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Utilization of an unstable plasmid and the I-SceI endonuclease to generate routine markerless deletion mutants in *Francisella tularensis*

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Abstract

We engineered an efficient system to make *Francisella tularensis* deletion mutations using an unstable, poorly maintained plasmid to enhance the likelihood of homologous recombination. For counterselection, we adapted a strategy using I-SceI, which causes a double-stranded break in the integrated suicide vector, forcing a second recombination to mediate allelic replacement.

Keywords

Francisella; allelic replacement; molecular genetics

Francisella tularensis is a Gram-negative bacterium with bio-terror potential. To combat this threat, scientists must manipulate this organism's genomic content to understand its virulence and pathogenesis mechanisms. Recently, significant advances have been achieved in *F. tularensis* genetics (Rodriguez et al., 2008; Rodriguez et al., 2009), however opportunities exist to improve these molecular techniques. Specifically, generating routine *F. tularensis* markerless deletion mutants still remains a difficult task. This is likely due to low rates of homologous recombination and less than optimal counterselection methods to resolve merodiploids (Ludu et al., 2008). For example, successful recombination of suicide vectors requires relatively large fragments of homologous DNA (~1 kb) (Forslund et al., 2006; Golovliov et al., 2003). Once integrated, the occurrence of a second recombination is detected with a counterselection system involving expression of SacB (Golovliov et al.,

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2003), a *Bacillus subtilis* enzyme that confers sensitivity to sucrose resulting in lethality in most Gram-negative bacteria (Kaniga et al., 1991). The mutation rate of *sacB* is extremely high (Hashimoto et al., 2003) and this enzyme is not always lethal in the presence of sucrose, even when intact in *F. tularensis* (Ludu et al., 2008), further encumbering isolation of deletion mutants. Although deletion mutants of a related species, *F. novicida*, can be easily generated by utilizing PCR products (Lauriano et al., 2003), deleting genes in type A and B strains is more challenging.

The initial objective of this study was to identify *F. tularensis* shuttle vectors. Therefore, we transformed the LVS strain (ATCC 29684) with numerous plasmids harboring diverse replicons. One of these plasmids, pMQ131 (GenBank accession number EU546819.1, kanamycin (Km) resistance, pBBR1 replicon) (Shanks et al., 2009), consistently produced pinpoint colonies on agar plates with Km (10 µg/ml), that were not capable of subcultivation on this same medium (data not shown). The pBBR1 replicon is compatible with IncP, IncQ, IncW, ColE1 and p15a plasmids (Antoine and Loch, 1992), prompting others to postulate that it may belong to its own novel incompatibility group (Lefebvre and Valvano, 2002). We posed the following two hypotheses as potential explanations for the acquisition of pinpoint colonies. It was possible that the Km^R gene product of this plasmid was potentially produced at low levels, as this gene's promoter was not derived from *F. tularensis*. Ectopic promoter incompatibility has historically been a problem in *F. tularensis* genetics (Frank and Zahrt, 2007). The second hypothesis was that the pBBR1 replicon was unstable in *F. tularensis*, initially being maintained poorly until it was eventually lost. To test the first hypothesis, we generated the plasmid pMQ131hyg using standard recombinant DNA practices (Horzempa et al., 2008a). All cloning was conducted using *Escherichia coli* strains XL-10 Gold or EC100D. The plasmid, pMQ131hyg contained a hygromycin (Hm) resistance cassette under the control of the *Francisella groE* promoter (LoVullo et al., 2006). Electroporation of pMQ131hyg into *F. tularensis* LVS also resulted in the presence of pinpoint colonies on chocolate II agar plates containing Hm (200 µg/ml) that could not be subcultivated on this same medium (data not shown). As a control, pMP615 [Hm^R, pFNL10 replicon (LoVullo et al., 2006)] produced large colonies on chocolate II agar plates with Hm that were capable of subcultivation on media with Hm (data not shown). These data supported the second hypothesis – that the pBBR1 replicon is unstable in *F. tularensis*. This would present an optimal scenario for producing a recoverable merodiploid strain produced by a single recombination event if homologous DNA was present in the pBBR1-based vector. To confirm, we cloned two separate fragments of *F. tularensis* DNA each (a 560 bp or a 900 bp fragment) into pMQ131hyg, producing pMQ131hyg1581d and pMQ131hyg1664d respectively (Horzempa et al., 2008a). These vectors were subsequently mobilized into LVS by both conjugation and electroporation individually. Following recovery and selection, we still observed the presence of pinpoint colonies on selective media. However, in addition to the pinpoint colonies, transformation with pMQ131hyg1581d and pMQ131hyg1664d also produced numerous large colonies (a ratio of ~1 large colony per 2×10^4 pinpoint colonies), whereas transformation with the empty vector resulted in the presence of only the pinpoint colonies (data not shown). We verified that these vectors integrated into the chromosome at the specific homologous site by PCR using a primer adjacent to this vector's multicloning site paired with a primer that annealed just outside the site targeted for recombination (data not shown). Based on these data, the unstable nature of the pBBR1 replicon of pMQ131 in *F. tularensis* is robust for producing a homologous recombination event. In addition, the pBBR1 system allowed for chromosomal integration using substantially smaller fragments of homologous DNA (Horzempa et al., 2008a), compared to the 1 kbp used in previous reports (Golovliov et al., 2003).

Because the pBBR1 replicon supports favorable conditions for homologous recombination in *F. tularensis*, we decided to adapt this plasmid to generate marker-less deletion mutants.

Rather than develop a conventional counterselection scheme using *sacB*, we adapted a scheme that involves the expression of an intron-encoded homing restriction enzyme, I-SceI. This endonuclease recognizes and cleaves an 18 bp restriction sequence (Choulika et al., 1995). I-SceI has been used to cleave its cognate site that has been engineered on a suicide plasmid that has integrated into the chromosome by homologous recombination (Choulika et al., 1995; Janes and Stibitz, 2006; Rong et al., 2002). This causes a double-stranded break that is a favored substrate for host recombination systems. The recombination machinery can repair this break by recombining homologous regions flanking the break following the initial plasmid integration.

For this system to be successful in *F. tularensis*, we needed a pBBR1-based suicide plasmid containing the I-SceI site. We also required a second plasmid capable of replicating in *F. tularensis* that contained the coding sequence for the I-SceI enzyme under the control of a *Francisella* promoter. We therefore generated pJH1 (Figure 1A&C; pBBR1 replicon, Hm^R) and pGUTS [Figure 1B; pFNL10 replicon, *I-SceI* under the control of the *Francisella* glucose-repressible promoter (Horzempa et al., 2008b), Km^R]. Figure 1C shows that the I-SceI site introduced into pJH1 is digested by I-SceI protein in vitro.

To test the efficacy of this system in generating chromosomal deletion mutations, loci that flanked ORFs (~1 kb on either side) targeted for deletion were cloned in tandem in pJH1. These were mobilized to *F. tularensis* by triparental conjugation (Horzempa et al., 2008a). Merodiploid strains were recovered and transformed with pGUTS by electroporation. These colonies were screened by PCR using primers flanking the targeted site for deletion. Here, we saw 100% merodiploid resolution. Twenty-six percent of these resolved to retrieve the deletion mutation (15 / 58) while the remainder resolved to wild-type allele. Loss of pGUTS was achieved by passage in trypticase soy broth cultivated with 0.1% cysteine HCl. These cells were diluted and plated to achieve ~100–300 colonies per chocolate II plate. After 3 days at 37°C, these colonies were replica-plated onto chocolate II plates with and without Km (10 µg/ml). Km^S clones (approximately 1/100 colonies) were isolated and again tested for sensitivity to Km to confirm this phenotype. Altogether, we used this system to delete three genes in *F. tularensis* LVS (Figure 2). In addition, we deleted an ORF in the fully virulent type A strain, *F. tularensis* Schu S4 (Figure 2).

One of the genes that we deleted was FTL_1664, an ORF corresponding to *deoB* in *F. tularensis* LVS. We recently showed that this gene is important for uptake into host phagocytes and non-phagocytes using a disruption mutant of this ORF (Horzempa et al., 2008a). The Δ *deoB* mutant generated here also showed diminished uptake by HEK-293 cells and by the macrophage-like RAW 264.7 cells (Figure 3), determined by a gentamicin protection assay as we have previously detailed (Horzempa et al., 2008a). In this assay, mammalian cells were seeded into Primaria-coated 96-well dishes (Becton, Dickinson and Company) at a concentration of 5×10^4 cells per well in DMEM (Invitrogen) supplemented with 10% fetal calf serum, 25 mM HEPES, and 1% Glutamax. Bacteria were added at a multiplicity of infection of 500 and co-incubated with the mammalian cells for two hours. Subsequently, these cells were washed and treated with gentamicin (50 µg/ml) for 30 min. Cells were washed extensively with warm Hank's balanced salts solution (Gibco), lysed with 0.02% sodium dodecyl sulfate, diluted and plated to enumerate CFU. We typically observe 2 to 10 fold reduction in uptake of *F. tularensis deoB* mutant strains relative to wild type [unpublished observations and (Horzempa et al., 2008a)]. Upon complementation of LVS Δ *deoB* in trans, wild-type level of uptake was restored. This experiment confirms the previously published uptake data (Horzempa et al., 2008a), and provides phenotypic data to further support the functionality of the deletion strategy described here.

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Figure 1.

Maps of the plasmids used for generating *F. tularensis* deletion mutants. The unstable vector, pJH1 (A) and the plasmid encoding I-SceI, pGUTS (B) are depicted. Unique restriction sites and relevant loci are designated in both plasmid maps. The vector, pJH1 is also equipped with the necessary components for yeast recombination cloning (Shanks et al., 2006) and is transferrable to *F. tularensis* by conjugation. For pJH1 (A), *CEN/ARS*, yeast replication and segregation machinery; *LEU2*, Beta-isopropylmalate dehydrogenase (leucine biosynthesis); *oriT*, conjugal transfer origin. For pGUTS (B), Ap, β -lactamase (ampicillin resistance gene). Gel electrophoresis of pJH1 treated with the designated restriction enzymes (C).

Figure 2. Identification of *F. tularensis* markerless deletion mutants. Primer pairs (depicted by the small arrows) were used to PCR across the ORFs targeted for deletion in *F. tularensis*. Following PCR, amplicons were analyzed by agarose gel electrophoresis. The standards (arrowheads) are represented in kb. The ORF locus tag is represented below the gel image. FTL_# for strain LVS and FTT# for the fully virulent Schu S4. wt, wild type; Δ , deletion mutant.

Figure 3.

The diminished uptake of *F. tularensis* LVS Δ deoB by host cells confirms the deletion of this gene. RAW 264.7 murine macrophage-like cells or HEK-293 human embryonic kidney cells were infected with the strains indicated. These cells were co-incubated with bacteria at a multiplicity of infection of 500:1. After two hours at 37°C and 5% CO₂, supernatants were removed and the cells were treated with gentamicin to kill remaining extracellular bacteria, washed, and lysed. Lysates were diluted and plated to enumerate CFU which are displayed relative to LVS as % uptake relative to wild type. Data shown are mean $\pm$ SEM of six wells within one experiment. The abbreviation “comp” refers to the complementing construct (pF81664; contains the entire *F. tularensis* deoB gene with 600 bp upstream and 100 bp downstream in the pFNLTP8 backbone) and “vector” designates the empty shuttle plasmid, pFNLTP8. LVS vs. Δ deoB / vector, $P = 2.95 \times 10^{-6}$ (RAW 264.7) and $P = 4.65 \times 10^{-4}$ (HEK-293) as determined by a Student's t-Test.