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Improved Shuttle Vectors for *Francisella tularensis* Genetics

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Abstract

We previously described the construction and characterization of *E. coli-F. tularensis* shuttle vectors, derived from the cryptic *Francisella* plasmid pFNL10, for the genetic manipulation of *F. tularensis* subsp. *tularensis*. We now report further characterization of the biology of these shuttle vectors and the development of a new generation of *Francisella* plasmids. We show that the addition of ORF3 from pFNL10 can convert an unstable shuttle vector into a stable one, and that this is likely due to increased plasmid copy number. We also describe various improvements to the earlier generations of shuttle vectors, such as the addition of a multiple cloning site containing a novel *Rsr*II restriction endonuclease site for directional insertion of *Francisella* genes, and the inclusion of the *F. tularensis blaB* promoter for heterologous gene expression.

Keywords

Francisella; tularemia; plasmid

Introduction

Plasmids are extremely important for the genetic manipulation of bacteria as the cornerstone of systems for gene delivery and mutagenesis. The construction of novel plasmids is often the first step in the development of investigative tools for organisms lacking good genetic systems. A challenge is finding a suitable plasmid. This can be overcome by the use of broad host range plasmids, or the use of naturally occurring plasmids specific for the bacteria of interest (Schweizer, 2008). The latter approach was taken by several groups (Norqvist, *et al*., 1996, Maier, *et al*., 2004, LoVullo, *et al*., 2006) for the development of *E. coli* shuttle vectors capable of replication in *Francisella tularensis*, a Gram-negative pathogen responsible for the disease tularemia and a potential biological weapon (Dennis, *et al*., 2001, Titball, *et al*., 2003, Oyston, *et al*., 2004, Oyston, 2008). These shuttle vectors, along with others (Pavlov, *et al*., 1996, Bina, *et al*., 2006, Rasko, *et al*., 2007, Ludu, *et al*., 2008) and additional genetic tools for transposition (Quigley & Schwab, 1988, Kawula, *et al*., 2004, LoVullo, *et al*., 2006, Maier, *et al*., 2006, Qin & Mann, 2006, Buchan, *et al*., 2008) and allelic exchange (Golovliov, *et al*., 2003, Twine, *et al*., 2005, LoVullo, *et al*., 2006, Ludu, *et al*., 2008, Rodriguez, *et al*., 2008) have contributed to the recent expansion of research into *Francisella* biology and tularemia pathogenesis.

Most types of *E. coli-Francisella* shuttle vectors were built by fusion of the cryptic plasmid pFNL10, obtained from the '*F. novicida*-like' strain F6168 (Pomerantsev, *et al*., 2001b), to an *E. coli* cloning vector (Norqvist, *et al*., 1996, Maier, *et al*., 2004). The plasmid pFNL10 is

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3.9 kb in size and encodes six open reading frames: *repA*, ORF2, ORF3, ORF4, ORF5, and ORFM (Pomerantsev, *et al*., 2001b). These earlier shuttle vectors (for example, pTOPO/FNL10 or those derived from pFNL100) could replicate in both species and be recovered intact from *E. coli*, but suffered deletions during passage in *Francisella* bacteria (Pomerantsev, *et al*., 2001a, Pomerantsev, *et al*., 2001b, Maier, *et al*., 2004). These deletions removed all or part of the ORF4–ORF5 region encoding a putative toxin-antitoxin system. It was proposed that the cloning altered the regulation of this system in *Francisella*, leading to *Francisella*-specific plasmid toxicity that selected for derivatives in which the toxin-encoding part of the shuttle vector was deleted.

We previously reported on the development of novel *E. coli-Francisella* shuttle vectors made by starting with the minimal DNA from pFNL10 needed for replication in *F. tularensis* (LoVullo, *et al*., 2006). The initial vector, pMP393, included the pFNL10 *ori* and the *repA* and ORF2 genes, along with a ColE1 origin of replication for *E. coli* and the *aphA-1* gene from Tn*903* conferring kanamycin resistance. This plasmid was capable of highly efficient transformation of *F. tularensis* LVS, an attenuated vaccine strain, but the transformation efficiency of the plasmid with highly virulent *F. tularensis* subsp. *tularensis* strain Schu was several orders of magnitude lower. This problem was solved by replacing the native *aphA-1* promoter with the *F. tularensis groESL* promoter. This new, first generation vector, pMP527, could transform *F. tularensis* strains with high efficiency and did not undergo deletions. We then showed that this plasmid is unstable in the absence of antibiotic selection, but constructed a second generation of plasmids (pMP607 and pMP633) bearing the ORF4–ORF5 toxin-antitoxin module from pFNL10 that are stable in the population in the absence of antibiotic pressure (LoVullo, *et al*., 2006, Su, *et al*., 2007, Chakraborty, *et al*., 2008, Ludu, *et al*., 2008).

In this study, we report on the role of the ORF3 gene of pFNL10 in *Francisella* plasmid biology, determined the copy number of several shuttle vectors in *F. tularensis* LVS, and describe a new generation of shuttle vectors with improvements over the earlier vectors.

Materials and Methods

Bacterial strains, culture conditions, and transformation

Escherichia coli strain DH10B (F- *mcrA* Δ(*mcrBC-hsdRMS-mrr*) [Δ80dΔ*lacZ*ΔM15] Δ*lacX74 deoR recA1 endA1 araD139* Δ*(ara,leu)7697 galU galK* λ- *rpsL nupG*) was used for routine cloning procedures and was grown in Luria-Bertani (LB) broth (BD Biosciences) or on LB agar. *F. tularensis* subsp. *holarctica* LVS and *F. tularensis* subsp. *tularensis* Schu were grown at 37°C in modified Mueller-Hinton broth (MMH), which is Mueller Hinton broth (BD Biosciences) supplemented with 1.0% (w/v) glucose, 0.025% (w/v) ferric pyrophosphate, and 0.05% (w/v) L-cysteine or on MMH agar, which is the MMH medium above supplemented with 1.0% (w/v) proteose peptone (BD Biosciences), 2.5% (v/v) defibrinated sheep blood (Remel) and 1.5% (w/v) bacto-agar (BD Biosciences). When necessary, kanamycin (Sigma-Aldrich) was used at 50 μg ml^{-1} for *E.* coli, 5 μg ml^{-1} for LVS and Schu. Kanamycin stock solutions were corrected for the concentration of active kanamycin in each lot. Hygromycin B (Roche Applied Science) was initially used at 200 μg ml^{-1} for all species and strains, however we later found that 100 μg ml^{-1} is sufficient for LVS and Schu. Electroporations were done as previously reported (LoVullo, *et al*., 2006).

Plasmid stability tests were performed by growing strains overnight in media supplemented with antibiotic, then diluting the cultures 1:20 in fresh medium lacking antibiotic. The cultures were incubated at 37° C and samples taken after 6 hours and 24 hours, diluted, and plated onto media lacking antibiotics. Fifty to a hundred clones from these plates were then

patched onto MMH media with and without antibiotics and scored for resistance. A strain carrying a known unstable plasmid was used as a control.

DNA manipulation—DNA fragments were isolated using QIAquick spin columns (Qiagen Inc.). Invitrogen Life Technologies synthesized the oligonucleotides used in this work, while New England Biolabs or Fermentas supplied all of the restriction endonucleases and DNA modifying or polymerase enzymes. PCR reactions were performed with Iproof™ High-Fidelity DNA Polymerase (BIO-RAD). All plasmids used in this study (Table 1) were from the corresponding author's collection, unless otherwise noted. Preparation of plasmid DNA from *E. coli* and *F. tularensis* was done as previously reported (LoVullo, *et al*., 2006).

Plasmid copy number analysis—Copy number of plasmids in LVS was determined using an agarose gel-based assay as previously described (Pushnova, *et al*., 2000), except that lysozyme treatment at 37°C was omitted. This modification was needed because we found that *Francisella* lysed very quickly in the presence of lysozyme and this resulted in the degradation of the genomic and plasmid DNA. The procedure entailed the extraction of total DNA from the cells, serially dilution of the preparations and separation of the genomic and plasmid DNA species using a 0.8 % (w/v) SeaKem Gold agarose (Cambrex) gel in 1 X TBE. The gels were stained in ethidium bromide, destained, and the DNA visualized on an UV transilluminator. The gel images were digitally photographed and inverted images scanned with UN-SCAN-IT software version 4.3 (Silk Scientific Corporation), and data were obtained from the quantification of pixels in each peak. The linear ranges for both the genomic and plasmid DNA were used to quantify each DNA species from the gel images in order to calculate the plasmid copy number per genome using the equation previously described (Pushnova, *et al*., 2000). Three cultures were assayed for each plasmid and all samples for a given plasmid were separated on the same agarose gel. In most cases, samples of different plasmids were run together on a large gel for comparison.

Plasmid construction—Plasmids used in this study are in Table 1 and are briefly described below. Detailed information about the construction of the plasmids can be obtained from the corresponding author.

ORF3 plasmid modifications

ORF3 plasmid pMP716—PCR was used to obtain the ORF3 gene from pFNL10, which was then ligated into pMP527, mirroring its location in pFNL10, to form pMP716.

ORF3 frameshift mutation in pFNLTP1—The shuttle vector pFNLTP1 was digested with the restriction enzyme *Cla*I, which linearized the vector near the middle of the ORF3 gene. The ends were blunted with T4 DNA polymerase and religated to form pMP773. This alteration generates a unique *Nru*I site and a frameshift mutation, which was confirmed by DNA sequencing of pMP773.

Generation of P_{blaB} multiple cloning site cassette—Plasmid pMP615 (LoVullo, *et al*., 2006) was restriction digested to obtain the *F. tularensis blaB* promoter. This was cloned upstream of the multiple cloning site in the suicide vector pMP590 (LoVullo, *et al*., 2006) to form pMP649, bearing the P_{blaB}-mcs cassette.

Construction of intermediate plasmids with the P_{blaB} multiple cloning site

Stable Hyg^R shuttle vector, pMP656—The P_{blaB}-mcs cassette was moved from pMP649 into pMP633 (LoVullo, *et al*., 2006) forming the stable hygromycin plasmid pMP656.

Unstable Hyg^R shuttle vector, pMP658—This plasmid was made by introducing the P$_{blaB}$-mcs cassette from pMP649 into pMP529.

Stable Km^R shuttle vector, pMP662—pMP607 was originally modified by eliminating the restriction sites *Sal*I, *Cla*I, and *Pst*I by restriction digestion and religation to form pMP657, which was then modified with the P$_{blaB}$-mcs cassette from pMP649 to generate pMP662.

Construction of plasmids with the P$_{blaB}$-*Rsr*II multiple cloning site

Generation of a mutated *hyg1* gene lacking *Rsr*II sites—We utilized multiple site-directed mutagenesis (Seyfang & Jin, 2004) to remove three *Rsr*II restriction sites in the hygromycin resistance gene, *hyg*, and introduce silent mutations to produce the mutant allele, *hyg1*, which was cloned into pBluescript II KS(+) to form pMP738.

Stable Km^R shuttle vector, pMP814—We used inverse PCR to introduce a unique *Rsr*II restriction site to the multiple cloning site of pMP662, generating the P$_{blaB}$-*Rsr*II-mcs cassette in pMP814.

Stable Hyg^R shuttle vector, pMP822—The *hyg* gene in pMP656 was replaced with the *hyg1* gene from pMP738 to form pMP739. The P$_{blaB}$-*Rsr*II-mcs cassette from pMP814 was used to replace the P$_{blaB}$-mcs of pMP739 to generate pMP822.

Unstable Hyg^R shuttle vector, pMP823—The *hyg* gene in pMP658 was replaced with *hyg1* gene from pMP738 to form pMP740. The P$_{blaB}$-*Rsr*II-mcs cassette from pMP814 was used to replace the P$_{blaB}$-mcs of pMP740 resulting in pMP823.

Construction of plasmids with the *Rsr*II multiple cloning site

Stable Km^R Shuttle vector, pMP828—Utilizing inverse PCR, the *blaB* promoter was removed upstream the multiple cloning site of pMP814 to form pMP828.

Unstable Hyg^R shuttle vector, pMP829—The *Rsr*II-mcs was removed from pMP828 and used to replace the P$_{blaB}$-*Rsr*II-mcs of pMP823 forming pMP829.

Stable Hyg^R shuttle vector, pMP831—The *Rsr*II-mcs was removed from pMP828 and used to replace the P$_{blaB}$-*Rsr*II-mcs of pMP822 forming pMP831.

Results and Discussion

ORF3 of pFNL10 confers plasmid stability in the absence of selection

Our approach to *E. coli-Francisella* shuttle vector construction was to build vectors from the "bottom up", starting with the minimal requirement for a *Francisella* replicon taken from the cryptic plasmid pFNL10. Our first generation were not stable in the absence of antibiotic selection, but introduction of the ORF4–ORF5 region from pFNL10 resulted in stable second generation plasmids. A puzzling observation was that one widely used shuttle vector, pFNLTP1, lacks an intact ORF4–ORF5 region but is still stable in the absence of selection (Maier, *et al*., 2004). A major difference between pFNLTP1 and our vectors is that pFNLTP1 has an intact copy of the ORF3 gene of pFNL10. This gene encodes a hypothetical protein with homology to putative integrases/resolvases found in other plasmids but its function in plasmid biology is unknown (Pomerantsev, *et al*., 2001a, Pomerantsev, *et al*., 2001b).

We tested the role of ORF3 in plasmid maintenance by cloning a PCR product bearing ORF3 from pFNL10 into the unstable plasmid pMP527. The construction was designed to recapitulate the ORF3 location in pFNL10 within the context of the pFNL10 components of pMP527 (Fig. 1). We found that the new plasmid, pMP716, was capable of efficient transformation of *F. tularensis* strains LVS and Schu similar to that of pMP527, and was stable in the absence of antibiotic selection in both strains.

Shuttle vector copy number

The function of the ORF3 product is unknown, but it may play a role in the separation of concatamers as suggested by its homology to putative plasmid integrases/resolvases. However, this function was hypothesized to be the role of the ORF2 gene product, a putative helicase, since plasmids lacking ORF2 form higher-order concatamers (Pomerantsev, *et al*., 2001a, Pomerantsev, *et al*., 2001b). None of our plasmids that lack ORF3 form multimers (data not shown).

Alternatively, ORF3 might affect plasmid copy number, which could increase the chance of proper segregation of plasmids into daughter cells. To explore this, we determined the average copy number of these plasmids in LVS (Table 2). We found that the unstable plasmid pMP527 has an average copy number of 12 copies/genome, while that of pMP716, the stable derivative of pMP527 bearing ORF3 is 26/genome. We also determined that pFNLTP1 has an average copy number of 171 copies/genome (Table 2). The instability of pMP527 is puzzling, as one would not expect that a plasmid with a copy number of ~12 to be easily lost. Thus, there may be additional factors that contribute to plasmid segregation in this organism. However, the presence of ORF3 doubled the copy number of pMP716 compared to its parent pMP527, which could explain why the former plasmid is stable in the absence of selection.

To test this idea, we mutagenized ORF3 in pFNLTP1 by taking advantage of a unique *Cla*I restriction endonuclease site situated near the middle of the gene. The plasmid was digested with *Cla*I, blunted and self ligated to generate pMP773. This modification generated a frameshift mutation resulting in termination in the middle of the ORF3 coding sequence. As shown in Table 2, the mutation decreased the copy number of pMP773 to 60 copies/genome. This is still higher than the ORF3 bearing plasmid pMP716 and, consistent with the view that higher copy number plasmids are difficult to lose, we found that pMP773 is stable in the absence of antibiotic selection.

The addition of ORF3 to the unstable plasmid pMP527 doubled the copy number, while mutation of ORF3 in the high copy number plasmid pFNLTP1 decreased the copy number by almost three-fold but still within the range that ensures stability in the absence of selection. Variations in DNA structure, supercoiling, and subsequent replication dynamics may explain why pFNLTP1 and pMP773 have higher copy numbers compared to pMP527 and pMP716.

We previously reported that pFNLTP1 transformants of *F. tularensis* Schu grow more slowly on plates than transformants bearing pMP527 derivatives, and we hypothesized that this might be due to presence of ORF3 in pFNLTP1 (LoVullo, *et al*., 2006). However, we have found that Schu transformants bearing pMP716 (pMP527 with ORF3) grow as well as clones bearing pMP527, but that transformants bearing pMP773 (pFNLTP1 with a mutated ORF3) still have a growth defect (data not shown). Perhaps the relatively higher copy number of pFNLTP1 and pMP773 contributes to the growth phenotype of Schu clones transformed with these plasmids.

Shuttle vector improvements

In this work, we describe new, third generation shuttle vectors (shown in Fig. 2), derived from the Hyg^R plasmids pMP529 (unstable), pMP633 (stable) or the Km^R plasmid pMP607 (stable) (LoVullo, *et al*., 2006). In the case of the stable plasmids, all are second-generation vectors bearing the ORF4–ORF5 toxin-antitoxin genes. We chose to use these stable plasmids instead of the stable ORF3-bearing plasmids because of concern for the higher copy number of the ORF3 plasmids. We thought it best to refrain from using the ORF3-bearing vector as the primary platform for new vectors as its higher copy number might be detrimental in the cloning of genes with potentially toxic products. The copy number of the ORF4–ORF5 plasmid could not be formally measured because the amount of plasmid DNA in the nucleic acid preparations was too low to yield the necessary data needed to calculate copy number. We noted that DNA preparations from cells bearing the ORF4–ORF5 plasmid had less DNA than samples prepared from cells containing the unstable, low copy number vector pMP527 (data not shown). We think that the lower yield is due to a decrease in copy number brought about by the presence of the *hyg* gene, which has a higher G+C content than that found in the genome of *Francisella* and could possibly affect plasmid replication dynamics. Thus, we chose the ORF4–ORF5 Hyg^R plasmid as the backbone upon which new vectors would be built.

The first modification is the inclusion of the promoter region of the *F. tularensis blaB* gene. We chose this particular promoter because we have found that it is not recognized in *E. coli* (data not shown) and would therefore be beneficial for the cloning of *F. tularensis* genes that are toxic to *E. coli*. This promoter region was modified by the addition of the multiple cloning site (mcs) taken from our earlier *sacB* vector pMP590 (LoVullo, *et al*., 2006). A cassette consisting of P_{blaB}-mcs was introduced into a set of three intermediate plasmids (pMP656, pMP658, and pMP622, see Table 1) that were used for subsequent modifications.

The multiple cloning site of the P_{blaB}-mcs cassette was altered to include the recognition site for the endonuclease *Rsr*II. This is an unusual enzyme that recognizes the sequence CGGWCCG, where W is either a T or A. Because of this particular specificity, it is possible to use this single site for directional cloning of PCR fragments (Fig. 3). We took advantage of this since there is only one *Rsr*II site in the genomes of *F. novicida* Utah112, LVS, and *F. tularensis* Schu S4 located in the gene FTT_1110 (or relevant homologs). Thus, the novel nature of this enzyme allows one to use it for the directional cloning of almost all *Francisella* genes. To incorporate this recognition site into the multiple cloning site of our Hyg^R plasmids, we had to remove three *Rsr*II sites present within the *hyg* gene by site-directed mutagenesis, as described in the methods section. The *Rsr*II site was engineered into the mcs of the P_{blaB}-mcs plasmid set to generate pMP822 (stable, Hyg^R), pMP823 (unstable, Hyg^R), and pMP814 (stable, Km^R). Maps of these plasmids are shown in Fig. 2.

Additional plasmids were constructed, each of which contained the *Rsr*II-mcs but lacked the P_{blaB} promoter. These were built for use in cases where the P_{blaB} promoter would not be desirable, for example, in situations where screening for gene expression in *E. coli* is required, or when the native promoter is more appropriate for the particular gene in question. These plasmids, pMP831 (stable, Hyg^R), pMP829 (unstable, HygR), and pMP828 (stable, Km^R) are shown in Figure 2. The modifications to these plasmids do not affect their ability to transform *E. coli* or *Francisella* bacteria (data not shown).

We have shown here that the biology of *Francisella* plasmids is complex. There are at least two ways to stabilize shuttle plasmids in this organism: one can use the toxin-antitoxin genes to maintain the plasmid in the population, or one can increase the copy number of the plasmid by adding the ORF3 gene and presumably improve segregation into daughter cells. In addition, our results support the idea that high-copy number plasmids attenuate the

growth of *F. tularensis* subsp. *tularensis*. We envision that the improved shuttle vectors described in this manuscript will be helpful to others working on the genetics of *Francisella* bacteria.

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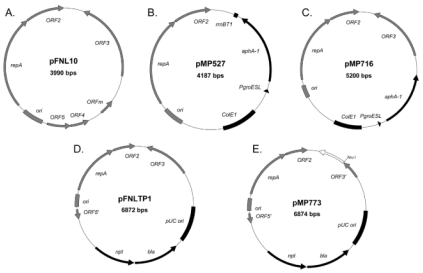

Figure 1. Maps of the ORF3 plasmids

(A) Cryptic plasmid pFNL10, (B) First generation, unstable *E. coli-Francisella* shuttle vector pMP527, (C) pMP716, the stable ORF3-bearing variant of pMP527, (D) *E. coli-Francisella* shuttle vector pFNLTP1, and (E) pMP773, the derivative pFNLTP1 with the ORF3 frameshift mutation.

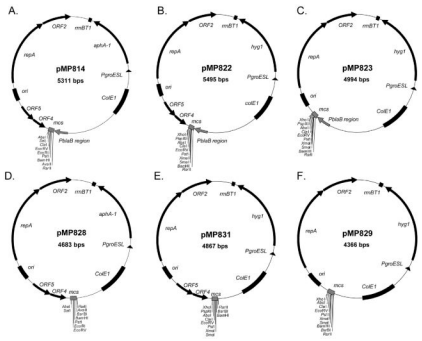

Figure 2. Maps of new shuttle vectors

A, B, and C show maps for plasmids bearing both the *Rsr*II multiple cloning site (mcs) and the *Francisella blaB* promoter. pMP814 (A) and pMP822 (B) are both stable plasmids, but pMP814 is Km^R and pMP822 is Hyg^R, while pMP823 (C) is unstable and Hyg^R. D, E, and F show maps for plasmids pMP828, pMP831, and pMP829, that correspond to the plasmids in A, B, and C, except that they lack the *blaB* promoter.

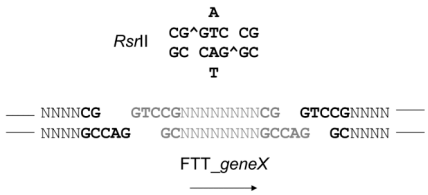

Figure 3. *Rsr*II cloning

The *Rsr*II restriction endonuclease site is shown at the top of the figure, and the bottom shows how a given *Francisella* gene can be engineered with *Rsr*II sites for directional cloning.

Table 1

Plasmids used in this study

Plasmids	Description	Reference or Source
pBluescript II KS (+) pFNL10	Ap^R, cloning vector cryptic plasmid from the "*F. novicida* like" F6168 strain	Stratagene (Pomerantsev, *et al.*, 2001a, Pomerantsev, *et al.*, 2001b)
pFNLTP1	Km^R, Ap^R *E. coli-F. tularensis* shuttle vector	(Maier, *et al.*, 2004)
pMP527	Km^R, *E. coli-F.tularensis* shuttle vector, unstable	(LoVullo, *et al.*, 2006)
pMP529	Hyg^R, *E. coli-F.tularensis* shuttle vector, unstable	(LoVullo, *et al.*, 2006)
pMP607	Km^R, *E. coli-F.tularensis* shuttle vector, stable	(LoVullo, *et al.*, 2006)
pMP633	Hyg^R, *E. coli-F.tularensis* shuttle vector, stable	(LoVullo, *et al.*, 2006)
pMP649	Km^R, source of P_{blaB}-mcs	This work
pMP656	Hyg^R, *E. coli-F.tularensis* shuttle vector pMP633 with P_{blaB}-mcs	This work
pMP657	Km^R, *E. coli-F.tularensis* shuttle vector pMP607 minus *Pst*I, *Cla*I, *Sal*I sites	This work
pMP658	Hyg^R, *E. coli-F.tularensis* shuttle vector pMP529 with P_{blaB}-mcs	This work
pMP662	Km^R, *E. coli-F.tularensis* shuttle vector pMP657 with P_{blaB}-mcs	This work
pMP716	Km^R, *E. coli-F.tularensis* shuttle vector pMP527 with ORF3 of pFNL10	This work
pMP738	pBluescript II KS (+) with *hyg1*	This work
pMP739	Hyg^R, *E. coli-F.tularensis* shuttle vector pMP656 with *hyg1* replacing *hyg*	This work
pMP740	Hyg^R, *E. coli-F.tularensis* shuttle vector pMP658 with *hyg1* replacing *hyg*	This work
pMP773	Km^R, Ap^R *E. coli-F. tularensis* shuttle vector pFNLTP1 with ORF3Δ*Cla*I frameshift mutation	This work
pMP814	Km^R, *E. coli-F.tularensis* shuttle vector pMP662 with P_{blaB}-*Rsr*II-mcs	This work
pMP822	Hyg^R, *E. coli-F.tularensis* shuttle vector pMP739 with P_{blaB}-*Rsr*II-mcs, stable	This work
pMP823	Hyg^R, *E. coli-F.tularensis* shuttle vector pMP740 with P_{blaB}-*Rsr*II-mcs, unstable	This work
pMP828	Km^R, *E. coli-F.tularensis* shuttle vector pMP814 with *Rsr*II-mcs, stable	This work
pMP829	Hyg^R, *E. coli-F.tularensis* shuttle vector pMP823 with *Rsr*II-mcs, unstable	This work
pMP831	Hyg^R, *E. coli-F.tularensis* shuttle vector pMP822 with *Rsr*II-mcs, stable	This work

Table 2

Plasmid copy number determinations

Plasmid	pFNL10 "genotype"[a]	Copy number/genome[b]
pMP527	*ori repA* ORF2	8, 10, 15 (12)
pMP716	*ori repA* ORF2 ORF3	25, 24, 25 (26)
pFNLTP1	*ori repA* ORF2 ORF3 ORF5'	186, 156 (171)
pMP773	*ori repA* ORF2 ORF3Δ*Cla*I(FS) ORF5'	64, 59, 58 (60)

[a] Indicates genes originally from pFNL10.

[b] Copy number/genome determination replicates for each plasmid with the average in parentheses.