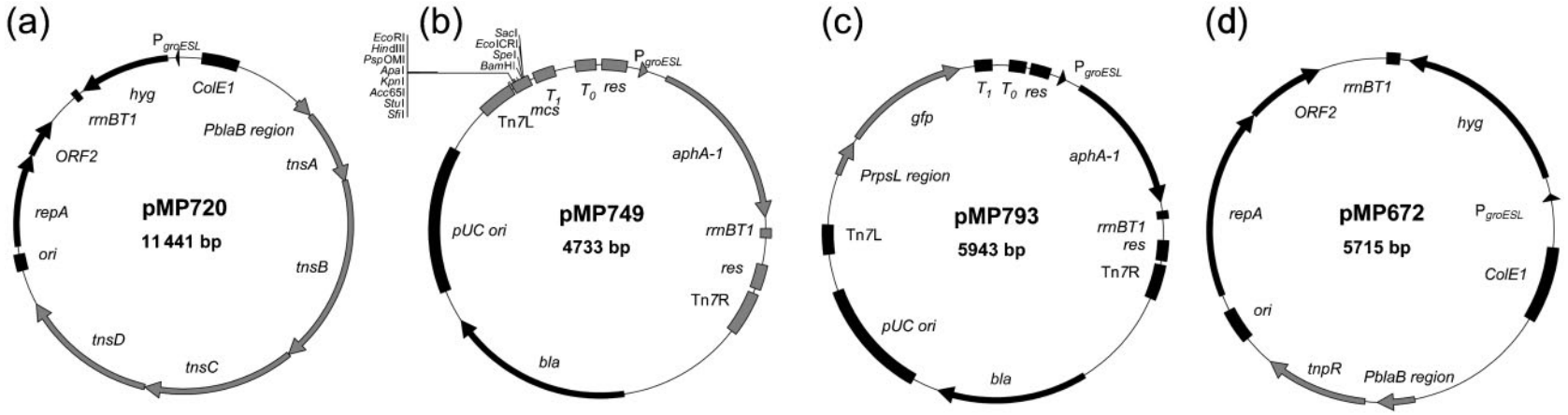

Fig. 1. Maps of the Tn7 system vectors. (a) The helper plasmid pMP720 is an unstable *E. coli–F. tularensis* shuttle vector that contains the *Francisella blaB* promoter driving the site- and orientation-specific transposase complex *tnsABCD* for integrating (b) the mini-Tn7 pMP749, which contains the transposon end Tn7L, a multiple cloning site, two terminators (T_1 and T_0) to limit readthrough from the *glmS* promoter on insertion, P_{groESL}–*aphA-1* (conferring resistance to kanamycin flanked by the γδ-sites for resolution of the cassette) and the other transposon end Tn7R, or (c) pMP793, which has the *Francisella rpsL* promoter driving *gfp* expression in the multiple cloning site of pMP749. (d) The resolvase plasmid pMP672 is an unstable *E. coli–F. tularensis* shuttle vector which has *tnpR*, encoding γδ-resolvase, driven by the *blaB* promoter for resolution of the kanamycin cassette after insertion into the *Francisella* genome.

form pMP780. Inverse PCR was performed as above to eliminate 192 bp of unnecessary DNA upstream of P_{dnaK} to produce pMP812.

Plasmid for Δ*murI* allelic exchange, pMP884. A DNA fragment containing *murI* was obtained from strain LVS genomic DNA using PCR and cloned into pMP812 to yield pMP880. An in-frame deletion of 768 bp within *murI* was made using PCR to yield pMP884. There are 962 bp upstream and 982 bp downstream of the Δ*murI1* allele in this plasmid.

Non-replicative mini-Tn*7* *murI* complementing vector, pMP890. The *F. tularensis murI* gene was amplified from LVS genomic DNA by PCR and placed downstream of P_{rpsL} in pMP767, forming pMP889. This P_{rpsL}–*murI* fragment was then inserted into the multiple cloning site of pMP749, generating pMP890.

***blaB*::*murI*$^+$ integration vector, pMP895.** The P_{rpsL}–*murI* fragment was amplified from pMP889 by PCR and inserted into the multiple cloning site of pMP815, generating pMP895.

RESULTS AND DISCUSSION

Development of the mini-Tn*7* system

The most common implementation of the Tn*7* system is to simultaneously deliver both transposon and transposase to the cells on separate suicide plasmids (Choi *et al.*, 2005). This allows for transient expression of the transposase and subsequent integration of the transposon in the chromosome without replication of the delivery plasmids. This approach did not work with *F. tularensis* using the suicide transposase plasmid pTNS2 (Table 1) and an early generation Tn*7* suicide plasmid. We first hypothesized that the *lac* promoter driving the 6 kb *tnsABCD* operon in the helper plasmid was not functional in *F. tularensis*. We created another suicide helper plasmid with the operon cloned downstream of the *Francisella groESL* promoter, which is the same promoter that we have used for the expression of selectable markers. However, this approach was also unsuccessful. We found that the *tnsABCD* genes are not optimal for the codon preference of *F. tularensis* and this, coupled with the size of the operon, probably prevented the cells from producing enough transposase proteins to catalyse transposition during the transient expression period. We then hypothesized that expressing the operon from a replicating plasmid prior to introduction of the Tn*7* suicide plasmid would allow time for the cell to produce sufficient amounts of transposase. A similar method using a temperature-sensitive helper plasmid has been reported to express the transposase in *E. coli* and *S. typhimurium* (McKenzie & Craig, 2006). Toward this end, we created the helper plasmid pMP720 (Fig. 1a) from the unstable hygromycin-resistant shuttle plasmid pMP658 (LoVullo *et al.*, 2008), which contains the transposase operon expressed from the *Francisella blaB* promoter. This revised strategy proved successful, as described below.

In addition to the helper plasmid, we created a mini-Tn*7* element on a suicide vector. The plasmid pMP749 (Fig. 1b) contains the kanamycin-resistance marker *aphA-1* driven by the *Francisella groESL* promoter, flanked by γδ-*res* DNA binding sites for the site-specific γδ-resolvase of *E. coli* transposon Tn*1000* (Bardarov *et al.*, 2002). It also contains two terminators (T_0 and T_1) to prevent read-through from the *glmS* promoter after chromosomal insertion (Choi *et al.*, 2005) and a multiple cloning site for cloning DNA elements. An additional mini-Tn*7* construct, pMP793 (Fig. 1c), was made to express GFP, and has a *Francisella rpsL* promoter driving *gfp* cloned into the multiple cloning site of pMP749.

The methodology we developed is shown in Fig. 2. First, the helper plasmid pMP720 is electroporated into *F. tularensis* and transformants selected by hygromycin resistance. One clone is prepared for electroporation while maintaining hygromycin selection. We then transform the strain with the mini-Tn*7* plasmid pMP749, and select for kanamycin-resistant clones. We routinely obtain ~10^4 kanamycin-resistant LVS transformants per electroporation with ~1 μg pMP749 DNA. We obtained similar results with the mini-Tn*7* plasmid expressing GFP, pMP793, with both LVS and Schu, resulting in ~10^4 kanamycin-resistant transformants per electroporation. We grew kanamycin-resistant transformants overnight in liquid media lacking selection and these were subcultured 1 : 10 and grown for an additional 24 h, after which they were plated on medium lacking antibiotics. The antibiotic-resistance phenotypes of the resulting clones were then screened, and we found that the hygromycin-resistance helper plasmid pMP720 was lost from the population at a frequency of 50–80 %, while kanamycin resistance, encoded in Tn*7*, was maintained at 100 % in the population. This confirmed our expectations that the helper plasmid would be readily lost from the population but that the Tn*7* would be stably maintained. We confirmed the presence of the kanamycin-resistant transposon insertion at the *att*Tn*7* site using PCR with primers attF and attR (see Methods), which lie outside the *att*Tn*7* site (Fig. 3). Sequence analysis of five LVS and five Schu clones determined that the insertion site occurs at either 25 bp (eight insertions) or 26 bp (two insertions) downstream of the *glmS* stop codon (data not shown), regardless of strain. This is similar to the behaviour of Tn*7* in *P. aeruginosa*, in which the transposon inserts at two sites, either 24 or 25 bp downstream of *glmS* (Choi *et al.*, 2005). In contrast, Tn*7* inserts into a single site 25 bp downstream of *glmS* in *Y. pestis* and *E. coli* (Choi *et al.*, 2005; DeBoy & Craig, 1996). Southern blot analysis using the *aphA-1* gene as a probe confirmed that there were no additional insertions in the LVS chromosome (data not shown). This is in agreement with the observations that transposition mediated by TnsABCD yields insertions only at *att*Tn*7* (Peters & Craig, 2001), and that Tn*7* also confers immunity, whereby it blocks transposition into a site already occupied by a Tn*7* element (DeBoy & Craig, 1996). We used confocal microscopy to visualize the GFP in the LVS Tn*7* strain, but only ~10 % of cells in each field expressed GFP at one time (data not shown). We believe

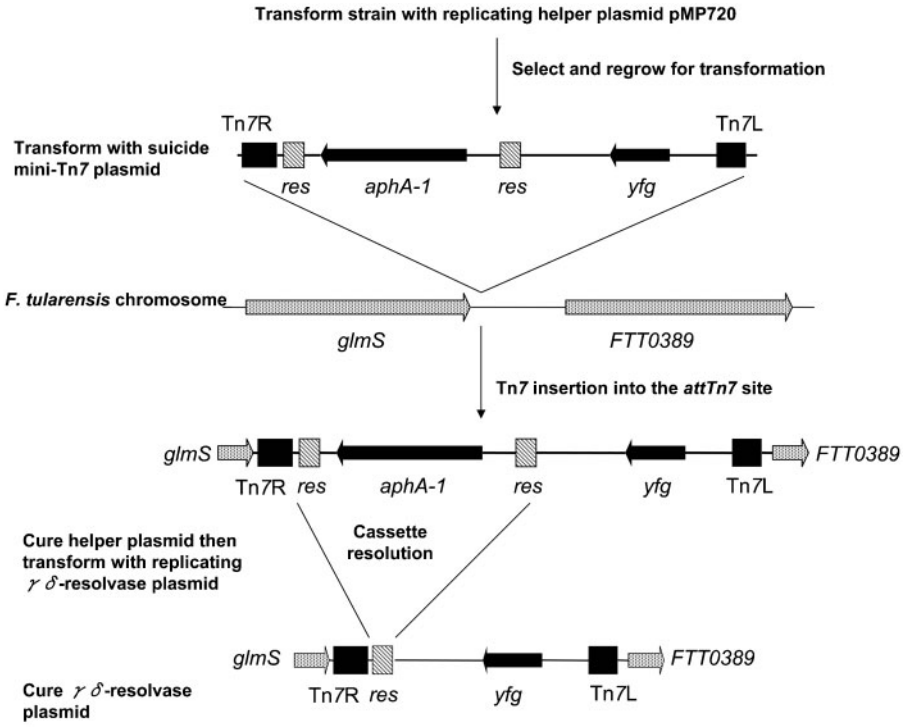

Fig. 2. Tn7 system. A typical experimental procedure is performed by first electroporating the helper plasmid pMP720 into the strain of interest and selecting for hygromycin-resistant transformants. One transformant is electroporated with the plasmid containing the mini-Tn7 element (based on pMP749) containing your favourite gene (*yfg*), and transposon insertions are selected on medium containing kanamycin. After curing a clone of the helper plasmid, the Tn7 insertion strain is ready for use. If desired, the kanamycin marker can be deleted using the γδ-resolvase plasmid pMP672.

that a multitude of factors could have been responsible for the poor visualization, including promoter strength, improper folding of GFP, degradation and photobleaching.

After confirming the loss of the helper plasmid we tested the γδ-resolvase system. We transformed LVS and Schu containing Tn*7* insertions with plasmid pMP672 (Fig. 1d), an unstable hygromycin shuttle vector expressing the γδ-resolvase from the *F. tularensis blaB* promoter. Select clones were then grown in liquid media containing hygromycin overnight and plated for single colonies on hygromycin medium. These were then screened for loss of kanamycin resistance, which occurred at a frequency of ~80 % in both LVS and Schu. Kanamycin-sensitive clones were cured of the γδ-resolvase plasmid in the same manner as the helper plasmid. We then confirmed the loss of the kanamycin marker with PCR, utilizing the Tn*7* attF and attR primers (Fig. 3). Sequence analysis of a resolved clone confirmed that the two γδ-*res* sites recombined into one γδ-*res* site with loss of the *aphA-1* marker (data not shown).

Development of the *blaB* integration system

We have previously shown that LVS and Schu contain only one functional β-lactamase, *blaB* (LoVullo *et al.*, 2006). The *blaB* gene lies in the Schu S4 chromosome with a hypothetical gene transcribed in the opposite direction 435 bp upstream of its start site and a potential DNA/RNA endonuclease family protein transcribed in the opposite direction overlapping the *blaB* stop codon by 10 bp (Larsson *et al.*, 2005). Based on our *sacB*-based suicide plasmid, we created a *blaB* integration vector that contains 1002 bp upstream of the *blaB* gene, a multiple cloning site, and 593 bp downstream of the *blaB* gene that includes the overlapping DNA/RNA endonuclease sequence.

The advantage of the *blaB* integration system is that we can quickly screen for the clones with the unmarked insertion in the secondary recombinant pool as they will be ampicillin-sensitive and kanamycin-sensitive. This is in contrast to the integration system developed for *Francisella novicida*, which integrates into a gene that is present only in the *F. novicida* chromosome and retains the kanamycin selectable marker (Ludu *et al.*, 2008).

Our *blaB* integration vector, pMP719 (Fig. 4a), is based on the suicide vector pMP671, a pFNL10 Δ*ori* derivative of pMP590 (LoVullo *et al.*, 2006). To test the ability of the system to integrate elements by allelic exchange, we cloned *lacZ* under the *Francisella rpsL* promoter into the multiple cloning site to form pMP790 (Fig. 4b). We selected the *rpsL* promoter because we knew from our studies with the P_{rpsL}–*gfp* cassette that the promoter is active in *E. coli* (data not

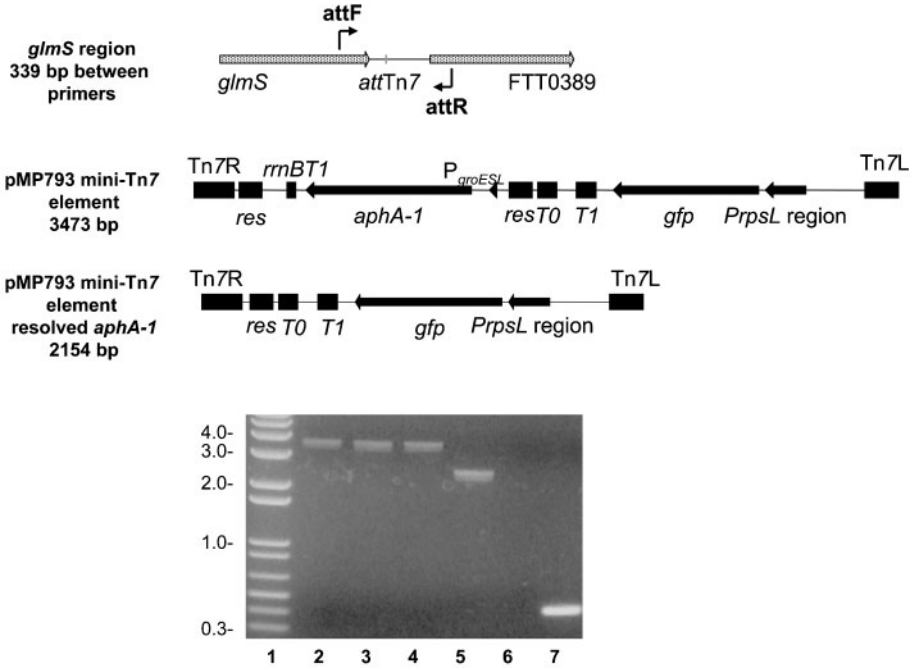

Fig. 3. pMP793 mini-Tn7 insertions into the *att*Tn7 site of Schu. Genomic Schu DNA analysed by PCR using *att*Tn7 outside primers attF and attR. Lanes: 1, DNA marker; 2, pMP793 mini-Tn7 element (Tn7) inserted into the *att*Tn7 of Schu with helper plasmid present (3.8 kb); 3, Tn7 strain after curing the helper plasmid (3.8 kb); 4, Tn7 strain passaged in broth in the absence of selection over 2 days (3.8 kb); 5, Tn7 strain transformed with the γδ-resolvase plasmid pMP672 that has undergone site-specific recombination of the γδ-*res* sites (2.5 kb); 6, no-DNA control; 7, wild-type Schu (0.34 kb). We obtained similar results with LVS.

shown), and therefore allowed us to confirm β-galactosidase production in *E. coli* before moving the system into *F. tularensis*. We performed an allelic exchange experiment similar to that shown in Fig. 5 for both LVS and Schu. We confirmed the expression of *lacZ* from the chromosomes of both strains by patching ampicillin-sensitive colonies onto medium with X-Gal and observing blue colonies (data not shown). We also confirmed the activity of the protein produced in LVS by performing β-galactosidase assays. We compared two Δ*blaB*::P_{rpsL}–*lacZ* strains with wild-type LVS. Three assays were performed on each strain: wild-type LVS averaged 4 Miller units and the two integrated strains averaged 300 Miller units, indicating expression of *lacZ* in the novel location within the chromosome.

Improved *sacB* suicide plasmid and *blaB* integration vector

Our previously described *sacB* suicide vector, pMP590 (LoVullo *et al.*, 2006), has proven itself useful for the sucrose counterselection-mediated construction of unmarked, in-frame deletions in both LVS and Schu, but needed improvement (LoVullo *et al.*, 2006). This allelic exchange vector was developed from a shuttle vector that was made non-replicative in *F. tularensis* by replacement of the *repA* and ORF2 genes by the *sacB* gene, driven by the *repA* promoter. However, the pFNL10 *ori* sequence is still present in this plasmid, which could be problematic in experiments that test gene essentiality by deleting a gene in the presence of a plasmid carrying a wild-type copy of the gene. In such an experiment, *trans*-acting replication proteins from the plasmid could recognize the suicide vector-borne *ori* sequence in the chromosome and initiate replication that would likely be lethal due to incompatibility with the natural chromosomal origin of replication. To solve this problem, we removed the *ori* sequences and *repA* promoter and inserted the *F. tularensis dnaK* promoter region upstream of the *sacB* gene, which allowed for strong expression of *sacB* such that the concentration of sucrose in the selection medium could be reduced from 8 to 5 %, while maintaining a very clean selection (data not shown). This new plasmid was then subjected to inverse PCR to remove 192 bp of DNA upstream of the *dnaK* promoter region. This was done to prevent the integration of the suicide vector into the chromosomal *dnaK* region, which occurred often in test experiments (data not shown). The final suicide vector, pMP812, is shown in Fig. 6(a). Note that the *dnaK* promoter does not seem to be recognized by *E. coli*, as pMP812 transformants are not sensitive to sucrose. These improvements to our basic *sacB* vector led us to modify our original *blaB* integration vector, pMP719, in the same manner, resulting in pMP815 (Fig. 4c).

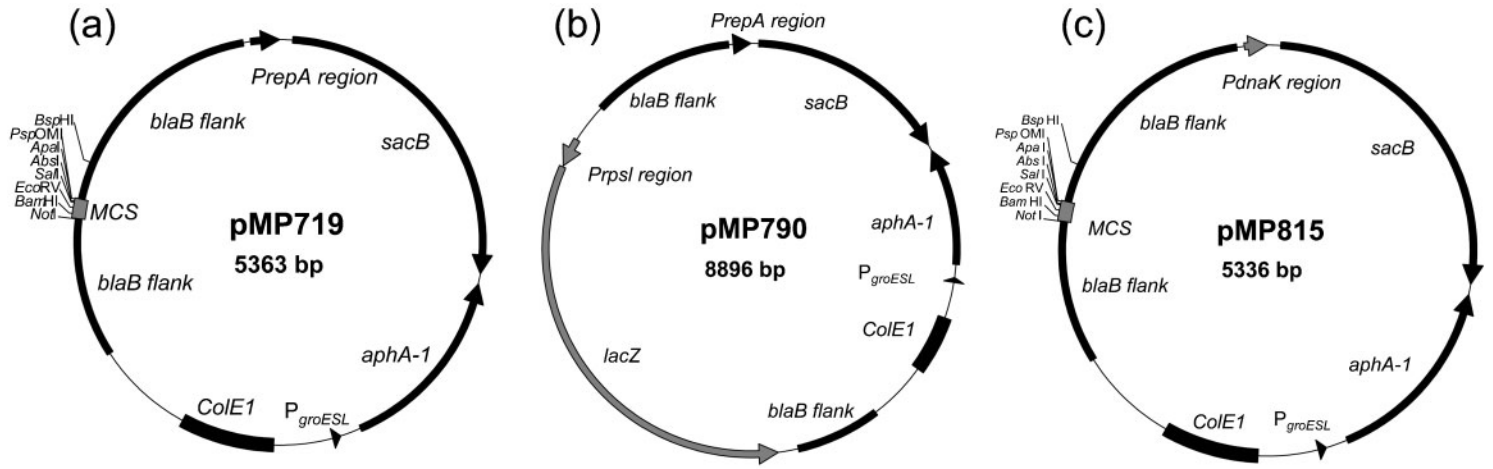

Fig. 4. Maps of the *F. tularensis blaB* integration vectors. (a) pMP719 is the first *blaB* integration vector based on the *sacB*-based suicide plasmid pMP671 that contains 1002 bp upstream of the *blaB* gene, a multiple cloning site, and then 593 bp downstream which includes the overlapping DNA/RNA endonuclease sequence. The *Bsp*HI site may be used in conjunction with a restriction enzyme in the multiple cloning site to remove the putative *blaB* promoter. (b) pMP790 is a Δ*blaB*::P_{rpsL}–*lacZ* integrating vector based on integration vector pMP719. (c) pMP815 is a modification of pMP719 in which the *repA* promoter upstream of *sacB* has been replaced with a *dnaK* promoter from *F. tularensis* Schu.

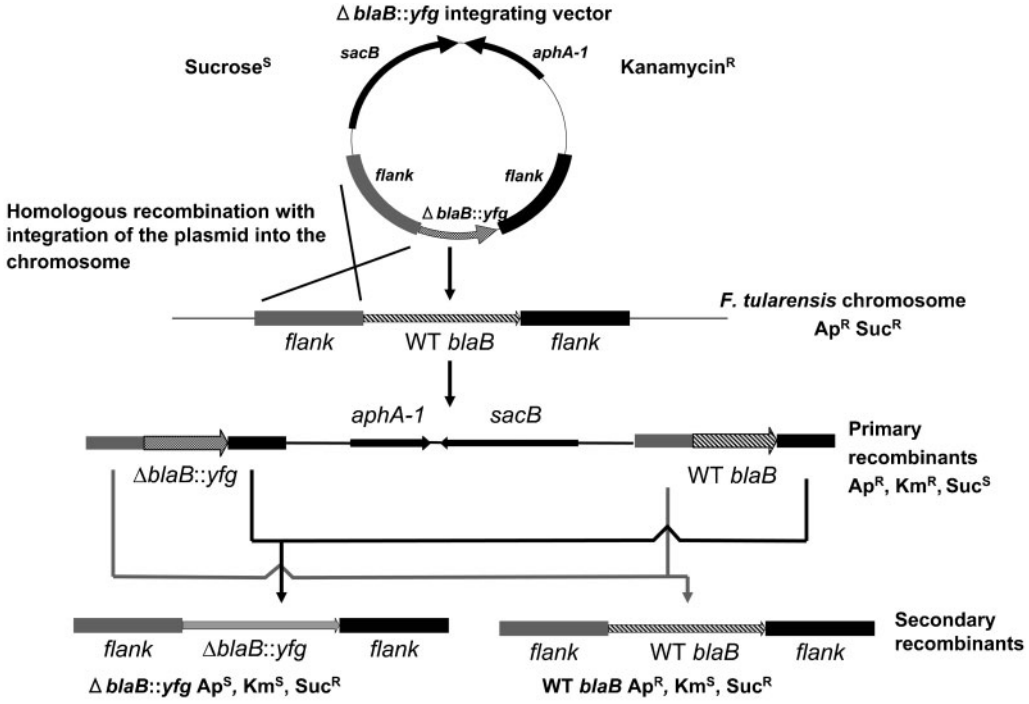

Fig. 5. *blaB* integration system. A typical allelic exchange procedure is done by electroporating the integration vector containing your favourite gene (*yfg*) into the strain of interest and plating on medium containing kanamycin. Each electroporation yields kanamycin-resistant recombinants at a frequency of 10^{-5}–10^{-6} relative to the transformation efficiency of our replicating plasmids. The kanamycin-resistant primary recombinants are then screened for sucrose sensitivity. Kanamycin-resistant, sucrose-sensitive primary recombinants are grown to saturation in medium lacking antibiotic and then plated onto sucrose media to select against clones that do not undergo a second recombination event. Sucrose-resistant clones arise from cultures at frequencies of 10^{-3}–10^{-4} relative to the viable counts. These putative secondary recombinants are then screened for loss of kanamycin resistance and for ampicillin sensitivity as an indication that there is a successful recombination event and that insertion of your element is likely. In the Δ*blaB*::P_{rpsL}–*lacZ* experiment, ampicillin-sensitive colonies have ranged from 5 to 10 % of the population in LVS. The frequency of ampicillin-sensitive colonies is lower than expected, probably because of the difference in length of the DNA flanking the Δ*blaB* allele (593 versus 1002 bp).

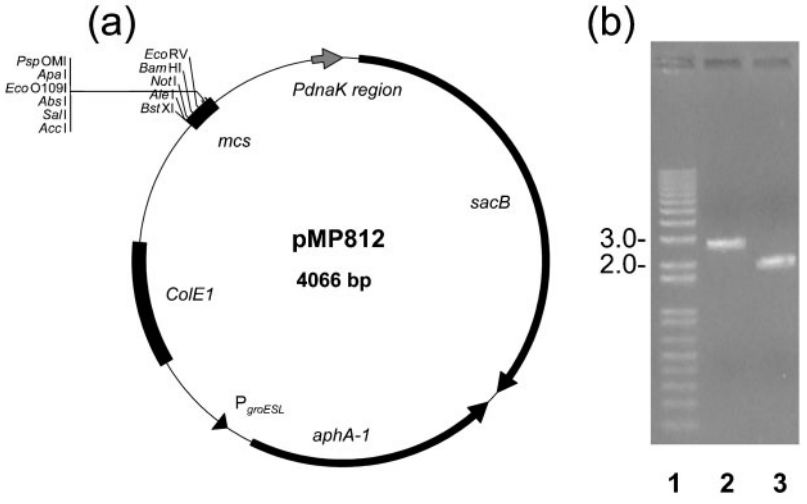

Fig. 6. Improved *sacB* suicide vector and *murI* deletion. (a) Improved *sacB* vector pMP812. The *ori* and *repA* promoter of pMP590 were replaced with the *Francisella dnaK* promoter to produce pMP780, and DNA upstream of the *dnaK* promoter was removed by PCR to produce pMP812. The multiple cloning site (mcs) is also shown. A Δ*murI1* allele cloned within pMP812 was used to delete the wild-type allele. (b) PCR amplicons obtained from genomic LVS DNA with primers specific to the *murI* region. Lanes: 1, DNA marker; 2, wild-type LVS (2.7 kb); 3, LVS Δ*murI1* strain PM2181 (2.0 kb).

Allelic exchange of *murI* and complementation with the single-copy integration systems

To test these new tools, we sought to construct a strain with a novel mutation, characterize it, and then complement it with a wild-type copy of the gene inserted into the chromosome using each integration system. We chose to interrupt the production of D-glutamate by deleting the *murI* gene, which encodes a glutamate racemase that is essential to *E. coli* (Doublet *et al.*, 1993). D-Glutamate is indispensable for the biosynthesis of peptidoglycan in most eubacteria, and is produced through two known routes: by D-amino acid transferase (D-AAT), which converts α-ketoglutarate to D-glutamate by transamination with D-alanine provided by the alanine racemase reaction; and by glutamate racemase, which produces D-glutamate through the racemization of L-glutamate (Liu *et al.*, 1998). In a number of bacteria, most notably in certain *Bacillus* species, D-glutamate is also shuttled into the production of poly-γ-D-glutamic acid (PGA). In *Bacillus anthracis*, there are two glutamate racemases (RacE1 and RacE2) that produce D-glutamate for peptidoglycan as well as the biosynthesis of PGA, which constitutes an antiphagocytic capsule, one of the two major virulence factors of *B. anthracis* (Dodd *et al.*, 2007; Mock & Fouet, 2001). *F. tularensis* has *capB* and *capC* genes, which share sequence homology at the amino acid level of 38 and 29 % with the CapB and CapC proteins of *B. anthracis* that synthesize PGA (Su *et al.*, 2007), and a number of groups have shown that the *capBC* genes of *F. tularensis* are important for tularemia pathogenesis (Maier *et al.*, 2007; Su *et al.*, 2007; Weiss *et al.*, 2007). However, there is as yet no evidence that *F. tularensis* produces PGA.

Our inspection of the genomes of *F. tularensis* strains and of *F. novicida* indicates that the gene FTT1197c, annotated as *murI*, is the only glutamate racemase present in these organisms, and we could find no genes encoding a D-AAT, suggesting that there is only one source of D-glutamate in these bacteria. However, a comprehensive transposon library of the *F. novicida* genome has one mutant with an insertion in the middle of the *murI* gene, and D-glutamic acid was not included in the selection medium (Gallagher *et al.*, 2007). This might indicate that *murI* is dispensable.

To clarify this matter, we sought to determine whether a *murI* deletion mutant would be auxotrophic for D-glutamate, indicating the lack of any additional amino acid racemase or D-AAT capable of compensating for the loss of *murI*. We constructed an in-frame deletion of *murI* in our improved *sacB*-based suicide vector, pMP812. Since it has been shown that it is possible to rescue D-glutamate auxotrophs with exogenous D-glutamate in *Bacillus subtilis* and *E. coli* B/r and K-12 strains (Ashiuchi *et al.*, 2007; Doublet *et al.*, 1993; Hoffmann *et al.*, 1972), we performed a standard two-step allelic exchange (LoVullo *et al.*, 2006) using pMP884 (Table 1) with D-glutamic acid present in the medium. We then picked and patched 24 sucrose-resistant secondary recombinants onto media with or without D-glutamic acid. This yielded two recombinants that could not grow on media lacking D-glutamic acid. PCR was used to confirm that the secondary recombinants auxotrophic for D-glutamic acid were *murI* deletions and that the D-glutamic acid prototrophs were wild-type recombinants (Fig. 6b). The *murI* deletion mutants formed smaller colonies than the wild-type, suggesting a growth defect. This is probably the reason why the phenotypes of the secondary recombinants were skewed towards the wild-type. One mutant, PM2181, was selected for further study.

We performed complementation studies on PM2181 utilizing the *Francisella rpsL* promoter driving *murI* in the Tn*7* system and the *blaB* system. The mini-Tn*7* vector pMP749 bearing P_{rpsL}–$murI^+$ was introduced into PM2181 as shown in Fig. 2. The resulting *att*Tn*7*::P_{rpsL}–$murI^+$ strain, PM2194, was prototrophic for D-glutamic acid. We then resolved the kanamycin marker to ensure that it had no effect on complementation of the *murI* lesion. As expected, the resolved strain, PM2209, was able to grow in the absence of D-glutamic acid. For the *blaB* system, a two-step allelic exchange as shown in Fig. 5 was performed with pMP815 bearing P_{rpsL}–$murI^+$. The resulting strain, PM2210, was also able to grow in the absence of D-glutamic acid. These results confirm that there is only one pathway for D-glutamate biosynthesis in wild-type *Francisella*. We do not know whether MurI also supplies D-glutamate for PGA biosynthesis, but this mutant could be used to identify such a polymer if it exists, since it should be possible to label PGA with radioactive D-glutamic acid supplied to PM2181 in culture.

The existence of a *murI* transposon mutant of *F. novicida* (Gallagher *et al.*, 2007) obtained without D-glutamate supplementation suggests that there may be an extragenic suppressor mutation in this mutant. To test this possibility we performed suppressor analysis of our LVS D-glutamate auxotroph. The strain was grown to saturation, washed, and serially diluted on plates with and without D-glutamic acid. We found that the strain produced suppressor mutants at a frequency of $\sim 1 \times 10^{-6}$ per viable D-glutamic acid-requiring colony-forming unit. This high frequency of suppression in PM2181 supports the idea that the *F. novicida* transposon mutant likely contains an extragenic suppressor.

We anticipate that these integration systems will be useful for studies requiring single-copy gene expression, such as the complementation of mutant genes when expression of the wild-type gene from multi-copy plasmids is toxic. Furthermore, these systems will be helpful where the use of multi-copy plasmids may not be suitable for cell culture or animal experiments. They may also be helpful for developing live vaccine strains containing additional antigens, where the use of antibiotic-resistance markers is undesirable.

ACKNOWLEDGEMENTS

This work was supported by the Molecular Pathogenesis of Bacteria and Viruses NIH grant T32 AI007362-18 to E. D. L., NIH grant

AI068013 to M. S. P., and NIH grants AI058141 and AI065357 to H. P. S. We wish to thank the members of the Pavelka lab for reviewing this manuscript.

REFERENCES

Ashiuchi, M., Nishikawa, Y., Matsunaga, K., Yamamoto, M., Shimanouchi, K. & Misono, H. (2007). Genetic design of conditional D-glutamate auxotrophy for *Bacillus subtilis*: use of a vector-borne poly-γ-glutamate synthetic system. *Biochem Biophys Res Commun* **362**, 646–650.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1987). *Current Protocols in Molecular Biology*. New York: Green Publishing & Wiley Interscience.

Bardarov, S., Bardarov, S., Jr, Pavelka, M. S., Jr, Sambandamurthy, V., Larsen, M., Tufariello, J., Chan, J., Hatfull, G. & Jacobs, W. R., Jr (2002). Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG and *M. smegmatis*. *Microbiology* **148**, 3007–3017.

Bina, X. R., Wang, C., Miller, M. A. & Bina, J. E. (2006). The Bla2 β-lactamase from the live-vaccine strain of *Francisella tularensis* encodes a functional protein that is only active against penicillin-class β-lactam antibiotics. *Arch Microbiol* **186**, 219–228.

Boyer, H. W. & Roulland-Dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J Mol Biol* **41**, 459–472.

Buchan, B. W., McLendon, M. K. & Jones, B. D. (2008). Identification of differentially regulated *Francisella tularensis* genes using a newly developed Tn*5*-based transposon delivery system. *Appl Environ Microbiol* **74**, 2637–2645.

Choi, K. H., Gaynor, J. B., White, K. G., Lopez, C., Bosio, C. M., Karkhoff-Schweizer, R. R. & Schweizer, H. P. (2005). A Tn*7*-based broad-range bacterial cloning and expression system. *Nat Methods* **2**, 443–448.

Choi, K. H., DeShazer, D. & Schweizer, H. P. (2006). Mini-Tn*7* insertion in bacteria with multiple *glmS*-linked *att*Tn*7* sites: example *Burkholderia mallei* ATCC 23344. *Nat Protoc* **1**, 162–169.

Choi, K. H., Mima, T., Casart, Y., Rholl, D., Kumar, A., Beacham, I. R. & Schweizer, H. P. (2008). Genetic tools for select-agent-compliant manipulation of *Burkholderia pseudomallei*. *Appl Environ Microbiol* **74**, 1064–1075.

Cormack, B. P., Valdivia, R. H. & Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**, 33–38.

DeBoy, R. T. & Craig, N. L. (1996). Tn*7* transposition as a probe of *cis* interactions between widely separated (190 kilobases apart) DNA sites in the *Escherichia coli* chromosome. *J Bacteriol* **178**, 6184–6191.

Dennis, D. T., Inglesby, T. V., Henderson, D. A., Bartlett, J. G., Ascher, M. S., Eitzen, E., Fine, A. D., Friedlander, A. M., Hauer, J. & other authors (2001). Tularemia as a biological weapon: medical and public health management. *JAMA (J Am Med Assoc)* **285**, 2763–2773.

Dodd, D., Reese, J. G., Louer, C. R., Ballard, J. D., Spies, M. A. & Blanke, S. R. (2007). Functional comparison of the two *Bacillus anthracis* glutamate racemases. *J Bacteriol* **189**, 5265–5275.

Doublet, P., van Heijenoort, J., Bohin, J. P. & Mengin-Lecreulx, D. (1993). The *murI* gene of *Escherichia coli* is an essential gene that encodes a glutamate racemase activity. *J Bacteriol* **175**, 2970–2979.

Ellis, J., Oyston, P. C., Green, M. & Titball, R. W. (2002). Tularemia. *Clin Microbiol Rev* **15**, 631–646.

Gallagher, L. A., Ramage, E., Jacobs, M. A., Kaul, R., Brittnacher, M. & Manoil, C. (2007). A comprehensive transposon mutant library of *Francisella novicida*, a bioweapon surrogate. *Proc Natl Acad Sci U S A* **104**, 1009–1014.

Golovliov, I., Sjostedt, A., Mokrievich, A. & Pavlov, V. (2003). A method for allelic replacement in *Francisella tularensis*. *FEMS Microbiol Lett* **222**, 273–280.

Hoang, T. T., Kutchma, A. J., Becher, A. & Schweizer, H. P. (2000). Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. *Plasmid* **43**, 59–72.

Hoffmann, B., Messer, W. & Schwarz, U. (1972). Regulation of polar cap formation in the life cycle of *Escherichia coli*. *J Supramol Struct* **1**, 29–37.

Kawula, T. H., Hall, J. D., Fuller, J. R. & Craven, R. R. (2004). Use of transposon–transposase complexes to create stable insertion mutant strains of *Francisella tularensis* LVS. *Appl Environ Microbiol* **70**, 6901–6904.

Larsson, P., Oyston, P. C., Chain, P., Chu, M. C., Duffield, M., Fuxelius, H. H., Garcia, E., Hälltorp, G., Johansson, D. & other authors (2005). The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. *Nat Genet* **37**, 153–159.

Liu, L., Yoshimura, T., Endo, K., Kishimoto, K., Fuchikami, Y., Manning, J. M., Esaki, N. & Soda, K. (1998). Compensation for D-glutamate auxotrophy of *Escherichia coli* WM335 by D-amino acid aminotransferase gene and regulation of *murI* expression. *Biosci Biotechnol Biochem* **62**, 193–195.

LoVullo, E. D., Sherrill, L. A., Perez, L. L. & Pavelka, M. S., Jr (2006). Genetic tools for highly pathogenic *Francisella tularensis* subsp. *tularensis*. *Microbiology* **152**, 3425–3435.

LoVullo, E. D., Sherrill, L. A. & Pavelka, M. S., Jr (2008). Improved shuttle vectors for *Francisella tularensis* genetics. *FEMS Microbiol Lett* **291**, 95–102.

Ludu, J. S., Nix, E. B., Duplantis, B. N., de Bruin, O. M., Gallagher, L. A., Hawley, L. M. & Nano, F. E. (2008). Genetic elements for selection, deletion mutagenesis and complementation in *Francisella* spp. *FEMS Microbiol Lett* **278**, 86–93.

Maier, T. M., Havig, A., Casey, M., Nano, F. E., Frank, D. W. & Zahrt, T. C. (2004). Construction and characterization of a highly efficient *Francisella* shuttle plasmid. *Appl Environ Microbiol* **70**, 7511–7519.

Maier, T. M., Pechous, R., Casey, M., Zahrt, T. C. & Frank, D. W. (2006). In vivo *Himar1*-based transposon mutagenesis of *Francisella tularensis*. *Appl Environ Microbiol* **72**, 1878–1885.

Maier, T. M., Casey, M. S., Becker, R. H., Dorsey, C. W., Glass, E. M., Maltsev, N., Zahrt, T. C. & Frank, D. W. (2007). Identification of *Francisella tularensis Himar1*-based transposon mutants defective for replication in macrophages. *Infect Immun* **75**, 5376–5389.

McKenzie, G. J. & Craig, N. L. (2006). Fast, easy and efficient: site-specific insertion of transgenes into enterobacterial chromosomes using Tn*7* without need for selection of the insertion event. *BMC Microbiol* **6**, 39.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Mock, M. & Fouet, A. (2001). Anthrax. *Annu Rev Microbiol* **55**, 647–671.

Peters, J. E. & Craig, N. L. (2001). Tn*7*: smarter than we thought. *Nat Rev Mol Cell Biol* **2**, 806–814.

Piuri, M. & Hatfull, G. F. (2006). A peptidoglycan hydrolase motif within the mycobacteriophage TM4 tape measure protein promotes efficient infection of stationary phase cells. *Mol Microbiol* **62**, 1569–1585.

Qin, A. & Mann, B. J. (2006). Identification of transposon insertion mutants of *Francisella tularensis tularensis* strain Schu S4 deficient in intracellular replication in the hepatic cell line HepG2. *BMC Microbiol* **6**, 69.

Rasko, D. A., Esteban, C. D. & Sperandio, V. (2007). Development of novel plasmid vectors and a promoter trap system in *Francisella tularensis* compatible with the pFNL10 based plasmids. *Plasmid* **58**, 159–166.

Rodriguez, S. A., Yu, J. J., Davis, G., Arulanandam, B. P. & Klose, K. E. (2008). Targeted inactivation of *Francisella tularensis* genes by group II introns. *Appl Environ Microbiol* **74**, 2619–2626.

Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennett, L. T., Bansal, G. P., Young, J. F., Lee, M. H. & other authors (1991). New use of BCG for recombinant vaccines. *Nature* **351**, 456–460.

Su, J., Yang, J., Zhao, D., Kawula, T. H., Banas, J. A. & Zhang, J. R. (2007). Genome-wide identification of *Francisella tularensis* virulence determinants. *Infect Immun* **75**, 3089–3101.

Titball, R. W., Sjostedt, A., Pavelka, M. S., Jr & Nano, F. E. (2007). Biosafety and selectable markers. *Ann N Y Acad Sci* **1105**, 405–417.

Twine, S., Byström, M., Chen, W., Forsman, M., Golovliov, I., Johansson, A., Kelly, J., Lindgren, H., Svensson, K. & other authors (2005). A mutant of *Francisella tularensis* strain SCHU S4 lacking the ability to express a 58-kilodalton protein is attenuated for virulence and is an effective live vaccine. *Infect Immun* **73**, 8345–8352.

Weiss, D. S., Brotcke, A., Henry, T., Margolis, J. J., Chan, K. & Monack, D. M. (2007). *In vivo* negative selection screen identifies genes required for *Francisella* virulence. *Proc Natl Acad Sci U S A* **104**, 6037–6042.

Edited by: P. van der Ley