Targeted Inactivation of *Francisella tularensis* Genes by Group II Introns

Stephen A. Rodriguez,1 Jieh-Juen Yu,1 Greg Davis,2 Bernard P. Arulanandam,1 and Karl E. Klose1*

South Texas Center for Emerging Infectious Diseases and Department of Biology, University of Texas San Antonio, San Antonio, Texas 78249,1 and Sigma-Aldrich Biotechnology, Genomics R&D, St. Louis, Missouri2

Received 21 December 2007/Accepted 21 February 2008

Studies of the molecular mechanisms of pathogenesis of *Francisella tularensis*, the causative agent of tularemia, have been hampered by a lack of genetic techniques for rapid targeted gene disruption in the most virulent subspecies. Here we describe efficient targeted gene disruption in *F. tularensis* utilizing mobile group II introns (targetrons) specifically optimized for *F. tularensis*. Utilizing a targetron targeted to *blaB*, which encodes ampicillin resistance, we showed that the system works at high efficiency in three different subspecies: *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, and *F. tularensis* subsp. *novicida*. A targetron was also utilized to inactivate *F. tularensis* subsp. *holarctica* *iglC*, a gene required for virulence. The *iglC* gene is located within the *Francisella* pathogenicity island (FPI), which has been duplicated in the most virulent subspecies. Importantly, the *iglC* targetron targeted both copies simultaneously, resulting in a strain mutated in both *iglC* genes in a single step. This system will help illuminate the contributions of specific genes, and especially those within the FPI, to the pathogenesis of this poorly studied organism.

*Francisella tularensis* is a highly infectious bacterium that causes tularemia, a zoonotic disease (11). Interest in this pathogen has been heightened due to its potential use as a biological weapon and the lack of a safe and effective vaccine approved for human use. The subspecies of *F. tularensis* differ in virulence in humans. *F. tularensis* subsp. *tularensis* is the most virulent of the subspecies (39) and has been associated historically with bioweapons development (30). *F. tularensis* subsp. *holarctica* maintains high virulence in humans, but infections by this organism are reported to be less severe (40). An attenuated form of *F. tularensis* subsp. *holarctica*, the “live vaccine strain” (LVS) (10), is used frequently as a laboratory model for the more virulent *F. tularensis* strains. Finally, *F. tularensis* subsp. *novicida* is generally the least virulent to humans. All three subspecies are closely related at the genomic level, with the less virulent *F. tularensis* subsp. *novicida* showing less genomic decay than the virulent *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis* (34, 36).

*F. tularensis* is able to survive and replicate within macrophages, an ability that has been linked to its virulence (1, 5, 13). A cluster of genes that constitute the *Francisella* pathogenicity island (FPI) are required for intramacrophage survival and growth, including the *iglC* gene (15, 16, 23, 32, 38), Transcription of the FPI genes is regulated by MglA (7, 24), a global transcription factor that associates with RNA polymerase (8). The entire FPI is duplicated within the genomes of the virulent *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* strains, unlike the case for *F. tularensis* subsp. *novicida* strains, which have a single FPI (22).

Most studies utilizing targeted gene knockouts have been performed with *F. tularensis* subsp. *novicida*, due to ease of genetic manipulation, more established genetic tools, and the presence of a single FPI (1–4, 23, 25). For example, for unknown reasons, *F. tularensis* subsp. *novicida* can take up and integrate linear DNA into its genome, an ability that appears to be lacking in *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*. A technique for targeted mutagenesis within *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis* has been developed, based on conjugation or transformation with a nonreplicative plasmid followed by sacB counterselection (15). However, this technique is time-consuming and relatively inefficient; moreover, targeted mutagenesis of the duplicated FPI genes requires twice the effort. The cumbersome nature of biosafety containment required for the more virulent subspecies has added another hurdle to the development of efficient genetic manipulation techniques for *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*. Thus, to date, there are only a few examples of successful targeted mutagenesis in *F. tularensis* subsp. *tularensis* (15, 41).

Group II introns have been exploited for targeted gene disruption in various bacteria. The *Lactococcus lactis* LtrB (LtrB1) intron has been developed into a “targetron” that can be retargeted to inactivate specific genes of interest (14, 20, 31, 33, 42, 43). This system involves homing of a ribonucleoprotein complex (RNP) that consists of the group II intron RNA molecule (LtrB1) and the associated LtrA protein. Base pairing between exon binding site 1 (EBS1), EBS2, and δ of the RNA molecule with intron binding site 1 (IBS1), IBS2, and δ′ within the target gene confers specificity upon the subsequent integration event (Fig. 1). Thus, the intron can be targeted to specific genes by replacing EBS1 and EBS2 with sequences complementary to the insertion site within the gene of interest. LtrB1 and LtrA are expressed from a donor plasmid, from which the LtrB1 intron splices out of its flanking 5′ and 3′ exons and forms an RNA lariat that associates with LtrA (20, 26, 29). The LtrA protein facilitates formation of the intron’s catalytic structure and recognition of the target gene.
reverse splices into the insertion site, while LtrA reverse transcribes the intron RNA sequence, synthesizing an antisense copy of the RNA. Because the ltrA gene is not encoded within the inserted sequence, the resultant insertion mutation is stably maintained in the absence of LtrA.

In the present study, we have adapted the TargeTron group II intron mutagenesis system for *F. tularensis*. We demonstrate that this optimized targetron system works at high efficiency in *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, and *F. tularensis* subsp. *novicida*. Moreover, the intron is capable of inactivating two identical genes simultaneously within the same strain in the absence of selection, which will facilitate the study of the duplicated FPI genes in the more virulent subspecies.

### MATERIALS AND METHODS

#### Bacterial strains and media.

The bacterial strains used in this study are listed in Table 1. *Francisella tularensis* strains were grown on TSAP medium (tryptic soybean agar powder [40 g/liter] with 0.1% cysteine, 25 µg/ml ferrous sulfate, 25 µg/ml sodium pyruvate, and 25 µg/ml sodium metasulfite). *Escherichia coli* strain DH5α was grown on Luria-Bertani (LB) medium. The concentrations of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal), kanamycin (Kan), and ampicillin (Amp), when used, were 40 µg/ml X-Gal, 50 µg/ml Kan, and 1 mg/ml Amp, respectively.

#### Construction of the targetron plasmid.

The oligonucleotides used in this study are listed in Table 2. The *Francisella* plasmid pKK214 (21) was PCR amplified with primers pKK214EcoRI and pKK214EcoNotI, and then the PCR product was digested with EcoRI and religated to form pKEK648. This step removed the *cat* gene from pKK214 and introduced an EcoRI restriction site. Next, *F. tularensis* subsp. *novicida* KKF24 chromosomal DNA using primers groELpDownBglINotI and groELpUpEcoRIXhoI, digested with EcoRI and religated to form pKEK648. This step amplifies the *groEL* gene from pKK214 and introduced a NotI restriction site. Finally, the *F. tularensis* subsp. *novicida* KKF24 chromosomal DNA was amplified from pKEK898 (25) using primers pET15BUpSalI and FpKanUpNotI, digested with SalI and NotI, and ligated to pKEK995 digested similarly, resulting in pKEK995. The product was digested with EcoRI and religated to form pKEK648. This step amplified the duplicated FPI genes in the more virulent subspecies.

#### Table 1. Bacterial strains used in this study

| Subspecies or species and strain | Genotype | Source or reference |
|----------------------------------|----------|---------------------|
| *F. tularensis* subsp. *novicida* |          |                     |
| U112                             | Wild type | ATCC 15482          |
| KKF23                            | U112 *blaB::ltrB*1 | This study          |
| KKF24                            | U112 *ΔiglC::ermC* | 23                  |
| *F. tularensis* subsp. *holarctica* |          |                     |
| LVS                              | Live vaccine strain | ATCC 29684          |
| KKL4                             | LVS *blaB::ltrB*1 | This study          |
| KKL1                             | LVS *iglC1::ltrB*1 | This study          |
| *F. tularensis* subsp. *tularensis* |          |                     |
| Schu4                            | Wild type | CDC                 |
| KKT1                             | Schu4 *blaB::ltrB*1 | This study          |
| E. coli DH5α                     | *ΔdeoR endA1* | 17                  |
|                                  | LsdR17 supE44 |                     |
|                                  | *Δthi-1 recA1* |                     |
|                                  | *ΔargF-lacZYA* |                     |
|                                  | F. tularensis |                     |

FIG. 1. (A) Targetron plasmid. Plasmid pKEK1140 was constructed to adapt the TargeTron system to *F. tularensis*; the elements indicated are described in the text. (B) LtrB1 intron structure. The secondary structure of the LtrB1 targetron transcript is shown, with the EBS1 and EBS2 loops and δ site indicated. (The primary transcript also contains IBS1, IBS2, and δ’ sites identical to those found within the target gene; the intron excises from these prior to insertion into the target site within the chromosome.) (C) *F. tularensis* group II intron insertion sites. Shown are the targetron target sites within the *blaB* (102/103a) and *iglC* (427/428a) genes. The EBS1, EBS2, and δ elements within the respective targetrons are designed to base pair with the corresponding complementary sequences within the *blaB* and *iglC* genes, as indicated by hatch marks. The open triangles show the insertion site of the group II intron, while the elements recognized by the targetron RNP at −23, −20, and +5 with respect to the insertion site are depicted.
Targeted mutagenesis in *F. tularensis*. To target a specific *F. tularensis* gene for group II intron insertion, the LtrB *Ll* intron in pKEK1140 was retargeted by replacing lacZ, which is located between the Xhol and BsrGI sites, with a 350-bp PCR product. The PCR product was amplified using IntronXholF and IntronSpeIR. The resulting PCR fragment was digested with Xhol and Spl and ligated to pKEK1041 digested similarly, resulting in pKEK1058. Finally, the temperature-sensitive M120I mutation (28) was introduced into the 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to Western immunoblotting utilizing mouse monoclonal antibodies specific and intron-specific primers. Intron insertions were verified by DNA sequencing. Frequently the 915-bp intron insertion could be detected within a specific and intron-specific probe. Intron insertions were identified by PCR with chromosomal DNA from the target strain as template. The PCR products were digested with Xhol and BsrGI and ligated into pKEK1140 that was digested similarly.

**Construction of *F. tularensis* targetron insertion mutants.** *F. tularensis* strains were transformed with the targetron vectors via electroporation. Briefly, mid-log-phase *F. tularensis* cultures, grown in TSAP, were washed twice and resuspended in 0.5 M sucrose and then electroporated with 1 μg of targetron plasmid (600 Ω, 25 μF, 2.5 kV; Bio-Rad Gene Pulser II). Electroporated cells were inoculated into TSAP, incubated at 30°C 1 h, and then plated onto TSAP-Kan and incubated at 37°C. In some instances, we also utilized cryotransformation to introduce the targetron plasmid into *F. tularensis*; this procedure has been described previously (24). Intron insertions were identified by PCRs with chromosomal DNA isolated from transformant colonies, using combinations of gene-specific and intron-specific primers. Intron insertions were verified by DNA sequencing. Frequently the 915-bp intron insertion could be detected within a mixed population of wild-type and mutant cells; in these instances pure populations of the mutant strains were obtained by additional plate streaking and PCR screens. Insertion mutants were cured of the targetron plasmid by growth on TSAP at 37°C. Loss of the plasmid was detected by lack of growth on TSAP-Kan.

**Southern hybridization.** Southern hybridization was performed utilizing ECL detection systems (GE Healthcare) according to the manufacturer’s instructions. A 400-bp intron-specific probe that was amplified with oligonucleotides IntronSpeIR and IntronXhoIF (Table 2) was used for Southern blotting. A 400-bp intron-specific probe was used for Southern hybridization.
intramacrophage survival assay. Wild-type or mutant *F. tularensis* strains were used to infect the J774.1 macrophage cell line (ATCC) at a multiplicity of infection of ~10:1. Wells were seeded with ~10^6 J774 cells, and bacterial inocula ranged from 1.56 x 10^4 to 5.8 x 10^6. After 1 h of incubation at 37°C, gentamicin (50 μg/ml) was added to the medium to eliminate extracellular organisms. The macrophage cells were lysed with 0.2% deoxycholate at 24 h postinfection, the lysate was plated on TSAP and incubated at 37°C, and CFU were enumerated.

**RESULTS**

Development of a targetron for targeted gene inactivation in *F. tularensis*. The TargeTron gene knockout system (Sigma-Aldrich) was optimized for use in *F. tularensis* (see Materials and Methods) (Fig. 1A). The targetron vector, pKEK1140, contains the following optimized attributes: (i) a plasmid origin for replication in *E. coli* (ori*)F*; (ii) a plasmid origin for replication in *F. tularensis* (ori*)F*; (iii) an antibiotic resistance gene (Kan*) optimized for expression in *F. tularensis* and permitted for use in select agent *F. tularensis* strains; (iv) the RNP (lbrB* and LtrA) driven by the *F. tularensis* groEL promoter for optimized expression; (v) a lacZ* “stuffer” within lbrB* to facilitate cloning via blue-white screening; and (vi) a temperature-sensitive ori*F* to facilitate curing of the plasmid following mutagenesis.

The basic principle (Fig. 1B and C) of this system is that base pairing between the two loops (EBS1 and EBS2) and δ site within the LtrB RNA structure and the target DNA sequence dictate the subsequent insertion event. Thus, changing the (i.e., retargeting) the sequences of EBS1 and EBS2 to allow base pairing to the target site within the gene (IBS1 and IBS2) facilitates insertion of the intron into this specific sequence. Sequence elements that flank the IBS1 and IBS2 sites within the targeted gene are also recognized by the RNP complex. The TargeTron computer algorithm identifies potential insertion sites within any DNA sequence (33). For each *F. tularensis* gene targeted for inactivation, the TargeTron algorithm (Sigma-Aldrich) was used to identify potential insertion sites and to design primers necessary to target the LtrB intron. A PCR product that encompasses EBS1, EBS2, and δ in lbrB intron is amplified with specific primers utilizing splicing-by-overlap PCR. The PCR fragment is cloned between the XhoI and BsrGI sites in pKEK1140 to replace lacZ* and target the group II intron. Blue-white screening on X-Gal medium expedited the identification of correct clones, which were verified by sequencing.

**Efficiency of the targetron in three different subspecies of *F. tularensis*. ** *F. tularensis* strains express a β-lactamase, encoded by bla*B*, which confers Amp* (6, 23, 27). To test the efficiency of the targetron system in three *F. tularensis* subspecies (*F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. holarctica [LVS], and *F. tularensis* subsp. novicida), we utilized bla*B* as a target. The TargeTron algorithm identified nine potential insertion sites within bla*B*. We chose two targets, located between nucleotides 102 and 103 (102/103a) and between nucleotides 214 and 215 (214/215a). The RNP recognizes these targets on the antisense strand, and thus these targets are given the designation “a.” These target sites were chosen based on the score and e value derived from the algorithm (for 102/103a, score = 6.53 and e value = 0.313; for 214/215a, score = 8.48 and e value = 0.076). Both bla*B* target sites were identical in the genomes of *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. novicida, and *F. tularensis* subsp. holarctica (LVS), which allowed us to compare the efficiencies of targetron insertion across *F. tularensis* subspecies.

The two targetron plasmids targeting positions 102/103 (pKEK1181) and 214/215 (pKEK1180) in bla*B* were initially transformed into *F. tularensis* subsp. *novicida*. A primary transformant was screened for the Amp* phenotype on TSAP supplemented with 1 mg/ml Amp. No Amp* transformants (0/100) of the targetron target 214/215 were identified; however, 2/100 transformants of the targetron targeting 102/103 were Amp*. Utilizing gene-specific and insertion-specific sets of primers (Fig. 2), PCR screens detected the group II intron insertion mutation of 915 bp in bla*B* in the Amp* *F. tularensis* subsp. *novicida* colonies. Sequencing confirmed the insertion mutation at target 102/103, demonstrating that the targetron system is functional in *F. tularensis* subsp. *novicida*.

To test the efficiency of this system across *Francisella tularensis* subspecies, we utilized the 102/103 bla*B* targetron in *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. holarctica (LVS), and *F. tularensis* subsp. *novicida*. The same conditions were used in all three subspecies to transform pKEK1181 into the strains and evaluate targetron insertion in bla*B*, which allowed us to determine the relative efficiency of this system in the various subspecies. Ten primary transformants of each subspecies were screened by PCR with an intron-specific and gene-specific primer pair, to confirm the presence of the targetron insertion in bla*B* (Fig. 3). The bla*B* targetron inserted at high efficiency in all three subspecies (10/10 positive for *F. tularensis* subsp. *tularensis*, 9/10 positive for *F. tularensis* subsp. holarctica LVS, and 9/10 positive for *F. tularensis* subsp. *novicida*). These same primary transformants did not consistently give an Amp* phenotype in the presence of 1 mg/ml Amp and showed evidence of both wild-type and mutant bla*B*-
specific primers, indicating mixed populations of mutant and wild-type cells in the primary transformants. Pure populations of blaB targetron mutants in each subspecies were easily obtained by subsequent plate streaking of the primary transformants for isolated colonies, followed by PCR screening. Amp\(^r\) colonies of each subspecies were analyzed by PCR to confirm that this phenotype correlated with ltrB\(_{1,1}\) insertion into blaB (Fig. 2).

Because the experiments reported above were performed at 30°C due to the temperature-sensitive targetron ori, we wished to determine whether the relative efficiency of blaB inactivation would be altered at 37°C. For these experiments, the 102/103 blaB targetron lacked the temperature-sensitive ori\(_{Ft}\), enabling its use at 37°C. We found that performing the transformation and incubation at 37°C rather than 30°C had no apparent effect on the high efficiency of targetron insertion in any of the subspecies (data not shown).

For the blaB::ltrB\(_{1,1}\) insertion mutants in each subspecies, Southern hybridization analysis showed that a single intron insertion existed in the genome of each strain (Fig. 4) (an expected fragment of 2.9 kbp was detected for each strain). Thus, the blaB targetron proved to be an efficient means for targeted gene inactivation in the multiple subspecies of F. tularensis.

Simultaneous inactivation of duplicated FPI genes in F. tularensis. The FPI is duplicated within F. tularensis subsp. holarctica and F. tularensis subsp. novicida, necessitating step-wise inactivation of each copy of any specific FPI gene in order to study its function. The lack of efficient targeted mutagenesis tools combined with the duplication of the FPI genes has hampered efforts to study FPI genes in the virulent F. tularensis subsp. tularensis and F. tularensis subsp. holarctica strains; to date there has been only one report of an F. tularensis subsp. tularensis strain with both copies of the iglC gene inactivated (41). Because group II introns can insert and inactivate multiple genomic copies of their specific target sequence (35), we suspected that the targetron system would be useful in inactivating FPI genes. We constructed three vector types targeted to three sites in iglC (64/65s, 150/151s, and 427/428a). All three sites are identical in the three different F. tularensis subspecies and had favorable scores and e values derived from the TargeTron algorithm (for 64/65s, score = 6.76 and e value = 0.274; for 150/151s, score = 7.97 and e value = 0.111; and for 427/428a, score = 6.09 and e value = 0.404).

F. tularensis subsp. holarctica strain LVS was transformed with the three iglC targetron plasmids at 30°C. Kan\(^r\) colonies were screened by PCR, utilizing an intron-specific (EBS universal primer) and gene-specific (iglCNdeIF for 64/65 and 150/151 targets and iglCNcoIR for 427/428 target) primers to identify colonies in which the targetron insertion in iglC had occurred. Southern screening of LVS transformed with targetron plasmids targeting 64/65 and 150/151 revealed no evidence of targetron insertion in iglC. However, the first five LVS colonies (100%) transformed with the targetron plasmid targeting 427/428 gave a positive PCR for insertion in iglC (Fig. 5).

We further purified a single positive colony with evidence of an iglC::ltrB\(_{1,1}\) insertion and performed additional PCR analyses to determine whether both copies of iglC were inactivated by intron insertion in this strain. The two copies of the FPI (FPI-I and FPI-II) can be distinguished by PCR utilizing primers specific for the DNA flanking each FPI. Utilizing FPI-I- and FPI-II-specific primers together with an intron-specific primer, the PCR analysis revealed that the targetron inserted into both copies of iglC (Fig. 5). Additional evidence of insertion in both iglC genes was obtained by PCR amplification of each locus with FPI-I- and FPI-II-specific primers coupled with a primer that anneals downstream of the insertion site and then sequencing each iglC gene (Fig. 5). These experiments confirmed that iglC\(_1\) and iglC\(_2\) had been inactivated by simultaneous insertion of the intron in both genes.

The iglC\(_1::ltrB\(_{1,1}\) iglC\(_2::ltrB\(_{1,1}\) LVS mutant KKL1 was cured of the targetron plasmid by plate streaking and incubation at 37°C. Southern blot analysis confirmed that two intron insertions are present in the genome of this strain (Fig. 4) (expected fragments of 14.5 kbp and 12.3 kbp were detected). IgC is known to be required for F. tularensis intramacrophage growth, and an LVS strain with only one copy of the iglC gene mutated replicates within macrophages similarly to the wild-type LVS strain (15). The iglC\(_1::ltrB\(_{1,1}\) iglC\(_2::ltrB\(_{1,1}\) LVS mutant was defective for intramacrophage growth within the J774 cell line.
getron insertions in parent strains with anti-IglD antibodies revealed that the tar-

2624 RODRIGUEZ ET AL. APPL. ENVIRON. MICROBIOL.

whole-cell lysates from the macrophage growth (37). Western immunoblot analysis of iglC, and IglD has also been shown to be necessary for intra-

line (Fig. 6A). The iglD gene is immediately downstream of iglC, and IgID has also been shown to be necessary for intramacrophage growth (37). Western immunoblot analysis of whole-cell lysates from the F. tularensis subsp. holarctica strains LVS (wild type [WT]) (lanes 2, 4, 6, 8, and 10) and KKL1 (lanes 3, 5, 7, 9, and 11) (B). Primers (Table 2): a, iglCNcoIF; b, iglCNdeIR; c, iglC427 428a-EBS2; d, FTL_0105R; e, FTL_1152R.

FIG. 5. PCR analysis of the F. tularensis subsp. holarctica (LVS) iglC::ltrB1,2 iglC::ltrB1,2 mutant. Using combinations of gene-specific primers (primers a and b), an intron-specific primer (primer c), and FPI-I- and FPI-II-specific primers (primers d and e) (A), PCR analysis was performed on genomic DNA from F. tularensis subsp. holarctica strains LVS (wild type [WT]) (lanes 2, 4, 6, 8, and 10) and KKL1 (lanes 3, 5, 7, 9, and 11) (B). Primers (Table 2): a, iglCNcoIF; b, iglCNdeIR; c, iglC427 428a-EBS2; d, FTL_0105R; e, FTL_1152R.

FIG. 6. Intramacrophage growth of the F. tularensis subsp. holarctica (LVS) iglC::ltrB1,2 iglC::ltrB1,2 mutant. (A) The F. tularensis subsp. holarctica LVS wild-type and KKL1 (iglC::ltrB1,2 iglC::ltrB1,2) strains were inoculated at a multiplicity of infection of ~10:1 into J774 cells, and intracellular bacteria were enumerated at 3 and 24 h. The assay was performed in triplicate. (B) Western immunoblotting was performed on whole-cell lysates of F. tularensis subsp. holarctica LVS wild-type and KKL1 (iglC::ltrB1,2 iglC::ltrB1,2) strains with mouse monoclonal anti-IgID antiserum. Error bars indicate standard deviations.

DISCUSSION

F. tularensis is a potentially dangerous pathogen for humans, and yet very little is known about how it causes disease. The reasons for our lack of understanding are partly historical, and yet very little is known about how it causes disease. The single technique that has been successfully employed to construct targeted mutations in F. tularensis subsp. tularensis and F. tularensis subsp. holarctica (15, 41) is a relatively inefficient and time-consuming process. This technique is frequently used to generate targeted mutations in a number of different bacteria, and it involves conjugation or transformation with a nonreplicative plasmid, selection for a plasmid cointegrate, and subsequent counter-selection to achieve a second recombination and loss of the plasmid. We describe here a new system for targeted mutagenesis in F. tularensis that utilizes a retargeted group II intron for gene inactivation.

The targetron system has been optimized for use in F. tularensis, and we have demonstrated that it is functional in three different subspecies: F. tularensis subsp. tularensis, F. tularensis subsp. holarctica, and F. tularensis subsp. novicida. One of the advantages of group II introns is that, once expressed, the RNP is relatively host factor independent (9). Additionally, high levels of DNA transformation are not necessary, as they are for efficient homologous recombination procedures, because a single targetron plasmid can express multiple copies of the group II intron. Thus, the critical adaptation to making this system functional in F. tularensis was to ensure the expression of the targetron in F. tularensis. Importantly, the retargeted introns show high specificity for their target sequences and are unmarked (i.e., do not contain any antibiotic resistance gene), allowing for the constructed F. tularensis strains to be further genetically manipulated. The targetrons insert at high efficiency, so very little screening was required to identify the unmarked insertion mutation in iglC. The efficiency of insertion, at least with the blaB targetron, was equivalent for the
more virulent *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* strains and the less virulent *F. tularensis* subsp. *novicida* strain. To our knowledge, this is the first genetic technique developed for *F. tularensis* that shows this pattern, since previous targeted knockout techniques have all shown lower efficiency in the more virulent subspecies.

We chose at least two different target sequences within each gene inactivated which were identified by the Targetrion computer algorithm (Sigma-Aldrich). This algorithm evaluates potential insertion sites within a given gene based on recognition preferences of the group II RNP complex, and it provides scores and e values that are based on known group II insertion sites. For the two genes we targeted in this report, we were able to create a targetron vector that inserted at relatively high efficiency. However, we also created targetron vectors for each gene that inserted at lower efficiencies. A comparison of the successful target sites allowed us to refine target site selection within *F. tularensis* (Fig. 1C). Targeted potential regions that consisted of a T, G, A, and T at positions −23, −21, −20, and +5, respectively, were successfully mutated. Additionally, the targetron can insert in a sense or antisense orientation relative to the coding sequence, and our successful insertions were all antisense. Using these general rules, we have also successfully utilized targetrons to inactivate the *pilE2* and *uovA* genes in LVS and the *mglA* gene in Schu4 (data not shown).

One of the greatest potential advantages of the group II introns is their ability to inactivate all target sites within their host. This capacity typically would have little relevance in haploid bacteria, but in the more virulent *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* strains, the FPI is found in two identical copies within the genome. Traditional techniques of gene inactivation targeting the duplicated FPI genes would be cumbersome and time-consuming. We demonstrate here that the targetron targeted to the FPI *iglC* gene inserted in both copies of this gene simultaneously in *F. tularensis* subsp. *holarctica*, resulting in an *iglC1 iglC2* mutant in a single step. The targetron system will be helpful in constructing additional mutations in the duplicated FPI genes to elucidate the function of these critical virulence factors.

The potential drawback of group II intron insertion, as with most insertional mutagenesis techniques (e.g., transposons), is the possibility of polar effects on downstream gene expression within operons. The inserted group II intron sequence encodes the possibility of polar effects on downstream gene expression by insertion mutations. The polar nature of targetron insertions could be used to advantage in knocking out the expression of an entire operon by a single insertion in the first gene of the operon. Additionally, newer-generation targetrons that contain various inserted elements within the group II intron have been developed; it should be possible to insert an *F. tularensis* promoter into the intron sequence to enhance expression of downstream genes. Finally, insertions in monocistronic operons or in the terminal genes within operons will not be affected by polarity issues. The relative ease and efficiency of targetron insertions in three different *F. tularensis* subspecies should make this a valuable new tool for targeted mutagenesis of *F. tularensis*.

**ACKNOWLEDGMENTS**

This study was supported by NIH grant PO1 AI57986 to K.E.K. and NIH grant GM060655 to S.A.R.

**REFERENCES**

1. Anthony, L. S., D. R. Burke, and F. E. NANO. 1991. Growth of *Francisella* spp. in rodent macrophages. Infect. Immun. 59:3291–3296.
2. Baron, G. S., S. V. Myltseva, and F. E. NANO. 1995. Electrophoresis of *Francisella tularensis* subsp. *holarctica* and *tularensis* subsp. *novicida*. Appl. Environ. Microbiol. 61:140–145.
3. Bhatnagar, N., E. Getachew, S. Straley, J. Williams, M. Meltzer, and A. Fortier. 1994. Reduced virulence of rifampicin-resistant mutants of *Francisella tularensis*. J. Infect. Dis. 170:841–847.
4. Bina, X. R., C. Wang, M. A. Miller, and J. E. Bina. 2006. The Bla 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. 186:219–226.
5. Eigelsbach, H. T., and C. M. Downs. 2001. Group II introns as controllable gene targeting vectors for genetic manipulation of bacteria. Nat. Biotechnol. 19:451–456.
6. Fortier, A. H., S. J. Green, T. Polsinelli, T. R. Jones, R. M. Crawford, D. A. Leibler, L. E. Elkins, M. S. Meltzer, and C. A. Nacy. 1994. Life and death in an intracellular pathogen: *Francisella tularensis* and determination of the T-cell response to the proteins in individuals vaccinated with *F. tularensis*. Infect. Immun. 62:1824–1829.
7. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166:577–580.
8. Ho, S. N., B. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 75:51–59.
9. Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 75:53–56.
10. Karberg, M., H. Guo, J. Zhong, R. Coo, J. Perutka, and A. M. Lambowitz. 2003. Group II introns as controllable gene targeting vectors for genetic manipulation of bacteria. Nat. Biotechnol. 19:1162–1167.
11. Kuoppa, K., A. Forsberg, and A. Norqvist. 2001. Construction of a reporter plasmid for screening in vivo promoter activity in *Francisella tularensis*. FEBS Microbiol. Lett. 205:77–81.
12. Larsson, P., P. C. Oyston, P. Chain, M. M. Chu, M. Duffield, H. H. Fuxelius,
E. Garcia, G. Halltorp, D. Johansson, K. E. Isherwood, P. D. Karp, E. Larson, Y. Liu, S. Michell, J. Prior, R. Prior, S. Malaffi, A. Sjostedt, K. Svensson, T. Vanek, L. Vergez, J. K. Wagg, B. W. Wren, L. E. Lindler, S. G. Anderson, M. Forsman, and R. W. Titball. 2005. The complete genome sequence of Francisella tularensis, the causative agent of tularemia. Nat. Genet. 37:153–159.

23. Laurianno, C. M., J. R. Barker, F. E. Nano, B. P. Arulanandam, and K. E. Kloos. 2005. Alelic exchange in Francisella tularensis using PCR products. FEMS Microbiol. Lett. 229:195–202.

24. Laurianno, C. M., J. R. Barker, S. S. Yoon, F. E. Nano, B. P. Arulanandam, D. J. Hassett, and K. E. Kloos. 2004. MgIA regulates transcription of virulence factors necessary for Francisella tularensis intraepithelial and intracellular survival. Proc. Natl. Acad. Sci. USA 101:8246–8249.

25. Liu, J., X. Zogaj, J. R. Barker, and K. E. Kloos. 2007. Construction of targeted insertion mutations in Francisella tularensis subsp. novicida. BioTechniques 43:87–92.

26. Long, M. B., J. P. Jones III, B. A. Sullenger, and J. Byun. 2003. Ribozyme-mediated revision of RNA and DNA. J. Clin. Invest. 112:312–318.

27. LoVullo, E. D., L. A. Sherrill, L. L. Perez, and M. S. Pavelka, Jr. 2000. Group II introns in the bacterial translation shuttle plasmid. Appl. Environ. Microbiol. 66:167–185.

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

29. Martinez-Abarca, F., and N. Toro. 2006. Fluorescent in situ techniques for highly pathogenic Francisella tularensis subsp. tullarensis. Microbiology 152:3425–3435.

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

31. Moore, C. M., J. R. Barker, S. S. Yoon, F. E. Nano, B. P. Arulanandam, D. J. Hassett, and K. E. Kloos. 2004. MgIA regulates transcription of virulence factors necessary for Francisella tularensis intraepithelial and intracellular survival. Proc. Natl. Acad. Sci. USA 101:8246–8249.

32. Liu, J., X. Zogaj, J. R. Barker, and K. E. Kloos. 2007. Construction of targeted insertion mutations in Francisella tularensis subsp. novicida. BioTechniques 43:87–92.

33. Long, M. B., J. P. Jones III, B. A. Sullenger, and J. Byun. 2003. Ribozyme-mediated revision of RNA and DNA. J. Clin. Invest. 112:312–318.

34. LoVullo, E. D., L. A. Sherrill, L. L. Perez, and M. S. Pavelka, Jr. 2000. Group II introns in the bacterial translation shuttle plasmid. Appl. Environ. Microbiol. 66:167–185.

35. Mohr, G., D. Smith, M. Belfort, and A. M. Lambowitz. 2000. Rules for DNA target-site recognition by a lactococcal group II intron enable retargeting of the intron to specific DNA sequences. Genes Dev. 14:539–573.

36. Nano, F. E., N. Zhang, S. C. Cowley, K. E. Kloos, K. K. Cheung, M. J. Roberts, J. S. Ludu, G. W. Letendre, A. L. Meierovics, G. Stephens, and K. L. Elkins. 2004. A Francisella tularensis pathogenicity island required for intramacrophage growth. J. Bacteriol. 186:6430–6436.

37. Perutka, J., W. Wang, D. Goerlitz, and A. M. Lambowitz. 2004. Use of computer-designed group II introns to disrupt Escherichia coli DHFR-TD box protein and DNA helicase genes. J. Mol. Biol. 336:421–439.

38. Petrosino, J. F., Q. Xiang, S. E. Karpathy, H. Jiang, S. Yerrapragada, Y. Liu, J. Gioia, L. Hemphill, A. Gonzalez, T. M. Raghavan, A. Uzman, G. E. Fox, S. Highlander, M. Reichard, R. J. Morton, K. D. Clinkenbeard, and G. M. Weinstock. 2006. Chromosome rearrangement and diversification of Francisella tularensis revealed by the type B (OSU18) genome sequence. J. Bacteriol. 188:6977–6985.

39. Rawsthorne, H., K. N. Turner, and D. A. Mills. 2006. Multiplicity integration of heterologous genes, using the lactococcal group II intron targeted to bacterial insertion sequences. Appl. Environ. Microbiol. 72:6088–6093.

40. Rohmer, L., C. Fong, S. Abmayr, M. Wassnick, T. J. Larson Freeman, M. Radey, T. Guina, K. Svensson, H. S. Hayden, M. Jacobs, L. A. Gallagher, C. Mansol, R. K. Ernst, B. Dres, D. Buckley, E. Haugen, D. Bovee, Y. Zhou, J. Chang, R. Levy, R. Lim, W. Gillett, D. Guenthener, A. Kang, S. A. Shaffer, G. Taylor, J. Chen, B. Gallis, D. A. D’Argenio, M. Forsman, M. V. Olson, D. R. Goodlett, R. Kaul, S. I. Miller, and M. J. Brittbrunner. 2007. Comparison of Francisella tularensis genomes reveals evolutionary events associated with the emergence of human pathogenic strains. Genome Biol. 8:R102.

41. Santic, M., M. Molmeret, J. R. Barker, K. E. Kloose, A. Dekanic, M. Doric, and Y. Abu Kwaik. 2007. A Francisella tularensis pathogenicity island protein essential for bacterial proliferation within the host cell cytosol. Cell. Microbiol. 9:2391–2403.

42. Santic, M., M. Molmeret, K. E. Kloos, S. Jones, and Y. A. Kwaik. 2005. The Francisella tularensis pathogenicity island protein IgIC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. Cell. Microbiol. 7:960–979.

43. Sadowski, S., and H. N. Carlisle. 1961. Studies with tularemia vaccines in volunteers. IV. Brucella agglutinins in vaccinated and nonvaccinated volunteers challenged with Pasteurella tularensis. Am. J. Med. Sci. 242:166–172.

44. Sjostedt, A. 2003. Virulence determinants and protective antigens of Francisella tularensis. Curr. Opin. Microbiol. 6:66–71.

45. Twine, S., M. Bystrom, W. Chen, M. Forsman, I. Golovliov, A. Johansson, J. Kelly, H. Lindgren, K. Svensson, C. Zingmark, W. Conlan, and A. Sjostedt. 2005. A mutant of Francisella tularensis strain SCHU S4 lacking the ability to express a 56-kilodalton protein is attenuated for virulence and is an effective live vaccine. Infect. Immun. 73:8345–8352.

46. Yao, J., and A. M. Lambowitz. 2007. Gene targeting in gram-negative bacteria by use of a mobile group II intron (“targetron”) expressed from a broad-host-range vector. Appl. Environ. Microbiol. 73:2735–2743.

47. Yao, J., J. Zhong, Y. Fang, E. Geisinger, R. P. Novick, and A. M. Lambowitz. 2006. Use of targetrons to disrupt essential and nonessential genes in Staphylococcus aureus reveals temperature sensitivity of LtrB group II intron splicing. RNA 12:1271–1281.