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*, tularemia, 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 & Kongsbavn, 1987; Allen, 1961; Eigelsbach *et al.*, 1975; Kostiala *et al.*, 1975; Claflin & Larson, 1972). The cellular response is H-2 restricted (Anthony & Kongsbavn, 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...
Table 1. Bacterial strains, plasmids and bacteriophage

<table>
<thead>
<tr>
<th>Bacterial strains, plasmids and bacteriophage</th>
<th>Relevant characteristics</th>
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<tr>
<td><strong>F. novicida</strong></td>
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<tr>
<td>U112</td>
<td>Wild-type</td>
<td>Larson et al. (1955)</td>
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<tr>
<td>KM14</td>
<td>U112, duplication of valAB region, zaa-1::mTn10Km</td>
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<tr>
<td>KM14S</td>
<td>KM14, valAB duplication resolved, KmR</td>
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<tr>
<td>KM21</td>
<td>U112, zkb-1::mTn10Km</td>
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<td>KM14S-C1</td>
<td>KM14S, polA::mTn10Km</td>
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<tr>
<td>KM14S-C3</td>
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<tr>
<td>GB1</td>
<td>U112, polA::mTn10Km</td>
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<td><strong>E. coli</strong></td>
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<tr>
<td>DH5a</td>
<td>F- Δgal galZ::Km15 endA1 recA1</td>
<td>Hanahan (1983)</td>
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<tr>
<td>MLK53</td>
<td>W3110 (wild-type) btrB1::Tn10</td>
<td>Karow et al. (1991)</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pTZ18U</td>
<td>ApR phagemid</td>
<td>Mead et al. (1986)</td>
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<tr>
<td>pRL498</td>
<td>KmR</td>
<td>Elhai &amp; Wolk (1988)</td>
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<tr>
<td>pNK862</td>
<td>ApR KmR, source of Km cassette</td>
<td>Way et al. (1984)</td>
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<td>pKEM14</td>
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<td>pRL498::SalI (14 kb)</td>
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<td>pKEM14-5</td>
<td>pTZ18U::5 kb SalI fragment from pKEM14-12</td>
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<td><strong>Bacteriophage</strong></td>
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<td>1105</td>
<td>Replication-defective λ carrying mTn10Km</td>
<td>Way et al. (1984)</td>
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_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-
ponents 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 *PstI*. 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 *BamHI*, and then ligated to a *BamHI* 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 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 11 10⁵ 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 *Sau3AI* into the *BamHI* 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 [³²P]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.
(Intelligenees). 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 x 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 x 10^8 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 log₈ 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 10^9 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/6NCrICR 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 x 10^9 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.

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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.

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RESULTS

Mutagenesis and isolation of mutants

A cassette mutagenesis protocol, designed originally for Haemophilus influenzae (Sharetzsky 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
We studied only mutants which had growth defects described here. The growth rates of these 12 mutants were measured in macrophages, and the wells were monitored microscopically for the destruction of the macrophage monolayers as a result of 100 isolates showing delayed cytopathic effects on the resident peritoneal or bone-marrow-derived macrophages. 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 $\text{valB}$ mutants that appeared to destroy the macrophage monolayer more slowly was then measured by viable counting. This revealed 12 mutants of $\text{F. novicida}$ 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).
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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 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-solubilized 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 BclI-cut DNA flanking the cassette revealed a deduced polypeptide capable of serving as part of ribosome binding regions were transcriptional terminator regions are evident.
Francisella ABC transporter

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 (Ochvinnikov 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 BstI-digested KM41 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 gene 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 XbaI site and the BamHI site of pKEM14-8; the DNA sequence of the 3263 bp region to the right of the left-most XbaI 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 '+'.
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 BgII-BglII 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 BamHI 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 htrB; therefore we tested a clone containing valA for its ability to complement E. coli htrB mutants. The E. coli htrB 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 htrB 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 rightmost XbaI 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±sd) in serum after 3 h at 37°C was 0±0.034, 1±0.02, 2.4±1.2, 15.8±1.1 and 36.0±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. XbaI-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-C4; 3, KM14S-C1; 4, wild-type (U112). Arrow 1, a restriction fragment that is apparently the 8 kb XbaI site, and presumably there has been a simple duplication of part or all of the 8 kb fragment.](image-url)
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 val/AB region (Fig. 6). Thus, it appears that a functional copy of val/AB 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 val/AB 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 val/AB 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::mTn10Km, provides evidence that lesions in val/AB contributed to the serum-sensitive phenotype, since the only functional *F. novicida* genes that were introduced to generate KM14S-C1 were val/AB. Moreover, Southern blot evidence suggests that the only events that occurred in generating KM14S were the integration and resolution of DNA in the val/AB 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 val/AB 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, val/AB suppresses mutant alleles of htrB.

The apparent operon organization of val/AB is not unexpected given the organization of msbA and orfE. In contrast to the conserved operon organization of val/AB with respect to msbA and orfE, the genome organization is apparently quite different between *F. novicida* and *E. coli*. In *F. novicida*, val/AB, 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 val/AB 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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