Serum-sensitive mutation of *Francisella novicida*: association with an ABC transporter gene

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***Francisella novicida* is a facultative intracellular pathogen that can survive and grow in macrophages by preventing phagolysosomal fusion. In this study *in vitro* cassette mutagenesis was used to generate a library of insertion mutants of *F. novicida*. Two related mutants, KM14 and KM14S, initially identified as defective for growth in macrophages, were found to be sensitive to serum. These mutants were also found to grow approximately 1000-fold less well in the livers and spleens of infected mice. We cloned a genetic locus that was presumably mutagenized in these mutants and found that it included genes that had high similarity in their deduced amino acid sequence to those of *msbA* and *orfE* of *Escherichia coli*. The former is a member of the superfamily of ABC transporter proteins. We named the corresponding genes in *F. novicida*, *valAB*. Integration of a cloned *valAB* locus into the chromosome of KM14S partially restored the serum resistance phenotype found in wild-type *F. novicida*.**

Keywords: *Francisella*, tularaemia, complement, ABC transporter, macrophage

INTRODUCTION

Francisella tularensis is a facultative intracellular bacterial pathogen that causes a granulomatous, febrile illness in a wide range of animals. The pathology of human infection by virulent *F. tularensis* is limited in about two-thirds of the cases to a localized lymphadenopathy; in the remainder of the cases a typhoidal type disease results with pathological lesions similar to those seen in tuberculosis (Dienst, 1963; Evans *et al.*, 1985; Goodpasture & House, 1928). Rodent studies with virulent and attenuated strains of *F. tularensis* suggest that the course of disease is dependent on the route of infection, which in turn may determine the type and rapidity of the immune response (Downs *et al.*, 1947; Fortier *et al.*, 1991). Animal studies and autopsy of human cases also show that *F. tularensis* grows in several organs in the body, especially the liver, spleen and, in typhoidal cases, the lungs. The normal course of disease in humans, without antibiotic treatment, runs approximately 32 d, with no long-term sequelae evident (Dienst, 1963).

In experimentally infected animals, *F. tularensis* is found both inside macrophages and extracellularly (White *et al.*, 1964). Unlike macrophages, polymorphonuclear leucocytes (PMNs) are unable to phagocytose *F. tularensis* in the absence of antiserum (Proctor *et al.*, 1975). *Francisella* can grow *in vitro* in rodent resident peritoneal, thioglycollate-elicited, or bone-marrow-derived macrophages (Anthony *et al.*, 1991a). There is some evidence that, *in situ*, *F. tularensis* can also invade non-professional phagocytic cells such as hepatocytes (Conlan & North, 1992). The growth of *Francisella* inside macrophages is similar to that of *Mycobacterium tuberculosis* and *Legionella pneumophila* in that they prevent phagolysosomal fusion (Anthony *et al.*, 1991a).

Consistent with intracellular replication is the observation that cell-mediated immunity is needed to clear *F. tularensis* infections (Anthony & Kongshavn, 1987; Allen, 1961; Eigelsbach *et al.*, 1975; Kostiala *et al.*, 1975; Clafin & Larson, 1972). The cellular response is H-2 restricted (Anthony & Kongshavn, 1988; Surcel *et al.*, 1989), and involves the activation of macrophages via the action of the cytokines interferon- γ and tumour necrosis factor- α (Anthony *et al.*, 1989; Fortier *et al.*, 1992; Leiby *et al.*, 1992). Growth of *F. tularensis* in murine macrophages *in*

The GenBank accession number for the nucleotide sequence reported in this paper is L17003.

Table 1. Bacterial strains, plasmids and bacteriophage

Relevant characteristics		Source/reference
<i>F. novicida</i>		
U112	Wild-type	Larson <i>et al.</i> (1955)
KM14	U112, duplication of <i>valAB</i> region, <i>zaa-1::mTn10Km</i>	This study
KM14S	KM14, <i>valAB</i> duplication resolved, Km ^R	This study
KM21	U112, <i>zbb-1::mTn10Km</i>	This study
KM14S-C1	KM14S, <i>polA::mTn10Km</i>	This study
KM14S-C3	KM14S, <i>polA::mTn10Km</i>	This study
KM14S-C4	KM14S, <i>polA::mTn10Km</i>	This study
GB1	U112, <i>polA::mTn10Km</i>	This study
<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	Hanahan (1983)
MLK53	W3110 (wild-type) <i>htrB1::Tn10</i>	Karow <i>et al.</i> (1991)
Plasmids		
pTZ18U	Ap ^R phagemid	Mead <i>et al.</i> (1986)
pRL498	Km ^R	Elhai & Wolk (1988)
pUC18	Ap ^R	Vieira & Messing (1982)
pNK862	Ap ^R Km ^R , source of Km cassette	Way <i>et al.</i> (1984)
pKEM14	pUC18:: <i>Bcl</i> I insert from KM14 containing the Km cassette and flanking DNA	This study
pKEM14-1	pUC18:: <i>Eco</i> RI– <i>Bam</i> HI fragment (left-hand) from pKEM14	This study
pKEM14-12	pRL498:: <i>Sau</i> 3AI (14 kb) <i>F. novicida</i> DNA	This study
pKEM14-2	pTZ18U:: <i>Bgl</i> II fragment from pKEM14-12	This study
pKEM14-2-3	pTZ18U:: <i>Xba</i> I– <i>Xho</i> I fragment from pKEM14-12	This study
pKEM14-5	pTZ18U::5 kb <i>Sa</i> I fragment from pKEM14-12	This study
pKEM14-8	pTZ18U::8 kb <i>Xba</i> I fragment from pKEM14-12	This study
Bacteriophage		
λ 1105	Replication-defective λ carrying mTn10Km	Way <i>et al.</i> (1984)

vitro is halted by the pre-treatment of macrophages with interferon- γ and lipopolysaccharide (LPS), resulting in the production of nitric oxide (Anthony *et al.*, 1992; Fortier *et al.*, 1992).

Although the immune response to *F. tularensis* infection has been studied considerably, very little is known about the virulence properties of the bacterium. Natural isolates vary in their virulence for different animal hosts. The North American type A strain is highly virulent in primates, rabbits and rodents, whereas the type B strain, found throughout the world, is mildly virulent in primates and rabbits. A capsule that is apparently needed for infectivity and virulence has been found on both a virulent strain (Hood, 1977) and the live vaccine strain (Sandstrom

et al., 1988). The capsule prevents phagocytosis by PMNs and may have a role in protecting against complement (Sandstrom *et al.*, 1988); the role of the smooth-type LPS has not been studied. Eigelsbach *et al.* (1951) described opacity variants of a highly virulent strain that are avirulent in mice; these variants are resistant to serum and still grow in macrophages (L. S. D. Anthony & F. E. Nano, unpublished data).

F. novicida is a close relative of *F. tularensis*, being indistinguishable by 16S RNA sequence or by DNA hybridization. We have chosen to use *F. novicida* as a model of infection since it is virulent for mice (Owen *et al.*, 1964) and grows in macrophages (Anthony *et al.*, 1991a), yet does not have the fastidious growth require-

Fig. 1. (a) Possible genetic events leading to mutants KM14 and KM14S. *In vitro* ligation of Km cassette between *Bam*HI sites located in *polA* gene and *rpoC* genes. Transformation of *F. novicida* probably led to integration by a single cross-over event of ligation products, leading to duplication of a portion of the chromosome. The duplication apparently resolved, leaving a mutation undetectable by Southern blotting. (b) Southern blot of *Bcl*I-digested chromosomal DNA probed with insert of pKEM14-1. Lane 1, KM14S, lane 2, KM14, lane 3, U112. The lower band in lane 2 is approximately 3 kb and co-migrates with a band in KM14 that hybridizes with the Km cassette. The upper band is estimated to be 6 kb.

ments of *F. tularensis*, is not infectious for humans, and is amenable to basic genetic manipulation.

METHODS

Bacteria. Bacterial strains are listed in Table 1. *F. novicida* strains were cultured at 37 °C in tryptic soy broth containing 0.1% (w/v) cysteine hydrochloride (TSB-C), or on cysteine heart agar (Difco) containing 5% (v/v) defibrinated horse blood (CHA-B). Kanamycin sulfate (Km) was added to 15 µg ml⁻¹ as required. *Escherichia coli* strains were grown in LB broth (Sambrook *et al.*, 1989) supplemented with sodium ampicillin (250 µg ml⁻¹) or Km (30 µg ml⁻¹) as required.

Genetic manipulation of *F. novicida*. Random insertional mutagenesis of *F. novicida* was accomplished essentially as described by Sharetsky *et al.* (1991). Briefly, chromosomal DNA from wild-type *F. novicida* was digested to completion by the restriction endonuclease *Pac*I. The DNA was ligated at a concentration of 1 µg ml⁻¹ to favour intramolecular ligation, thus generating circularized DNA. The circularized fragments were partially digested by *Bam*HI, and then ligated to a *Bam*HI fragment containing the kanamycin resistance gene from plasmid pNK862 (Way *et al.*, 1984). The ligation mixture was used to transform *F. novicida* by a modification of a method described previously (Anthony *et al.*, 1991b). Briefly, *F. novicida* was grown in Chamberlain's medium to about 10⁹ cells ml⁻¹, concentrated by centrifugation at 5000 *g*, and resuspended in 0.1 vol. of transformation buffer. Approximately 0.5 µg DNA

was added to 0.1 ml cells, and this mixture was incubated at 37 °C for 1 h with shaking (100 r.p.m.). Chamberlain's medium (2 ml) was added prior to a 2 h expression period in which the cells were incubated with shaking (250 r.p.m.). Kanamycin-resistant clones were selected by plating on CHA-B containing kanamycin sulfate (15 µg ml⁻¹). Plasmid clones were mutagenized with the transposase-less mTn10Km by delivering the transposon with λ1105 as described previously (Berg *et al.*, 1992).

Recombinant techniques and DNA sequencing. Standard recombinant DNA procedures were used (Sambrook *et al.*, 1989). Plasmid pKEM14-12 was isolated from a recombinant clone bank, constructed by ligating DNA partially digested by *Sau*3AI into the *Bam*HI site of pRL498 (Elhai & Wolk, 1988). DNA was sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977), utilizing phagemid pTZ18U (Bio-Rad) clones to provide single- and double-stranded templates. A commercially available T7 DNA polymerase ('Sequenase', US Biochemicals) was used for the sequencing reactions according to the manufacturer's instructions. Radiolabelled [³⁵S]dATPαS (1 Ci mmol⁻¹; 37 GBq mmol⁻¹) was obtained from Dupont. Both strands of the 3 kb fragment that encompasses the *valAB* operon were sequenced in their entirety, using the universal sequencing primer to sequence deletion subclones generated with the cyclone system (IBI), or using custom-designed primers to sequence from internal sites in the fragment.

DNA and deduced amino acid sequence information were analysed using the family of programs included in GeneWorks

(Intelligenetics). BLASTP (Altschul *et al.*, 1990) was used to search for amino acid sequence similarities among six protein databases available on-line through the National Library of Medicine (USA); the SwissProt release 24.0 was included in the databases.

Screening for mutants defective in intracellular growth, and intracellular growth assay. Insertional mutants were screened for their ability to induce a cytopathic effect in macrophage monolayers, using a modification of an intracellular growth assay, which has been described previously (Anthony *et al.*, 1991a). Briefly, macrophages were harvested from mice by peritoneal lavage 3 d after intraperitoneal injection of 4% (w/v) Brewer's thioglycollate medium (Difco), and adhered to flat-bottomed 96-well microtitre plates at a density of about 2×10^5 macrophages per well. *F. novicida* strains were grown in TSB-C and diluted in Dulbecco's Modified Eagle's Medium (DMEM) (ICN Flow) containing 10% (v/v) bovine foetal serum (Hyclone) to a concentration of about $2-5 \times 10^6$ c.f.u. ml⁻¹. The macrophage monolayers were seeded with 150 µl of this bacterial suspension, and the plates were then centrifuged (600 g, 10 min) to accelerate the association between bacteria and macrophages. The cultures were incubated for 1 h at 37 °C in a humidified atmosphere containing 5% (v/v) CO₂, after which the wells were washed three times and replenished with 150 µl DMEM. The plates were incubated and the monolayers examined over a period of 48 h for microscopic evidence of cytopathic effect, i.e. rounded or lysed cells. This screening method was repeated with clones which displayed delayed evidence of cytopathic effect.

To monitor intracellular growth, resident peritoneal, thioglycollate-elicited or bone-marrow-derived macrophages were infected with the *F. novicida* strains as described above. Instead of observing microscopic evidence of cytopathic effect, the macrophage monolayers were lysed at various times following infection with 0.1% sodium deoxycholate dissolved in saline. Results are expressed as the mean ± 1 SD log₁₀ c.f.u. *F. novicida* per macrophage monolayer, based upon triplicate wells.

Serum-sensitivity testing. Bacteria were cultured overnight, washed and resuspended three times in sterile PBS. A portion (100 µl) of this bacterial suspension at a concentration of about 10^6 c.f.u. ml⁻¹ was plated in each well of a 96-well microtitre plate. To this was added 100 µl guinea pig serum (Cedarlane Laboratories), either normal or heat-inactivated (56 °C, 30 min). The concentration of the bacteria was measured in the inoculum ($t = 0$ h) and in the wells after 3 h incubation at 37 °C.

Mice and experimental infections. Female, specific-pathogen-free C57BL/6NCrlBR strain mice were purchased from Charles River Canada. They were housed in barrier-topped cages under conventional conditions and given food and water *ad libitum*. For infection studies, *F. novicida* strains were cultured in TSB-C to a density of about 10^9 c.f.u. ml⁻¹ and stored in aliquots at -80 °C. Prior to infection, an aliquot was thawed at 37 °C and diluted in sterile physiological saline to a concentration of $1-5 \times 10^8$ c.f.u. ml⁻¹. The number of *F. novicida* c.f.u. in the inoculum was verified retrospectively by plating on CHA-B. Mice were inoculated via the lateral tail vein with 0.2 ml of this suspension. At the indicated times following infection, the mice were killed by decapitation, and the livers and spleens dissected and homogenized individually in sterile 0.85% saline. Serial dilutions of organ homogenates were plated on CHA-B and c.f.u. per organ determined after overnight incubation at 37 °C. Results are expressed as the mean ± 1 SD log₁₀ c.f.u. *F. novicida* per organ, based on four mice per group.

RESULTS

Mutagenesis and isolation of mutants

A cassette mutagenesis protocol, designed originally for *Haemophilus influenzae* (Sharetzky *et al.*, 1991), was tested for its usefulness in mutagenizing *F. novicida*. As described in Methods this scheme uses ligation of an antibiotic cassette to DNA prior to transformation; integration of the ligated DNA should result in replacement of a chromosomal allele by a fragment containing the antibiotic cassette. After carrying out this mutagenesis protocol using a kanamycin resistance gene (Km) cassette, we examined 20 independent colonies for insertion of the Km cassette. Southern blot analysis showed that each

Fig. 2. (a) Growth *in vitro* of *F. novicida* mutants in thioglycollate-elicited murine macrophages. Bacteria were enumerated by plating serial dilutions. Results are the mean of three determinations and the error bars indicate ± 1 SD log₁₀. □, Wild-type (U112); ○, KM21; △, KM14S. (b) Growth of *F. novicida* mutants in livers from infected mice. At different times following intravenous inoculation, livers were homogenized and liberated bacteria were enumerated by plating serial dilutions. □, U112 and ○, KM14. Results are the mean of four determinations and the error bars indicate ± 1 SD log₁₀ (error bars shown only when their size exceeds that of the symbol). Similar results were obtained with the spleens from infected mice.

Fig. 3. Restriction map and ORF organization of the *valAB* region. The 14 kb insert in pKEM14-12 was generated by ligating DNA partially digested by *Sau3A*I into pRL498; the area to the left of the double slashed lines contains approximately 4 kb of uncharacterized DNA. All of the other plasmids that are shown are subclones of pKEM14-12. Open triangles indicate Km cassette as they were located in the recombinant clones in *E. coli* prior to transformation of *F. novicida*. The inserts designated KM14S-C1 and GB1 were made in pKEM14-8 and used to generate strains KM14S-C1 (and -C3) and GB1, respectively. The unmarked insertion indicates the location of an insertion in pKEM14-2; this mutagenized derivative of pKEM14-2 was used in attempts at gene replacement. Several *Hind*III sites in the left-most portion of the restriction map were not localized. Letters at the end of pKEM14-12 indicate restriction sites as follows: X, *Xba*I; S, *Sall*; P, *Pst*I; Sp, *Sph*I; H, *Hind*III. The region from the left-most *Xba*I site to approximately the *Kpn*I site was sequenced; the area to the right of *valB* was not completed and several small errors remain. However, several ORFs of 200–400 bp had deduced amino acid sequences with approximately 50% identity with DNA polymerase I of *E. coli*. These ORFs were arranged left to right to align N-terminus to C-terminus with DNA polymerase I. The hatched arrow indicates the probable location of the putative DNA polymerase I gene.

clone contained one cassette insertion, and that the different insertions were located on separate restriction fragments (data not shown).

After we had determined that this procedure resulted in random insertion of the antibiotic cassette, a pilot study to assess whether the protocol could generate a variety of avirulent mutants was carried out. However, since we judged that this mutagenesis scheme was generating less than 500 random mutants, we screened only 1000 mutants in the pilot study. These mutants were inoculated into microtitre wells containing thioglycollate-elicited murine macrophages, and the wells were monitored microscopically for the destruction of the macrophage monolayers as a result of *F. novicida* intracellular growth. Approximately 100 isolates showing delayed cytopathic effects on the macrophage monolayers were found, and these mutants were subjected to the same analysis a second time in duplicate experiments. Growth of those *F. novicida* strains that appeared to destroy the macrophage monolayer more slowly was then measured by viable counting. This revealed 12 mutants of *F. novicida* that appeared to grow more slowly in macrophages than did the parent strain. The growth rates of these 12 mutants were measured in bacteriological medium, and all but three were found to grow more slowly than the wild-type (data not shown). We studied only mutants which had growth defects specifically for macrophages. One of these, KM14, is described here.

Phenotypic characterization of KM14 and KM14S

The original mutant, KM14, was first analysed for the stability of its phenotype, particularly since we had

evidence from the Southern blots that DNA had been duplicated (Fig. 1b). We found that the kanamycin-resistance phenotype was quickly lost, and we named a KM-sensitive variant KM14S. Its Southern blot profile showed one band hybridizing with pKEM14-1, indicating that the presumed duplication present in KM14 had been resolved (Fig. 1b).

KM14 and KM14S were analysed for a variety of phenotypes, including virulence in mice, growth in macrophages, and deoxycholate- and serum-sensitivity. The parent, wild-type U112 and a strain with a random, stable Km cassette (KM21) were used as controls. The growth of KM14 and KM14S in thioglycollate-elicited, resident peritoneal or bone-marrow-derived macrophages yielded viable counts after 48 h that were approximately 25-fold lower than wild-type or KM21 (Fig. 2a, and data not shown). KM14S grew similarly to U112 over the first 10 h but then stopped growing. When treated with 50% serum, the plating efficiency of strains KM14 and KM14S was about 100-fold less than the control strain (data not shown). The mutant strains were essentially identical to the wild-type strain in their sensitivity to deoxycholate (data not shown).

As an indicator of virulence in the whole animal, we tested the growth of KM14 in the liver (Fig. 2b) and spleen (data not shown) from infected animals. We found that KM14 grew 1000–10000-fold less well than the wild-type. In separate experiments, we demonstrated that KM14S grew identically to KM14 and that KM21 grew slightly more slowly than the wild-type, but had 1000–10000-fold more growth than KM14 (data not shown).

1	TCTAGAGTTATATATTTTGAATAAACTTCAAGCCATGATAGCCATTGCGAGTTTTC	60	2161	AGGCTCTATCAATCGGGACTTCAATAGTACATATGCTAGATAAGATTGGTACAGATCAA	2220
	GluLeuThrIleTryLysSerPheAsnValGluPheGlyHisTyrGlyAsnArgThrLys			GlySerIleAsnArgAspPheAsnSerThrTyrAlaArgEnd	
61	TTTTCTGATCTTGTGTAAGCATACTCTTGTGGTGGTGGGTAAGAACTGGGAAG	120		*** * valB→ MetLeuAspLysIleTryTryArgSerLys	
	LysGluSerArgAlaLeuAlaTryGluGlnLysThrGlnProHisIlePheValProPhe				
121	TTGGCTGCTATTTTATAAATCCATTTCGTAGCATCTGCTTCAGTTGCACCAACAAC	180	2221	AACCAAACTGGCTTAGTCGGGTGCTACAACCAATATCTTGGTTTTATAGATATTGCAA	2280
	AsnAlaAlaIleLysIlePheGlyAsnAlaLeuMetGluAspGluThrAlaGlyValVal			ProAsnLeuLeuSerArgValLeuGlyProIleSerLeuValPheIleAspIleAlaAsn	
181	ACCCAACTCCTGTCTTATAGTGTATGCCTAATAACATATCTCTAACGGCCGCCCAACT	240	2281	ATAAAGCTAAAAATAAACAGCAACTCAAGCAATATAAATCAAAAATTCCTATAATAGTTG	2340
	ValTrpAspLysAspLysPheThrIleGlyLeuLeuMetAspArgValAlaGlyGlyVal			LysArgLysIleLysGlnGlnLeuLysGlnTyrLysSerLysIleProIleIleValVal	
241	AAGTAAATTTTACCTGCTTAAATAGTTAAACATCTCTTTGATTACTTTATGTTAGCAGA	300	2341	TTGGCAATATCTCTGTTGGCGGTACTGGCAAACTCCAGTTGTTAGAATGTTGGCTCAGC	2400
	LeuTyrPheLysMet ← cca * * * -10			GlyAsnIleSerValGlyGlyThrGlyLysThrProValValArgMetLeuAlaGlnGln	
301	AAAAATGTGTATAATTTATTGTTATCTGTCACCTTTTAAACAACCTTTATAAATTTT	360	2401	AATATTAGCACAAAGTAAAAAACAGCTATAATAGTCGTGGATATGGTGCAAGGCTG	2460
	-35 -35 -10			TyrLeuAlaGlnGlyLysLysProAlaIleIleSerArgGlyTryGlyAlaLysAlaAsp	
361	TGGCTAATATGATTGATAAAATAGATCTTAAGTCTCAGGATCTAGTAATCTAAGTGGAG	420	2461	ATAATTATCTCTTTGAAGTAAACAGTGGTACTCTAGCAACTCAATGGCGGATGAGCCTG	2520
	-10 *** * valA→			AsnTyrProPheGluValThrSerGlyThrLeuAlaThrGlnCysGlySerAspGluProAla	
421	AAATGACAAACCATCAGAAAGTAGGCACCTTTATAAAGGTTGTTACTCCAAGTTAAGC	480	2521	CGATGTTATTGATGCTTTGCAAGCACAGGTTCTATTGTTATTGCTCCAGAGAGAGTTC	2580
	*** * valA→			MetLeuPheAspAlaLeuGlnAlaGlnValProIleValIleAlaProGluArgValGln	
481	ATTTATGGCATTCTCTGTTTGGCTGCTATCGGAAGTATATTTTCTCAGCGCTGATGC	540	2581	AGGCTGTTAAATACATTGAAAGAAATTTCTGATACAGATATAATTATATCTGATGATG	2640
	MetAlaPheSerAlaPheGlyCysTyrArgLysTyrIlePheLeuSerAlaAspAla			AsnTyrProPheGluValThrSerGlyThrLeuAlaThrGlnCysGlySerAspGluProAla	
541	CTCAATGATATATCTGATTAAATCCGATTTTGAATTTATGGTTTGGTCTGGTGGCGGAT	600	2641	GCTTGCAACATTATAAATTAGCTCGAGATAAGGAAATAGTGGTCTGATGCTATTAGAA	2700
	SerMetIleTyrLeuIleAsnProIleLeuAsnTyrGlyPheGlyProGlyGlyLysIle			LeuGlnHisTryLysLeuAlaArgAspLysGluIleValValValAspAlaIleArgMet	
601	TACTAAACAAAGCGCTACTATACTAATGCTTATGGTGTGGCATGGTGGTTTCATAGC	660	2701	TGTTTGGCAATAAATATGTTTGGCTGCTGCTGCTGATGAGAGAACCGATGAGAGATTA	2760
	ThrLysGlnSerAlaThrIleLeuMetLeuMetGlyValGlyMetValGlyPheIleAla			PheGlyAsnLysLeuCysLeuProAlaGlyProLeuArgGluProIleGluArgLeuLys	
661	GTAAGATCAGTAGGTTTCATTTGTCTCAGACTCTTATAGGTTCTTATAGTCAAAAGT	720	2761	AAGAAGTAGATCAAAATATAGTTATAGTTGCTCAGATAAAGATAAAGAGTTACTCA	2820
	LeuArgSerValGlySerPheValSerGlnTyrPheIleGlySerLeuGlyGlnLysVal			GluValAspGlnIleIleValIleValIleGlyAsnHisGlnAsnValIleAlaIleAlaGlyLys	
721	TGTTTATAAATTTAGGAAGATATTTATAAAGGTTAATGGATCTACCACTAGTTTCTT	780	2821	AAAACATAAAATATGACTTATGCAAAAGTCGTAGTACTGAAATTTGTTAATATATTA	2880
	ValTyrLysPheArgLysAspIleTyrLysArgLeuMetAspLeuProAlaSerPhePhe			AsnTyrLysAsnValThrTyrAlaLysValValAlaThrGluPheValAsnIleLeuThr	
781	TGATAAGCACTCAACAGCAGATATCTCAAGATATTATACAATGTTGATCAGGTTAC	840	2881	CAGCTAAAAAGTAGCTAAGACTGAATTTAATCATCAAAATGTAATAGCTATAGCAGGA	2940
	AspLysHisSerThrGlyGlnIleIleSerArgLeuLeuTyrAsnValAspGlnValThr			AlaLysValAlaLysThrGluPheAsnHisGlnAsnValIleAlaIleAlaGlyLys	
841	AGAGGCTACTCTACAGCAATPATAAGTTGTCAGGATGGAATCTTTGTTATGGGCT	900	2941	TTGGCAATCCAAACAAATTTTAAAGCTTTAGAGAGAGTGCTATAACATAACAGCTA	3000
	GluAlaThrSerThrAlaIleIleThrValValGlnAspGlyThrPheValIleGlyLeu			GlyAsnProThrLysPhePheLysThrLeuGluGluSerAlaIleAsnIleThrAlaLys	
901	AATGTTGTAAATGTTGTCTCAAGCTGGCAATGTCATATTTTAAATAGTTGTCGGACC	960	3001	AAAAAGTTTTTAAAGATCACCATAAGTTTACTCAGAGTGATTTGAGGGTATAGATAGT	3060
	IleValValMetPheValSerSerTrpGlnLeuSerLeuPheLeuIleValValGlyPro			LysValPheLysAspHisHisLysPheThrLeuSerAspPheGluGlyIleAspSerAsp	
961	ATTTCTGGGATTTTATATGATCATTAACAAAAATTTAGGAATTTAAGTAGAATAAC	1020	3061	ACATAACTGTAGTGATGACATATAAAGATGCTATTAAATGCAAAATTTTCGTAAGCTA	3120
	PheLeuGlyLeuPheIleSerIleIleAsnLysLysPheArgAsnLeuSerArgAsnThr			IleThrValValMetThrTryLysAspAlaIleLysCysLysAsnPheAlaLysAlaAsn	
1021	TCAGTCATCAATGGGTAATGTTACACATACTGCTGAAGAGACGATTAGAACTATAAGA	1080	3121	ATTGGTGGTATCTGGATATAGCTTTAGATATCAATGTTTAAAAATCTCAAAAACAACAT	3180
	GlnSerSerMetGlyAsnValThrHisThrAlaGluGleThrIleArgAsnTyrLysGlu			TrpTrpTryLeuAspIleAlaLeuAspIleAsnValEnd	
1081	AATAAGAAATTTTGGTGGCACAACAAAAACAATAAATCTTTAAAAATCTTGATTA	1140	3181	GTTACACAAATGAATTTGCTATATAAGTATATATATCTATTAATGATAAAAAA	3240
	IleArgIlePheGlyAlaGlnGlnLysGlnAsnLysPhePheLysAsnLeuAspTyr			-35 -10	
1141	TACATACTCAGCAAAATTAGAAGTAGCATTAGATGCGCTGACATCACCAGTAATACA	1200	3241	TTGCAATAAAATCTATGAAAAA 3263	
	ThrTyrSerGlnGlnIleArgThrIleAlaLeuAspAlaLeuThrSerProValIleGln			* * polA→ MetLysLys	
1201	AATTATAGCTCTTCTGTTTATGCTTTCTCATTTATTTACAATGCTATATTTGGTACTAA	1260			
	IleIleAlaSerLeuValLeuAlaPheSerLeuPheThrIleAlaIlePheGlyThrAsn				
1261	TGATGGTGGGCTTCATCTGTTGTCAGCAGAGGCTCTTGTCTCTCTTCTTGGCAGTGC	1320			
	AspGlyGlyGlySerSerTrpLeuThrAlaGlySerPheAlaSerPhePheAlaAlaAla				
1321	AGCGGCTATTCTAAACCGGTAAGAATCTTACGAAGTAAATGTTGTTATCAAAAGC	1380			
	AlaAlaIleLeuLysProIleLysAsnLeuThrLysValAsnValValIleGlnLysAla				
1381	TGTAGCAGCAACTGAAGATATATTTATCTCTGATTATCCAGCAGAGAAAGAACTGG	1440			
	ValAlaAlaThrGluAlaPheIlePheTyrIleLeuAspTyrProAlaGluLysLeuThrGly				
1441	TAGTAAAGAAATAGCTAAAGTTGATGTAATGTAACATCAAGATCTAAGTTTGGCTT	1500			
	SerLysGluLeuAlaLysValAspGlyAsnValThrIleLysAspLeuSerPheAlaPhe				
1501	TGTTGAACATAAAGTACTTAGTGGCGTAAGTGTGATATCAAGCAGGTCAGACTGTAGC	1560			
	GlyGluHisValLeuSerGlyValSerValAspIleLysAlaGlyGlnThrValAla				
1561	ATTTGTTGGTAAGTCAGGAAGTGTAAACTACTTTGACCACTATTATTCGAGATTTTA	1620			
	PheValGlyLysSerGlySerGlyLysThrThrLeuThrSerIleIleSerArgPheTyr				
1621	CACTCAGCATAAAGGTGAGATCTTCTGATGGAGTTGATACAAGAGAAATTAACTTTGA	1680			
	ThrGlnHisLysGlyGluIleLeuLeuAspGlyValAspThrArgGluLeuThrLeuGlu				
1681	GAATCTAAGGTCGCACCTGTCTATAGTTTACAGAAATGTTCAATTTATGATGATACAGT	1740			
	AsnLeuArgSerHisLeuSerIleValSerGlnAsnValHisLeuPheAspAspThrVal				
1741	TTATAATAATATAGCTTTTGGCCCTTCAAGAGAGGTTCCGAAGATGAAGTAATCGATGC	1800			
	TyrAsnAsnIleAlaPheGlyLeuSerArgGluValSerGluAspGluValIleAspAla				
1801	ACTAAAAAGAGCTAATGCATATGAGTTTGTCCCAAGAAATATCTGATGGTATTCATACCA	1860			
	LeuLysArgAlaAsnAlaTyrGluPheValGlnGluLeuSerAspGlyIleHisThrAsn				
1861	TATAGGCAATAATGGTTCAAGCTATCAGGAGGTCAGCGTCAAAAGATATCAATAGCAAG	1920			
	IleGlyAsnAsnGlySerLysLeuSerGlyGlyGlnArgGlnArgIleSerIleAlaArg				
1921	AGCTTTATTAATAAATGCTCTGTGCTGATATTGATGAGCAACTAGTGCTCTTGATAA	1980			
	AlaLeuLysAsnAlaProValLeuIlePheAspGluAlaThrSerAlaLeuAspAsn				
1981	TGAATCTGAGAGAGTAGTACAGCAAGCTCTGAAAGTTTGAATGATCATGTACTACGAT	2040			
	GluSerGluArgValValGlnGlnAlaLeuGluSerLeuThrGluSerCysThrThrIle				
2041	AGTTATAGCTCATAGGCTTAGTACTGTTGAAATGCTGATAAAATGTCGTGATGGATGG	2100			
	ValIleAlaHisArgLeuSerThrValGluAsnAlaAspLysIleValValMetAspGly				
2101	TGTAAGGTTGTTGAAAGTGGTGAAGCTCAAGAAATGCTAGAGCAGGTTGACCTTTATAC	2160			
	GlyLysValValGluSerGlyLysHisGlnGluLeuLeuGluGlnGlyGlyLeuTyrThr				

Fig. 4. DNA sequence and deduced amino acid sequence of 3263 bp region of pKEM14-12. *valA* and *valB* are arranged in an apparent operon that is transcribed left to right. A gene corresponding to *cca* of *E. coli*, which encodes tRNA nucleotidyltransferase, is transcribed right to left. Presumptive promoter regions are indicated by underlines, and bases capable of serving as part of ribosome binding regions are indicated by asterisks. No apparent rho-independent transcriptional terminator regions are evident.

The *F. novicida* mutants all had identical polypeptide profiles on Coomassie-stained SDS-polyacrylamide gels. Similarly, a silver-staining ladder pattern of proteinase K-treated samples separated by SDS-PAGE, indicative of a smooth-type LPS, was identical in all strains (data not shown).

Isolation of the mutagenized locus

Using the Km cassette as a probe of Southern blots of KM14 DNA, we identified and isolated the putative mutagenized locus in KM14, by ligating sized *Bcl*I-cut KM14 DNA to *Bam*HI-digested pUC18. Sequence analysis of the DNA flanking the cassette revealed a deduced amino acid sequence with similarity to *E. coli* DNA polymerase I (Joyce *et al.*, 1982) on one side, and amino

Fig. 5. Amino acid alignment between ValA and MsbA (a), and between ValB and OrfE (b). Identities are indicated by single-

acid similarity to the *E. coli* RNA polymerase β subunit (Ovchinnikov *et al.*, 1982) on the other side; we later concluded that an artifact of the mutagenesis procedure brought together the two polymerase genes (see Fig. 1a). Using the DNA polymerase gene fragment as a probe, we noted that two bands appeared on the Southern blot of *Bcl*I-digested KM14 DNA, one of about 6 kb that co-migrated with a band in U112 DNA, and one of about 3 kb (Fig. 1b). The 3 kb band co-migrated with a restriction fragment that hybridized with the Km cassette DNA (data not shown); this was the fragment that was cloned to generate pKEM14. Although it was apparent from this and other Southern blots (data not shown) that a portion of the chromosome was duplicated, these data did not easily explain the mutagenic events that resulted in the observed phenotypes. In order to decipher the genetic event and obtain a recombinant clone of the wild-type locus, we screened a clone bank (see Methods) using the DNA polymerase I fragment as a probe. Several clones of approximately 14 kb were isolated; one of these, pKEM14-12, and subclones of its insert DNA were studied in detail (Fig. 3). Part of pKEM14-12 was sequenced (Fig. 4).

DNA sequence analysis of the cloned region

We found four major open reading frames (ORFs, Fig. 4) between the left-most *Xba*I site and the *Bam*HI site of pKEM14-8; the DNA sequence of the 3263 bp region to the right of the left-most *Xba*I site is shown in Fig. 4. An ORF starting at bp 254 is apparently transcribed in a leftward direction, and has a deduced amino acid sequence with 58 % identity to the N-terminal 86 amino acids of the *E. coli* tRNA nucleotidyltransferase (Cudney *et al.*, 1986). Starting at bp 485 and proceeding to bp 3157 are two ORFs that are apparently arranged in an operon. We provisionally named the two genes represented by these two ORFs, *valAB* (for virulence-associated locus). Ninety-nine bp downstream of *valAB*, an ORF starts that has approximately 50 % identity to DNA polymerase I of *E. coli* (Joyce *et al.*, 1982).

The deduced amino acid sequence of *valAB* had significant similarity to the deduced amino acid sequence of two genes, *msbA* and *orfE*, in an operon found in *E. coli* (Karow & Georgopoulos, 1993). Both genes in this operon were shown to be essential for viability of *E. coli*. The putative protein ValA has 572 amino acids (molecular mass of 62555 Da), as compared to MsbA with 582 amino acids (64460 Da). ValA and MsbA are 42 % identical in their amino acid sequences (Fig. 5a) and both have Walker motifs A and B, commonly found in the superfamily of ATP-binding proteins (ABC proteins). Among the group B ABC proteins defined by Pugsley (1992), ValA and MsbA have 72 and 82 % identity with the consensus

letter code between the sequences; aligned amino acids that have a similar contribution to secondary structure are indicated with a '+'. (c) Alignment of ValA and MsbA to consensus Walker motifs A (top of c) and B (bottom of c). The ValA sequences are from residues 355–390 and 461–498.

sequence of Walker motifs A and B, respectively (Fig. 5c). The hydrophobicity plots of both proteins are similar (data not shown); visual inspection reveals the same six membrane-spanning regions in ValA as previously identified for MsbA (Karow & Georgopoulos, 1993).

The putative proteins ValB and OrfE have 39% identity in their amino acid sequences (Fig. 5b), but show no significant similarity to other proteins in any of the protein databanks, as determined by the BLASTP program (Altschul *et al.*, 1990). ValB has 322 amino acids and a molecular mass of 36103 Da as compared to 328 amino acids and molecular mass of 35588 Da for OrfE. The hydrophobicity plots of ValB and OrfE are similar (data not shown); although there are hydrophobic regions, no clear membrane-spanning regions could be found.

Lack of allelic replacement of *valAB*

Our previous experience with different loci (Anthony *et al.*, 1991b; Berg *et al.*, 1992) indicated that, upon transformation with mTn10Km-interrupted loci, Campbell-type double cross-over events occurred readily, resulting in one copy of the mTn10Km in the chromosome with inactivation of the target gene. However, six attempts to inactivate *valAB* through insertional mutagenesis failed; this contrasts with our ability to effect allelic replacement at three other loci with one transformation experiment for each locus, through the analysis of five or fewer colonies. Transposon mutagenesis of the *Bgl*II-*Bgl*II fragment in pKEM14-2 was performed, and allelic replacement with the transposon-mutagenized clones was attempted. Although we obtained kanamycin-resistant transformants of *F. novicida* when we transformed with the mutagenized clones, the resulting transformants had DNA rearrangements that left the wild-type *valAB* intact. Given that *msbA* and *orfE* are essential to the viability of *E. coli*, it is not surprising that we were unable to inactivate *valAB*, which are presumably essential to the viability of *F. novicida*. In contrast to the results with *valAB*, we were able to effect allelic replacement in the putative DNA polymerase I gene. We created Km insertions in the DNA polymerase I gene approximately 300 bp to the left of the *Bam*HI site in pKEM14-8. After transformation of *F. novicida*, we isolated one transformant, GB1, that had a Southern blot profile consistent with gene replacement (data not shown). This strain had no apparent defects for growth in macrophages, nor was it sensitive to serum (data not shown).

Complementation of KM14S and *E. coli* mutants with *valAB*

Recombinant clones of *msbA* were originally identified by their ability to suppress a lesion in *btrB*; therefore we tested a clone containing *valA* for its ability to complement *E. coli btrB* mutants. The *E. coli btrB* mutant strain MLK53 is unable to grow at 42 °C; however, when this strain harbours a recombinant plasmid encoding either the *E. coli msbA* locus or *valA* locus (pKEM14-2.3), it can grow at 42 °C (data not shown). Similarly, clones of

valAB were able to rescue strains of *E. coli* that were transduced with DNA containing null mutations in *msbA* (Karow & Georgopoulos, 1993) (data not shown). Since *valA* can substitute for *msbA* for survival and in suppressing the *btrB* mutant, it is likely that *valA* is functionally analogous to *msbA*.

Several attempts were made to complement the defects in KM14S by introducing wild-type alleles *in trans* on an IncQ plasmid. However, a broad-host-range plasmid known to replicate in *F. novicida* was not able to transform *F. novicida* when it carried *valAB*, or suffered deletions in *valAB* upon transformation of *F. novicida* (data not shown). However, we were able to introduce a wild-type allele of the *valAB* region *in cis*, taking advantage of the kanamycin-sensitivity of KM14S. A Km insert was placed by transposition into pKEM14-8 0.9 kb from the right-most *Xba*I site, and the DNA was used to transform KM14S to kanamycin-resistance. Our goal was to find strains that had integrated a functional *valAB* locus either by a single or a double cross-over event; the Km-tagged locus was to serve as a co-transforming marker. Of four rare transformants, three were found to have increased serum-resistance relative to KM14S, but were less resistant than the wild-type. The percentage survival (mean $\pm$ SD) in serum after 3 h at 37 °C was 0.1 ± 0.034 , 1.0 ± 0.2 , 2.4 ± 1.2 , 15.8 ± 11.0 and $36.0 \pm 5.6\%$ for KM14S, KM14S-C4, KM14S-C3, KM14S-C1 and U112 (wild-type), respectively. Although KM14S-C1 was found to be about 100-fold more resistant to serum than KM14S, it failed to grow in macrophages. Southern blot analysis

Fig. 6. Southern blot analysis of transformants of KM14S. *Xba*I-cut chromosomal DNA was separated by gel electrophoresis and blotted onto a membrane prior to hybridization. Probes: (a) pKEM14-8; (b) pTZ18U; (c) Km cassette. Lanes: 1, KM14S-C3; 2, KM14S-C1; 3, KM14S; 4, wild-type (U112). Arrow 1, a restriction fragment that is apparently the 8 kb *Xba*I fragment that contains *valAB* and *polA*, and has acquired a Km cassette (1.6 kb). The Km cassette does not have an internal *Xba*I site. In KM14S-C2 this fragment is the only one that hybridizes with the pKEM14-8 insert, and presumably there has been a simple gene replacement event in the *polA* region in this strain. Arrow 2, restriction fragments that co-migrate with the one present in wild-type DNA and present in KM14S. Presumably this fragment in KM14S-C1 and -C3 is identical to the one in their parent strain, KM14S. Arrow 3, the 2.8 kb pTZ18U fragment. The appearance of these fragments in (a) is due to contaminating pTZ18U in the pKEM14-8 probe. The hybridization pattern of KM14S-C3 DNA is consistent with a single cross-over integration of pKEM14-8::mTn10Km. KM14S-C1 has a similar pattern with additional duplication of part or all of the 8 kb *Xba*I fragment and pTZ18U.

showed that KM14S-C1 and KM14S-C3 had incorporated the cloning plasmid and that these two strains had duplications of at least part of the *valAB* region (Fig. 6). Thus, it appears that a functional copy of *valAB* is able to substantially reverse the serum-sensitivity of KM14S. Since functional ABC transporter proteins are thought to be dimers, it may be that the partial complementation seen in KM14S-C3 is due to dimers being formed between functional and non-functional molecules of ValA.

DISCUSSION

In this work we generated mutants of *F. novicida* that were defective for growth in macrophages and sensitive to serum. Repeated attempts to generate allelic replacements in the *valAB* region have been unsuccessful, which is understandable given that the *E. coli* homologue has been shown to be essential for cell viability. The inability to effect gene replacement in *valAB* limits our ability to unequivocally attribute a particular mutation with the phenotypes of KM14 and KM14S. However, our success in partially reversing the serum-sensitive phenotype of KM14S, by transformation with pKEM14-8::mTn70Km, provides evidence that lesions in *valAB* contributed to the serum-sensitive phenotype, since the only functional *F. novicida* genes that were introduced to generate KM14S-C1 were *valAB*. Moreover, Southern blot evidence suggests that the only events that occurred in generating KM14S were the integration and resolution of DNA in the *valAB* region; no transpositional events or DNA rearrangements were detected. Despite these successes, further work is needed to address questions relating to the mutagenic events that resulted in the mutant phenotypes, especially the possibility that another mutation outside *valAB* is responsible for poor growth of KM14 and KM14S in macrophages.

We hypothesize that ValAB is responsible for secretion of LPS or another non-proteinaceous component to the outer membrane, since the *F. novicida* mutants have phenotypes consistent with compromised outer membranes. This hypothesis explains the sensitivity of the mutants to serum, but does not directly explain the lack of growth in macrophages. It may be that a reduction in the LPS content of the outer membrane, by as little as 10%, has profound effects on the outer membrane, affecting both serum-sensitivity and growth in macrophages. A role for the related gene, *htrB*, in LPS biosynthesis or export has recently been proposed (Schnaitman & Klena, 1993); as described here, *valAB* suppresses mutant alleles of *htrB*.

The apparent operon organization of *valAB* is not unexpected given the organization of *msbA* and *orfE*. In contrast to the conserved operon organization of *valAB* with respect to *msbA* and *orfE*, the genome organization is apparently quite different between *F. novicida* and *E. coli*. In *F. novicida*, *valAB*, *polA* and *cca* are all tightly linked on the chromosome, whereas in *E. coli*, *msbA* is located at 20 min, *polA* at 87 min and *cca* at 67 min on the chromosome.

Lastly, the possibility that mutations were induced during the integration of transforming DNA is unusual but not unprecedented. The phenomenon of induction of mutations accompanying transformation and integration of homologous DNA was observed in the cyanobacterium *Anacystis nidulans* (Herdman, 1973). Transformation with linear chromosomal DNA resulted in mutations tightly linked to the selected genetic markers, and occurred at a very high rate.

Although the essential nature of *valAB* and a lack of sophisticated genetic tools has hampered our analysis of KM14 and KM14S, these mutants and the information gathered in this study should aid future studies of *Francisella* intracellular growth. In particular, novel genetic approaches may be used to analyse KM14S to determine if more than one genetic lesion is responsible for both the serum-sensitivity and the macrophage growth phenotypes.

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