Transformation and allelic replacement in *Francisella* spp.

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We describe methods for transposon mutagenesis and allelic replacement in the facultative intracellular pathogen *Francisella*. Recombinant clones were constructed by insertion of partially cut *F. tularensis* or *F. novicida* DNA into pUC19 and then mutagenized with a mini-Tn10-Km transposon. *F. novicida* could be transformed with these plasmids either by a chemical transformation method or by electroporation, whereas *F. tularensis* could be transformed only by electroporation. Transformation of *F. tularensis* by electroporation was enhanced in the absence of the capsule. Southern blot analysis showed that the Km^R marker was rescued either by integration of the plasmid into the *Francisella* chromosome or by allelic replacement. Allelic replacement was found to be the mechanism underlying a site-specific mutation affecting FopA, an outer-membrane protein of *Francisella*. *F. novicida* could also be transformed with chromosomal DNA carrying the Km^R marker and the transformation frequency obtained using chromosomal DNA was generally greater than that obtained using plasmid DNA. *F. novicida* was also transformed by an IncQ plasmid containing an *F. novicida* DNA insert, which replicated autonomously in this host.

Introduction

Francisella tularensis, the causative agent of tularemia, is an encapsulated Gram-negative coccobacillus that is a facultative intracellular pathogen (Bell, 1981; Eigelsbach and McGann, 1984). *F. tularensis* infection has been used in several studies as a model of T-cell-mediated immunity (Allen 1961; Anthony & Kongshavn, 1987; Eigelsbach *et al.*, 1975; Kostiala *et al.*, 1975). The bacterium grows inside macrophages, and is eliminated from surviving animals by a mechanism which involves gamma interferon (Anthony *et al.*, 1989) and, presumably, activated macrophages. Although the immunological aspects of infection have received considerable attention, there has been little study of the bacterial factors that contribute to virulence.

Francisella novicida is a species that is closely related to *F. tularensis* (Larson *et al.*, 1955), but until recently it had been isolated only once from the environment. Both species cause identical diseases in experimental animals. *F. novicida* differs from *F. tularensis* in its ability to

ferment sucrose, in its expression of agglutinating surface antigen (Owen *et al.*, 1964) (probably capsule) and in its apparent lack of infectivity for humans, as surmised from the lack of infection in laboratory workers over a 30 year period. However, it was found recently that *F. novicida*-like strains have infected a small number of severely immunocompromised individuals (Wenger *et al.*, 1989; Hollis *et al.*, 1989). The geographical distribution of the infected patients indicates that *F. novicida*-like strains are widely distributed in nature.

In order to facilitate a genetic approach to the study of virulence in *Francisella* we analysed methods for introducing exogenous DNA, especially genetically engineered DNA from *Escherichia coli*, into *Francisella*. Tyeryar & Lawton (1969, 1970) showed that *F. novicida* can be transformed with chromosomal DNA from both *F. novicida* and *F. tularensis*. In this study, we describe the parameters necessary for the introduction of exogenous DNA into *F. novicida* and the construction of mutants by insertional inactivation of genes. Moreover, we describe a procedure for the transformation of *F. tularensis*, which requires different conditions from that for *F. novicida*.

This paper is dedicated to the memory of Bengtåke Jaurin.

Table 1. *Bacterial strains and plasmids*

Strain/plasmid	Relevant characteristics	Source/reference
<i>F. tularensis</i> LVS	Live vaccine strain	Eigelsbach & Downs, (1961); Salk Institute
<i>F. tularensis</i> LVSR	Capsule-negative variant of LVS	Sandström <i>et al.</i> (1988); G. Sandström
<i>F. tularensis</i> 504-3	<i>zsa::Tn10-Km</i>	This study
<i>F. novicida</i> U112	Wild-type strain	Larson <i>et al.</i> (1955); ATCC
<i>F. novicida</i> 68-1	<i>zbb::Tn10-Km</i>	This study
<i>F. novicida</i> 68-11	<i>fopA-1::Tn10-Km</i>	
<i>F. novicida</i> 68-17	<i>fopA-2::Tn10-Km</i>	
<i>E. coli</i> DH5 α	F Φ 80 <i>lacZ</i> Δ M15 <i>endA1 recA1 hsdR17 supE44 thi1 gyrA96 relA1</i> Δ (<i>lacZYA-argF</i>)U169	Bethesda Research Laboratories
pUC19	Ap ^R cloning vector	Vieira & Messing (1982)
pRL497	Km ^R , IncQ	Elhai & Wolk (1988)
pDSK519	Km ^R , IncQ	Keen <i>et al.</i> (1988)
pLA68	pUC19:: <i>Sau</i> 3AI (6.5 kb) <i>F. novicida</i> , clone of <i>fopA</i> .	This study
pLA68-1	pLA68::Tn10-Km	
pLA68-11	pLA68::Tn10-Km	
pLA68-17	pLA68::Tn10-Km	
pFEN504	pUC19:: <i>Sau</i> 3AI (11 kb) <i>F. tularensis</i> , LVS DNA	
pFEN504-3	pFEN504::Tn10-Km	
pFEN520	pRL497:: <i>F. novicida</i> <i>Sau</i> 3AI (4.9 kb) DNA	

Methods

Bacterial strains. Bacterial strains and plasmids are listed in Table 1. *Francisella* strains were grown routinely in Chamberlain's defined medium (Chamberlain, 1965) or tryptic soy broth containing 0.1% cysteine hydrochloride (TSB-C). Cystine-heart agar (Difco) supplemented with 5% defibrinated horse blood (CHA-H) and an appropriate antibiotic was used as the solid growth medium. Each lot of blood was tested for its ability to support growth of *F. tularensis*. *E. coli* strains were grown in LB (Sambrook *et al.*, 1989) medium. Sodium ampicillin (250 μ g ml⁻¹) and kanamycin sulphate (Km, 30 μ g ml⁻¹) were used for selection of the appropriate plasmids and transposons in *E. coli*. Biosafety procedures followed the guidelines of the two different host institutions; in general, biosafety level II containment and procedures were used.

DNA methylation analysis. DNA methylation studies were done by the Cancer Research Center (Columbia, MO, USA) using reverse phase HPLC. This analysis revealed no methylated deoxyribonucleosides, and integration of the nucleoside peaks showed that the G+C mol% of the *F. tularensis* DNA was 33%.

Western immunoblotting procedure. SDS-PAGE was done by the method of Dreyfuss *et al.* (1984). Proteins separated by SDS-PAGE were transferred to Nitroscreen West (Dupont) membrane using a discontinuous buffer system with a semi-dry immunoblotting apparatus (LKB) according to the manufacturer's instructions. The anti-*F. novicida* serum used to develop the immunoblots was raised in New Zealand White rabbits as described previously for *F. tularensis* (Nano, 1988). Blots were treated with ¹²⁵I-labelled protein A (Dupont) for autoradiography.

Recombinant DNA procedures. Standard recombinant techniques (Sambrook *et al.*, 1989) were used throughout. Clone banks were generated by ligating *F. novicida* chromosomal DNA, partially cut with *Sau*3AI, into the *Bam*HI site of cloning vectors. Transposon mutagenesis was done using λ 1105 to deliver a mini-Tn10-Km element (Way *et al.*, 1984) into *E. coli* strains harbouring recombinant plasmids. Standard Southern blotting techniques (Sambrook *et al.*, 1989) were

used to transfer DNA onto GeneScreen Plus (Dupont). DNA was labelled by random priming (Dupont kit) and separated from unincorporated ³²P-labelled deoxynucleotides on NENSORB columns (Dupont).

Transformation protocol. *F. novicida* were grown in Chamberlain's medium to a density of 1–3 $\times 10^9$ c.f.u. ml⁻¹. Cells were harvested from a 10 ml culture by centrifugation at 12000 *g* and resuspended in 1 ml of transformation buffer (Tyeryar & Lawton, 1970) modified to exclude Mg²⁺ and Mn²⁺ salts; 300 μ l of this concentrated cell suspension was added to a 1.5 $\times$ 15 cm glass culture tube along with 5 μ l of DNA. Plasmid DNA was isolated by gel-filtration using Sephacryl S-1000 (Pharmacia). Chromosomal DNA was isolated as described by Wilson (1987). The mixture was incubated at 37 °C for 1 h with shaking at 100 r.p.m. Chamberlain's medium (2 ml) was added and the shaking frequency increased to 250 r.p.m. After a 2 h expression period, appropriate dilutions were plated on CHA-H containing Km (10 μ g ml⁻¹) and incubated at 37 °C for 24–48 h.

Electroporation protocol. *F. novicida* and *F. tularensis* LVS were grown in TSB-C. Cells from 40 ml of early to mid-exponential phase cultures were centrifuged at 12000 *g* for 10 min and resuspended in 10 ml 500 mM-sucrose. The cells were washed again and resuspended in 1 ml of sucrose in a microfuge tube. After a 4 min centrifugation in a microfuge the cells were resuspended in 120 μ l of sucrose and kept on ice; 40 μ l of this cell suspension were then added to an electroporation cuvette (0.1 cm gap) and 2 μ l of DNA solution was added immediately before electroporation. Electroporation was done using a Gene Pulser apparatus (Bio-Rad) set at various voltages, with a 25 μ F capacitance setting. A pulse control unit was used with the Gene Pulser with the resistance level set at 400 Ω . The average time constants for the 7.5, 15 and 25 kV pulses were 7.5 ms, 5.9 ms and 0.8 ms, respectively. In some experiments, *F. tularensis* was treated prior to washing in sucrose with the detergent *N*-hexadecyl-*N,N*-dimethyl-3-ammonio-1-propane-sulphonate (HDAP), which was added directly to the *Francisella* culture to a final concentration of 2%. Within 90 s of electroporation 1 ml of TSB-C was added and the cells were transferred to 1.5 $\times$ 15 cm glass culture tubes. The cultures were shaken (250 r.p.m.) at 37 °C. *F. novicida* was grown for 2 h before plating on CHA-H containing Km

(10 µg ml⁻¹), while *F. tularensis* was grown for 6 h before plating on CHA-H containing Km (5 µg ml⁻¹). Under these growth conditions, the generation time for *F. novicida* was approximately 1 h while that of *F. tularensis* was approximately 3 h as determined by measuring turbidity with a Klett-Summerson colorimeter. Since the pulses frequently generated sparks, all electroporation experiments were done in type II biosafety cabinets.

Results and Discussion

Lack of restriction barrier

Analysis of *F. tularensis* LVS DNA indicated that it was not methylated. This suggested that there was no conventional DNA restriction/modification system in this strain. Previously published results (Tyeryar & Lawton, 1970) indicated that transformation of *F. novicida* is equally efficient whether the DNA is derived from *F. tularensis* LVS or from *F. novicida*. Moreover, transformation of *F. novicida* with a broad-host-range plasmid (see below) isolated from *F. novicida* or *E. coli* occurred at the same frequency. Taken together, these observations suggest that there is no restriction system in these two strains.

Transformation of *F. novicida* using recombinant plasmids and integrative rescue

Attempts were made to introduce non-*Francisella* DNA into *F. novicida* using autonomously replicating broad-host-range plasmids, or by rescue of antibiotic resistance markers by transposition. These attempts failed to yield any colonies (data not shown). Since transformation of *F. novicida* using its own chromosomal DNA was easily demonstrated, it was hypothesized that *Francisella* sequences are necessary for transformation. In order to include *F. novicida* sequences in the transforming DNA we constructed recombinants consisting of *F. novicida* DNA partially cut with *Sau3AI* inserted into the *Bam*HI site of pUC19. We then used λ1105 to transpose mini-Tn10-Km into the plasmid insert to provide a selectable marker.

One recombinant clone, pLA68, was chosen for detailed analysis (Figs 1 and 2a). This clone encodes a protein analogous to the FopA protein of *F. tularensis*, which is an outer-membrane protein and a major immunogen (Bevanger *et al.*, 1988; Nano, 1988). FopA migrates as a 43 kDa doublet on PAGE when heated to 95 °C or as a 34 kDa single band when solubilized at lower temperatures. Plasmid pLA68 was mutagenized by insertion of mini-Tn10-Km, and Km-resistant clones were examined for expression of FopA. Western blot analysis revealed that *E. coli* harbouring pLA68-1 retained expression of FopA, whereas *E. coli* harbouring

Fig. 1. Restriction map of pLA68. The solid black line indicates the *F. novicida* DNA insert. The stippled boxes represent the polylinker region of pUC19 (not drawn to scale). Triangles indicate the locations of mini-Tn10-Km insertions in plasmids pLA68-1, pLA68-11 and pLA68-17. The third *Hind*III site from the right may be located 350 bp to the left. The mini-Tn10-Km is 1675 bp and has a *Hind*III site at bp 1014 (from the left). Arrows indicate the direction of transcription of the Km^R gene. Abbreviations for restriction enzymes: H, *Hind*III; K, *Kpn*I; S, *Sst*I; Sp, *Sph*I.

pLA68-11 or pLA68-17 did not (Fig. 2a). Plasmid DNA from the pLA68::Tn10-Km derivatives were used to transform *F. novicida* selecting for Km resistance. Two distinct phenotypes were observed (Fig. 2b). *F. novicida* 68-17, which resulted from transformation with pLA68-17 (no expression of FopA in *E. coli*) did not produce FopA. However, a new immunoreactive peptide of 36 kDa was detected. This could be a truncated derivative of FopA resulting from the insertion of the mini-transposon. Transformation with plasmid pLA68-1 (expression of FopA in *E. coli*) or plasmid pLA68-11 (no expression of FopA in *E. coli*) yielded *F. novicida* strains that still expressed FopA.

The results of Southern blot analysis of genomic DNA of the *F. novicida* transformants provided possible explanations for the Western immunoblot results. Fig. 3(b) (lane 2) shows that when a Southern blot was probed with ³²P-labelled pUC19 DNA, a hybridizing band was found in the *F. novicida* 68-11B DNA. This indicates that pUC19 sequences, together with the transposon and some or all of the *F. novicida* recombinant DNA, integrated into the *F. novicida* chromosome by a Campbell-type recombination event. Such an event should not diminish expression of FopA since an intact copy of the *fopA* gene would be preserved in addition to the transposon-mutagenized copy. In pLA68-11 the mini-Tn10-Km insertion has caused the replacement of a 2.1 kb *Hind*III fragment by two fragments of 2.2 and 1.6 kb (the 1.7 kb mini-Tn10-Km carries a *Hind*III site). The sizes of the two bands containing sequences which hybridize with pNK862 (a pBR322-based plasmid carrying the mini-Tn10-Km element) in *F. novicida* 68-11 chromosomal DNA are the same size as in pLA68-11 plasmid DNA (lanes 2 and 6). This is consistent with the plasmid having integrated into the chromosome. Similarly, the fact that the transposon but not the plasmid sequences of pLA68-17 integrated in *F. novicida* 68-17 (lane 1, Fig. 3b, c) suggests that gene replacement caused loss of expression of FopA. This is consistent with the

Fig. 2. Western immunoblot of *E. coli* recombinants and *F. novicida* mutants. Blots were reacted with anti-*F. novicida* antiserum. (a) Immunoblot of extracts of *E. coli* strains harbouring recombinant plasmids containing *F. novicida* insert DNA, those recombinants mutagenized with mini-Tn10-Km and an *E. coli* control. Lane 1, DH5 α ; lane 2, DH5 α (pLA68); lane 3, DH5 α (pLA68-1); lane 4, DH5 α (pLA68-17); lane 5, DH5 α (pLA68-11). The arrow at the left indicates the mobility of the slowest-migrating FopA band. (b) Immunoblot of extracts of *F. novicida* strains that had been transformed with recombinant plasmids containing transposon inserts. Lane 1, *F. novicida*; lane 2, *F. novicida* 68-1; lane 3, *F. novicida* 68-11B; lane 4, *F. novicida* 68-17; lane 5, *F. novicida* with mini-Tn10-Km inserted in another region of the chromosome. The arrow at the right points to a 36 kDa peptide thought to be a truncated derivative of FopA.

Fig. 3. Southern blot analysis of transformants. Lanes 1, 2 and 3, *F. novicida* strains 68-17, 68-11B and 68-1; lane 4, 1 kb size standard; lane 5, blank; lane 6, plasmid pLA68-11; lane 7, plasmid pLA68. (a) Agarose gel of *Hind*III-cut chromosomal DNA from various transformant strains of *F. novicida* and *Hind*III-cut plasmid DNA. (b) Southern blot of the gel shown in (a) probed with 32 P-labelled pUC19. A 350 bp fragment which includes 34 bp of the pUC19 sequence appears as a weakly hybridizing band at the bottom of the blots in lanes 6 and 7. This fragment is also present in lane 2, but is too weak to be seen in this reproduction. (c) The same Southern blot as in (b), reacted instead with pNK862, a pBR322-derived plasmid that contains the mini-Tn10-Km element. Wild-type *F. novicida* DNA did not react with either probe (data not shown).

Table 2. Transformation of *F. novicida*

The values represent means of three experiments; standard deviations are indicated in parentheses.

Genetic locus	Transformants per µg DNA	
	Plasmid DNA	Chromosomal DNA
68-1	46 (0)	2.1×10^6 (1.2×10^6)
68-11B	6.1×10^5 (2.1×10^5)	2.6×10^6 (1.1×10^6)
68-17	61 (48)	2.2×10^6 (4.1×10^5)

appearance of the 36 kDa immunoreactive peptide in the Western blot. We found no evidence of extrachromosomal replication in *F. novicida* of the pUC19-derived recombinant plasmids.

Factors affecting transformation of *F. novicida*

Wide variations in the frequency of transformation were found when different plasmid constructs were used to transform *F. novicida*. Transformation with plasmids derived from pLA68 by mini-Tn10-Km insertion illustrate this variation (Table 2). One plasmid, pLA68-11, gave high frequency transformation (6×10^5 transformants μg^{-1}) whereas two other plasmids, pLA68-1 and pLA68-17 yielded about 10000-fold fewer transformants. Given the fact that the transposon inserts in pLA68-11 and pLA68-17 are approximately 300 bp apart (Fig. 1), it is particularly surprising that the plasmids transform *F. novicida* at such markedly different frequencies. The location of the transposon seems to be a major determinant in the transformation efficiency.

To examine other factors that might affect transformation efficiency, we isolated chromosomal DNA from the *F. novicida* strains 68-1, 68-11 and 68-17. When these chromosomal DNA preparations were used to transform *F. novicida* each yielded approximately the same number of Km-resistant colonies (Table 2), and the frequency of the transformation per μg DNA was similar to that of plasmid pLA68-11. Actually, the chromosomal DNA gave a much higher transformation frequency than pLA68-11 considering that a much smaller proportion of the chromosomal DNA contains the Km^R locus. Apparently, the length of DNA also influences the efficiency greatly, since transformation using plasmid pLA68-11 DNA that had been linearized by cutting with *Sph*I, which cuts only in the vector DNA, abolished transformation (data not shown). Lastly, chromosomal DNA was found to transform at a higher frequency (approximately 100-fold) when sheared fragments of 20–40 kb

were used, in comparison with high-molecular-mass DNA (data not shown).

Several parameters that might have affected transformation competence were investigated. *F. novicida* transformed equally well after incubation with DNA in the transformation buffer for 5 min (followed by addition of DNAase) or for 1 h. Heat shock, which enhances transformation of *E. coli* (Mandel & Higa, 1970) did not enhance transformation of *F. novicida* (data not shown).

Transformations with broad-host-range plasmids

Attempts to introduce broad-host-range plasmids into *F. novicida* and *F. tularensis* by conjugation or transformation failed. However, when IncQ plasmids containing *Francisella* DNA inserts were used, transformants could be recovered.

Several derivatives of pRL497 or pDSK519 carrying *Sau*3AI fragments from *F. novicida* cloned into the *Bam*HI site were constructed. Nine recombinant plasmids were isolated with the capacity to transform *F. novicida*. The sizes of the insertions in these clones ranged from 0.5–5.0 kb. The transformation frequency obtained using these plasmids was less than 10 transformants per μg DNA. Analysis of genomic DNA of the transformants suggested that the plasmid DNA was extrachromosomal and was present in multiple copies (data not shown). Both *E. coli* and *F. novicida* could be transformed with DNA isolated from *F. novicida* strains carrying the pRL497 recombinants, which supports the notion that the plasmids in *F. novicida* replicated autonomously.

The results reported here concerning transformation of *F. novicida* suggest that *Francisella* DNA is needed for efficient transformation to occur. In this regard, *F. novicida* is similar to *Haemophilis*, *Neisseria* and *Campylobacter* (Goodman & Scocca, 1988; Sisco & Smith, 1979; Wang & Taylor, 1990). In the case of *Haemophilis* and *Neisseria*, specific DNA uptake sequences are known to be required for transformation. However, transformation of *F. novicida* seems to be different in that it requires the presence of cations, whereas transformation of these other bacteria does not. Attempts to demonstrate *Francisella*-specific uptake sequences by competitively inhibiting transformation with *Francisella* DNA were unsuccessful. However, we have not ruled out the role of uptake sequences in a complicated transformation process.

Electroporation of *F. tularensis*

Attempts to transform *F. tularensis* with pUC19 recombinants containing *F. tularensis* DNA and marked with mini-Tn10-Km using the transformation protocol for *F.*

Table 3. Electroporation of *Francisella*

The values represent the results from individual experiments. Besides the variation in field strength all the conditions are as described in Methods. The numbers in parentheses represent the percentage of each culture surviving the electroporation pulse.

Field strength	Transformants per µg DNA*		
	7.5 kV cm ⁻¹	15 kV cm ⁻¹	25 kV cm ⁻¹
<i>F. tularensis</i> LVS	128 20 (82%)	2259 149 (39%)	41 46 (3%)
<i>F. tularensis</i> LVS + HDAP	3451 226 (125%)	2015 82 (16%)	231 5 (106%)
<i>F. tularensis</i> LVSR	431 5 (83%)	6052 303 (57%)	1594 4657 (60%)
<i>F. novicida</i>	<1†	<1†	<1†

* The DNA used to transform *F. novicida* was pLA68-11 and for *F. tularensis* pFEN504-3. In the absence of DNA no kanamycin-resistant *F. tularensis* colonies were found.

† Rare transformants; these transformants were verified by Southern blot analysis.

novicida were unsuccessful. However, both *F. tularensis* and *F. novicida* could be transformed with plasmid DNA by high-field-strength electroporation (Table 3), although transformation of *F. novicida* was inefficient. As with transformation of *F. novicida*, transformation frequency of *F. tularensis* by electroporation was highly dependent upon the plasmid DNA.

The effect of the capsule of *F. tularensis* on electroporation was investigated by removing the capsule by detergent treatment and by using a capsule-deficient strain. The detergent HDAP was found to enhance the electroporation efficiency at the lower electric field strengths without diminishing the viability of *F. tularensis* cultures (Table 3). Furthermore, the capsule-deficient strain of *F. tularensis* gave higher transformation frequencies than the encapsulated strain, except at the lower field strength. These results indicate that the capsule of *F. tularensis* does indeed constitute a significant barrier to the efficiency of transformation by electroporation. There was considerable variation in both the transformation frequency and the survival rate of the cultures undergoing electroporation. We attribute these differences to variations in the conductance of the solutions in the cuvettes resulting from leakage of ions from the very fragile *Francisella* cells.

In this work we have demonstrated that broad-host-range plasmids and chimeric narrow host range plasmids carrying *Francisella* chromosomal DNA fragments and an insert of mini-Tn10-Km can be transformed into *F. novicida* and *F. tularensis*. The transposon mutagenesis scheme described here demonstrates how mutants

lacking specific proteins can be isolated. This should facilitate an evaluation of the role of surface proteins and other potential virulence factors in the pathogenicity of *Francisella* infections. Although the transformation frequency of the broad-host-range plasmid recombinants was low, the frequency was sufficient for the construction of merodiploid strains. Such strains can be used to study gene expression or genetic complementation of genes involved in the control of virulence.

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