Attenuation and protective efficacy of an O-antigen-deficient mutant of Francisella tularensis LVS

Jiaxin Li,1† Cheryl Ryder,1† Manas Mandal,1‡ Farzana Ahmed,1 Parastoo Azadi,2 D. Scott Snyder,2 Roger D. Pechous,3 Thomas Zahrt3 and Thomas J. Inzana1

1Center for Molecular Medicine and Infectious Diseases, Virginia–Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA
2Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA
3Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, WI, USA

Francisella tularensis is a zoonotic, Gram-negative coccobacillus that causes tularemia in humans and animals. F. tularensis subspecies tularensis (type A) and F. tularensis subspecies holarctica (type B) are antigenically similar and more virulent than Francisella novicida in humans. The genetic locus that encodes the LPS O antigen was found to be substantially different between the type B live vaccine strain (LVS) and F. novicida. One LVS-specific gene with homology to a galactosyl transferase was selected for allelic replacement using a sacB–chloramphenicol expression suicide plasmid, and recombinants were screened for colony morphology on Congo red agar that matched that of F. novicida. Two mutants (WbtI S187Y and WbtI G191V) were isolated that contained substitutions in conserved motifs in the sugar transamine/perosamine synthetase (WbtI) of the O-antigen locus, and the latter mutant was extensively tested and characterized. WbtI G191V grew at the same rate as the parent strain in Chamberlain’s defined medium, completely lacked O antigen, was serum-sensitive but could grow in a mouse macrophage cell line, had increased resistance to sodium deoxycholate, and was highly attenuated in mice. Complementation of WbtI G191V with the wild-type wbtI gene in trans restored normal LPS synthesis, phenotypic properties similar to the parent, and virulence in mice. Immunization with WbtI G191V protected mice against a relatively low-dose intraperitoneal challenge with LVS, but was less protective against a high-dose challenge. These results indicate that complete loss of O antigen alters the surface phenotype and abrogates virulence in F. tularensis, but also compromises the induction of full protective immunity against F. tularensis infection in mice.

INTRODUCTION

Francisella tularensis is a Gram-negative, facultative intracellular coccobacillus and the aetiologic agent of tularemia for a wide variety of vertebrate and invertebrate animal species; tularœmia is a zoonotic disease for humans (Hopla & Hopla, 1994; Penn, 2005; Sjöstedt, 2005; Timoney et al., 1988; Titball & Sjöstedt, 2002). F. tularensis can be transmitted to humans by direct contact with infected animals, ingestion of contaminated food or water, bites from infected vectors such as ticks and mosquitoes, and inhalation of infectious aerosols or dust (Hopla & Hopla, 1994; Penn, 2005; Titball & Sjöstedt, 2002). There are four subspecies of F. tularensis: subs. tularensis (type A), subs. holarctica (type B), subs. mediasiatica and subs. novicida (Sjöstedt, 2005). However, the International Systematics Committee recognizes Francisella novicida as a separate species (http://www.bacterio.cict.fr/aldl.html). F. tularensis subs. tularensis is the most virulent subspecies for humans, with an infectious dose...
of as few as 10 cells (Dennis et al., 2001). Patients may present with several clinical forms of tularämia, the most common being ulceroglandular tularämia; other forms include ocułoglandular, oropharyngeal, intestinal, pneu-
monic and typhoidal tularämia. Pneumonic tularämia has a mortality rate of up to 30 % in the absence of antibiotics (Ellis et al., 2002; Penn, 2005). Based on these factors, F. tularensis has been classified as a category A select agent by the National Institutes of Health and the Centers for Disease Control (Darling et al., 2002; Dennis et al., 2001).

F. tularensis subsp. tularensis is predominately found in North America, and genetically falls into two subtypes: type A East and type A West (Staples et al., 2006). In contrast, subsp. holarctica is more widely distributed and has been isolated in Europe, Asia and North America. Type B strains are associated with morbidity and a somewhat lower mortality rate in humans that type A East strains (Staples et al., 2006). However, F. novicida is considerably less virulent in humans, and is rarely associated with severe disease, although it is highly virulent in mice (Ellis et al., 2002; Hopla & Hopla, 1994; Titball & Sjöstedt, 2002).

The factors that are associated with virulence in F. tularensis are not well known. Bacterial surface components that contribute to the disease process, or are important for host immunoprotection, have not been clearly identified. Most of the work on F. tularensis surface components has focused on the LPS. The LPS of F. tularensis types A and B is unusual in that the O antigen consists entirely of dideoxyxyloses, the core oligosaccharide contains manose in place of heptose (Vinogradov et al., 2002, 1991), and lipid A of the live vaccine strain (LVS) is tetraacylated and lacks phosphate (Vinogradov et al., 2002), while lipid A from a virulent type B isolate also contains a phosphate-linked galactosamine (Phillips et al., 2004). Furthermore, the LPS does not signal through TLR4, is not an agonist for TLR4, and does not induce an inflammatory response (Chen et al., 2005; Cole et al., 2006; Hajjar et al., 2006), which is probably due to the atypical structure of lipid A. However, apart from failing to incite an inflammatory response by the host, the role of the LPS in virulence and immunoprotection is unclear.

The genomes of at least five strains of F. tularensis types A and B and F. novicida have recently been sequenced (available at the National Center for Biotechnology Information), and the genome of type A strain Schu S4 has been annotated (Larsson et al., 2005). However, little information is available regarding genomic differences between the highly virulent F. tularensis and the much less virulent F. novicida. In order to identify novel genes that may be responsible for virulence in F. tularensis, we previously used suppression subtractive hybridization (SSH) with subsp. holarctica LVS as the tester and F. novicida strain U112 as the driver (Ahmed & Inzana, 2004). Of 76 LVS-specific genes identified, several were found in the wbt O-antigen locus of LVS that were absent in F. novicida. One LVS-specific gene, which encoded a galactosyl transferase, was selected for mutagenesis. The mutant was devoid of O antigen, was more resistant to the bactericidal effects of sodium deoxycholate than its parental strain, was serum susceptible and attenuated in mice, and provided partial protection against an intraperi-
itoneal (IP) high-dose challenge with the parental strain. The significance of O antigen in F. tularensis virulence and induction of host protection is discussed.

This work was presented, in part, at the mid-Atlantic Microbial Pathogenesis meeting (Wintergreen Conference Center, Charlottesville, VA, February 2004).

**METHODS**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this work and their sources are listed in Table 1. F. tularensis LVS was obtained as a vaccine vial from Dr May Chu, Centers for Disease Control (April, 2002), was subcultured on chocolate agar, and the cells were suspended in sterile skimmed milk and stored at ~80 °C.

**Cultivation of bacteria.** Escherichia coli DH5α and Mach1 T1R (Invitrogen) cells were grown at 37 °C in Luria–Bertani (LB) medium (Difco Laboratories) containing, as appropriate, 100 μg ampicillin (Amp) ml−1, 10 μg chloramphenicol (Cm) ml−1 or 50 μg kanamycin (Km) ml−1 for selection of recombinant strains. F. tularensis and F. novicida strains were grown in Difco Brain Heart Infusion (BHI) broth (Becton Dickinson) supplemented with 0.1 % L-cysteine hydrochloride monohydrate (Sigma) (BHI) at 37 °C. For culture on agar plates, 5 % (v/v) sheep blood was added to BHI agar (BHIBC), and the cultures were incubated at 37 °C in 5 % CO2, unless otherwise stated. Actinobacillus pleuropneumoniae was grown in supplemented BHI broth or agar, as described previously (Ward et al., 1998).

**DNA manipulation.** Plasmid isolation, DNA restriction endonuclease digestion, ligation and transformation procedures were carried out using standard protocols (Sambrook et al., 1989). Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. QIAprep Spin Miniprep and QIAquick gel extraction kits (Qiagen) were used to prepare E. coli plasmid DNA. A PUREGENE DNA isolation kit (Gentra Systems) was used to purify genomic DNA from F. tularensis.

**Isolation of LVS mutants.** Plasmid pPV, containing a sacB–CmR cassette that confers sucrose sensitivity and resistance to chlor-
amphenicol on Gram-negative cells (Golovilov et al., 2003), was used to mutagenize SSH-identified clone 2-042 (Ahmed & Inzana, 2004), which was later determined to be wbtB from the O-antigen gene cluster (Prior et al., 2003). A 1500 bp region upstream and a 1500 bp region downstream (Table 1) of wbtB were amplified by PCR using primers FA6-7-NF and FA6-7-NR, and FA6-7-CF and FA6-7-CR, respectively. A typical PCR reaction consisted of 1× PCR HIFI SuperMix (Invitrogen), 0.02 μg genomic DNA as template, and 0.4 μM of each oligonucleotide primer in 50 μl of reaction mixture. The PCR cycling parameters used were 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 68 °C for 2 min, and an additional extension for 5 min at 68 °C. The 5′ primers included a SalI restriction site and the 3′ primers incorporated either a BamHI restriction site or a PstI restriction site. The upstream and downstream PCR products were digested with SalI/BamHI and SalI/PstI, respectively, and separately cloned into pBluescriptKS+(Stratagene). The two recombinant clones were digested with SalI/...
Table 1. Bacterial strains, plasmids, and DNA primers used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid or primer</th>
<th>Characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F- φ80d lacZAM15A (lacZYA-argF)U169 recA1 endA1 hsdR17(rK mK+ ) phoA supE44i-</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>TOP10</td>
<td>F- mcrA Δ(mrr-hsdRMS merBC) φ80lacAM15 ΔlacX74 deor recA1 araD139</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Mach1 TI&lt;sup&gt;B&lt;/sup&gt;</td>
<td>F′ φ80(lacZ) ΔM15 ΔlacX74 hsdR17(rK mK+) ΔrecA1398 endA1 tonA</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>S17-1</td>
<td>thi thr leu tonA lacy supE recA</td>
<td>Dr May Chu</td>
</tr>
<tr>
<td>LVS</td>
<td>subsp. halocarta LVS</td>
<td>Dr Karen Elkins</td>
</tr>
<tr>
<td>U112</td>
<td>F. novicida wild-type strain</td>
<td>This study</td>
</tr>
<tr>
<td>Wbt&lt;sub&gt;LISY7&lt;/sub&gt;</td>
<td>LVS mutant deficient in O-antigen biosynthesis, with mutation in amino acid 187 (S187Y) of WbtI</td>
<td>This study</td>
</tr>
<tr>
<td>Wbt&lt;sub&gt;G191V&lt;/sub&gt;</td>
<td>LVS mutant deficient in O-antigen biosynthesis, with mutation in amino acid 191 (G191V) of WbtI</td>
<td>This study</td>
</tr>
<tr>
<td><strong>A. pleuropneumoniae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J45-100</td>
<td>Isogenic non-encapsulated, serotype 5 mutant with deletion in cps5ABC</td>
<td>Ward et al. (1998)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBlueScript SK&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Cloning vector; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pUC19</td>
<td>Cloning vector; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pCR 2.1-TOPO</td>
<td>Cloning vector for SSH PCR products; Amp&lt;sup&gt;+&lt;/sup&gt;, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pPV</td>
<td>Knockout vector; Amp&lt;sup&gt;+&lt;/sup&gt;, Cm&lt;sup&gt;+&lt;/sup&gt;, sacB, mob</td>
<td>Golovliov et al. (2003)</td>
</tr>
<tr>
<td>pFNLT6</td>
<td>Complementation shuttle vector; Km&lt;sup&gt;+&lt;/sup&gt;, Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Maier et al. (2004)</td>
</tr>
<tr>
<td>pTZ817</td>
<td>EcoRI- and Nhel-digested 2.0 kb wild-type wbtI PCR fragment cloned into pFNLT6 for complementation without groE promoter; Km&lt;sup&gt;+&lt;/sup&gt;, Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pTZ819</td>
<td>pTZ817 containing F. tularensis groE promoter in KpnI site</td>
<td>This study</td>
</tr>
<tr>
<td><strong>DNA primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA6-7-NF</td>
<td>5′-TAGCACTGTCGACGAAAGATGAACC-3′; Sall restriction site in bold type; PCR primer for wbtB knockout</td>
<td>This study</td>
</tr>
<tr>
<td>FA6-7-NR</td>
<td>5′-TAGAAAAGATCCTTATTAGGTCTATTATAT-3′; BamHI restriction site in bold type; PCR primer for wbtB knockout</td>
<td>This study</td>
</tr>
<tr>
<td>FA6-7-CF</td>
<td>5′-AAGAATTCTAGTCGAGTTTGGAGTAG-3′; PstI restriction site in bold type; PCR primer for wbtB knockout</td>
<td>This study</td>
</tr>
<tr>
<td>FA6-7-CR</td>
<td>5′-TTTTCCTGTCGACAAAACTATATATCAAGAC-3′; SalI restriction site in bold type; PCR primer for wild-type wbtI cloning for in trans complementation</td>
<td>This study</td>
</tr>
<tr>
<td>FA5-8NF1</td>
<td>5′-ATCAGCCAGCATATTCAAAAG-3′; PCR primer for confirming wbtB knockout</td>
<td>This study</td>
</tr>
<tr>
<td>FA5-8CR1</td>
<td>5′-CCTTGGATATGTGCCTTTTGAGC-3′; PCR primer for confirming wbtB knockout</td>
<td>This study</td>
</tr>
<tr>
<td>F-wbtI-EcoRI</td>
<td>5′-ACGGGAATTCGATCTATTTACATTACAAATG-3′; EcoRI restriction site in bold type; forward PCR primer for wild-type wbtI cloning for in trans complementation</td>
<td>This study</td>
</tr>
<tr>
<td>R-wbtI-Nhel</td>
<td>5′-ACGGGCTAGGTTCCCGCATTGTTAACATT-3′; Nhel restriction site in bold type; reverse PCR primer for wild-type wbtI cloning for in trans complementation</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Bam*HI and both the upstream and downstream insert fragments were ligated simultaneously into *Sal*I-digested pPV, resulting in plasmid pFA501, which was electroporated into *E. coli* S17-1. Conjugation of LVS with *E. coli* S17-1(pFA501) was carried out as described elsewhere (Golovliov et al., 2003). After 4 days of incubation, the colonies were transferred to BHIBC agar containing 100 μg Cm ml<sup>−1</sup>. Cm-resistant and polymyxin B-resistant colonies were screened for diminished iridescence (indicating a loss of surface carbohydrate) under incandescent light on BHIC agar, followed by further selection of the non-iridescent colonies on BHIC agar containing 75 mg Congo red 1<sup>−1</sup>. Screening for dark-red colonies on Congo red agar was used because colonies of *F. novicida* exhibited the dark-red phenotype, whereas colonies of LVS (and type A) were salmon to light pink, indicating surface-component-inhibited binding of Congo red by neutral carbohydrate structures (data not shown). Two colonies that were darker red than the LVS parent were isolated, and PCR (described above) using primers FA5-8NF1 and FA5-8CR1 and Southern blotting were performed to examine the deletion of *wbtB*.

**Plasmid construction and wbtB complementation.** A 2.0 kb DNA fragment containing *wbtI* from the LVS was amplified by PCR using primers F-wbtI-EcoRI and R-wbtI-Nhel, as described above. The resulting fragment was cloned into pCR2.1-TOPO, digested with EcoRI and Nhel, gel-purified, and subcloned into the corresponding sites in pFNLT6 (Maier et al., 2004), resulting in pTZ817. The
pTZ817 plasmid was then digested with KpnI and ligated with a KpnI-digested fragment carrying the F. tularensis groE promoter, resulting in pTZ819, which expressed wbtI from the groE promoter. All insert sequences were confirmed by DNA sequencing. Plasmids pTZ817 and pTZ819 were then electroporated into F. tularensis WbtIG191V. The bacteria were grown to a density of about 110 Klett units (correlating to about $10^8$ c.f.u. ml$^{-1}$), washed three times with 0.5 M sucrose, and suspended in 10 ml of 0.5 M sucrose. The plasmid DNA was then electroporated into the bacteria in a 0.1 cm cuvette using an Electro Cell manipulator ECM630 (BTX), as described previously (Maier et al., 2004). Immediately after electroporation, the cells were suspended into 1 ml of TSB without cysteine and incubated with shaking at 37°C for 4 h before selection on BHIBC agar containing 20 μg kanamycin ml$^{-1}$.

Sequence alignments and computational structural modelling. DNA and protein sequences were aligned with CLUSTAL W (Thompson et al., 1994). The tertiary structures of the wild-type protein WbtI, and mutants proteins WbtIG191V and WbtIG191V were modelled as previously described (Li et al., 2005) to predict the conformational changes caused by residue substitution. Helicobacter pylori amino-transferase Epsc (PDB_ID=2FNU, Chain_ID=2FNU_B) with 375 amino acids from the Protein Data Bank (PDB) was used as template for WbtI on the 3D-JIGSAW version 2.0 comparative modelling server (Bates & Sternberg, 1999) (http://www.bmm.icnet.uk/~3djig saw/). The coordinates in PDB formats for 3-D structures of WbtI were constructed and displayed with RasMol version 2.7.3 (Sayle & Milner-White, 1995).

Extraction of LPS. LPS was purified by aqueous phenol extraction and ultracentrifugation from killed cells, as described by Vinogradov et al. (1995), with modifications. The bacteria were scraped off BHIBC agar into PBS, and following phenol extraction, 4 vols distilled water was added and the mixture dialysed with tap water until no phenol odour remained. Sodium acetate (pH 7.0) was added to a final concentration of 30 mM, 2 μg DNase I ml$^{-1}$ from bovine pancreas (Sigma-Alrich) was added and the mixture was incubated for 2 h at 37°C, followed by an additional 2 h incubation with 2 μg RNase I ml$^{-1}$ from bovine pancreas (Amersham Pharmacia Biotech). Protease K (Sigma-Alrich) was then added at 20 μg ml$^{-1}$ and the mixture was incubated at 37°C for 2 h. Insoluble material was removed by centrifugation at 10 000 g at 4°C for 10 min. The LPS was sedimented by ultracentrifugation overnight at 100 000 g at 4°C, and resuspended in water. The low-speed/high-speed differential centrifugation process was repeated (the low speed was changed to 3000 g for 15 min) until the A$_{260}$ and A$_{230}$ of the supernatant were less than 0.02, and the LPS was lyophilized.

PAGE and Western blotting. The LPS electrophoretic profile was resolved by SDS-PAGE on Novex 16% Pre-Cast Tricine Gels (Invitrogen), as described by the manufacturer. Western blotting was carried out using a Trans-Blot SD semi-dry transfer cell (Bio-Rad), and the blots were developed with rabbit polyclonal antiserum to subsp. holarctica LVS (Inzana et al., 2004), rabbit polyclonal antiserum to F. novicida U112 (1:1000 dilution each) or murine mAb (Chemicon International) to F. tularensis O antigen at 1:500 dilution. Anti-rabbit IgG or anti-murine IgG coupled to horseradish peroxidase (HRP; Jackson ImmunoResearch Laboratories) were used as secondary conjugates at 1:2000 dilution, and colour was developed with 4-chloro-1-naphthol (Bio-Rad). Rabbit polyclonal antiserum to strain U112 (killed by irradiation) was raised in a New Zealand White rabbit, as previously described (Inzana et al., 2004).

LPS composition and structure. The glycosyl composition of LPS was determined by combined GC/MS of the per-O-trimethylsilyl derivatives of the monosaccharide methyl glycosides, which were produced by acidic methanalysis (Merkle & Poppe, 1994; York et al., 1985). The samples were then per-O-trimethylsilylated by treatment with Tri-Sil (Pierce Chemical) at 80°C for 30 min. GC/MS analysis of the per-O-trimethylsilyl methyl glycosides was performed on an HP 5890 GC interfaced to a 5970 MSD, using an All Tech EC-1 fused silica capillary column (30 m x 0.25 mm internal diameter).

For structural analysis LPS was hydrolysed 4 h in 0.1 M sodium acetate, pH 4.5, at 100°C. Lipid A was extracted using 2.5 vols chloroform:methanol (2:1), and the remaining aqueous phase was desalted on Dowex 50WX8 and lyophilized. The resultant oligosaccharide core was suspended in dry DMSO. The sample was then permethylated by the method of Ciucanu & Kerek (1984), suspended in 5 mM EDTA in methanol:water (1:1), and desalted by adding a few microlitres to a few grains of Dowex 50WX8, previously converted into the ammonium salt. The desalted sample was deposited, along with an equal volume of 20 mM dibasic ammonium citrate, onto a thin layer of matrix, whose components were 200 mg trihydroxycetophenone ml$^{-1}$ in methanol, and 15 mg nitrocellulose ml$^{-1}$ in acetonitrile:2-propanol (1:1, v/v), mixed in a 4:1 (v/v) ratio (Sturiale et al., 2005). MALDI-MS analysis was then performed using an Applied Biosystems 4700 mass spectrometer operating in the positive ion mode.

Sensitivity to sodium deoxycholate. The susceptibility of the LVS, mutant strain WbtIG191V and complemented strain WbtIG191V::pFNLTP/wbtI to the bactericidal activity of sodium deoxycholate was evaluated as described elsewhere (Cowley et al., 2000), with modifications. An equal volume of sodium deoxycholate (Sigma) in PBS was added to wells containing 5 x 10$^5$ c.f.u. of each strain in 100 μl to final concentrations (w/v) of 0, 0.01, 0.1, 1 or 10%. After 45 min incubation at 37°C, 20 μl of a 1:100 dilution of the mixtures was spread onto BHIBC agar, and bacterial viability was determined after up to 5 days incubation.

Serum bactericidal assay. The bactericidal activity of 0–40% pre-collostral calf serum (PCS, which contains no antibodies) for the LVS, mutant WbtIG191V and WbtIG191V::pFNLTP/wbtI was determined as previously described (Inzana & Anderson, 1985). Control tubes contained serum-sensitive A. pleuropneumoniae J45-100 in place of F. tularensis, or heat-inactivated PCS.

Survival in J774A.1 cells. Intracellular growth of the LVS, mutant WbtIG191V and the complemented mutant WbtIG191V::pFNLTP/wbtI was monitored in the murine macrophage-like cell line J774A.1 (American Type Culture Collection) by modification of published methods (Cowley & Elkins, 2003). The number of bacteria added was confirmed by viable plate counting, and used at a m.o.i. of 50:1 (bacteria:macrophages). After 2 h incubation of F. tularensis with J774A.1 cells at 37°C in 5% CO$_2$, extracellular bacteria were removed by washing the cells with PBS, and the medium was replaced with 1 ml complete Dulbeccos’s Modified Eagle Medium (DMEM) plus 50 μg gentamicin ml$^{-1}$ to eliminate extracellular bacteria. After 45 min incubation, the cells were washed three times with PBS, followed by the addition of complete DMEM without antibiotics. The cells were incubated at 37°C in 5% CO$_2$ for 72 h post-infection. The J774A.1 cells were washed in PBS and lysed by exposure to water for 3 min, and serial dilutions of the lysates were plated on BHIBC agar to determine the number of intracellular bacteria at 0 h and at the indicated time points.

Virulence and immunoprotection studies in mice. To assess virulence, groups of five BALB/c mice 6–8 weeks old (Jackson Laboratory) were challenged IP with various doses of exponential-phase LVS (200, 600 or 2000 c.f.u. per mouse), mutant WbtIG191V (10$^3$, 10$^5$, 5 x 10$^5$, 1.4 x 10$^6$ or 2.8 x 10$^7$ c.f.u. per mouse) or complemented mutant WbtIG191V::pFNLTP/wbtI (10$^5$ c.f.u. per mouse).
Characterization of an F. tularensis O-antigen mutant

Identification of LVS-specific genes involved in carbohydrate synthesis and generation of an LPS mutant

Following the use of SSH to identify LVS-specific genes, one gene (wbtB) was identified in a 17.4 kb region consisting of 15 carbohydrate biosynthesis genes (later determined to be the O-antigen locus), and was selected for allelic replacement with plasmid pFA501 (see Methods). However, PCR and Southern blotting of genomic DNA from these mutants indicated that wbtB was still present in the genome (data not shown). To determine if another gene in this region had mutated, 23 kb of DNA from both mutants was sequenced, which included the entire O-antigen locus. From this analysis it was determined that the two isolates differed from the parent by only a single nucleotide substitution in wbtB, a sugar transaminase/perosamine synthetase, required for biosynthesis of 4,6-dideoxy-4-formamido-D-glucose (Prior et al., 2003). A C→A transversion in mutant WbtIG191V caused a codon change of TCT→TAT, resulting in the change of Ser to Tyr in residue 187. A G→T transversion in mutant WbtIG191V causing a codon change of GGT→GGT resulted in residue 191 changing from Gly to Val. The wbtB gene from the LVS was amplified and sequenced, and neither of the substitutions found in the mutants was present in the parent strain. Aligning the amino acid sequence of LVS WbtI with its homologues from 25 species/subspecies indicated that Gly191 was highly conserved in all 25 enzymes (data not shown). Therefore, the phenotype of mutant WbtIG191V and its virulence in mice were further characterized.

Statistical analysis. The slope of the growth rate was determined from the formula:

\[
\frac{y_2 - y_1}{x_2 - x_1}
\]

where \(y\) represents Klett units and \(x\) represents time. Differences in susceptibility to serum or sodium deoxycholate were determined by Student’s \(t\) test. Attenuation of bacterial virulence and protective efficacy were determined by Fisher’s exact test of 2 \(\times\) 2 contingency tables. All statistical analyses were carried out using InStat software (Graphpad).

RESULTS

Characterization of an F. tularensis O-antigen mutant

Identification of LVS-specific genes involved in carbohydrate synthesis and generation of an LPS mutant

Following the use of SSH to identify LVS-specific genes, one gene (wbtB) was identified in a 17.4 kb region consisting of 15 carbohydrate biosynthesis genes (later determined to be the O-antigen locus), and was selected for allelic replacement with plasmid pFA501 (see Methods). However, PCR and Southern blotting of genomic DNA from these mutants indicated that wbtB was still present in the genome (data not shown). To determine if another gene in this region had mutated, 23 kb of DNA from both mutants was sequenced, which included the entire O-antigen locus. From this analysis it was determined that the two isolates differed from the parent by only a single nucleotide substitution in wbtB, a sugar transaminase/perosamine synthetase, required for biosynthesis of 4,6-dideoxy-4-formamido-D-glucose (Prior et al., 2003). A C→A transversion in mutant WbtIG191V caused a codon change of TCT→TAT, resulting in the change of Ser to Tyr in residue 187. A G→T transversion in mutant WbtIG191V causing a codon change of GGT→GGT resulted in residue 191 changing from Gly to Val. The wbtB gene from the LVS was amplified and sequenced, and neither of the substitutions found in the mutants was present in the parent strain. Aligning the amino acid sequence of LVS WbtI with its homologues from 25 species/subspecies indicated that Gly191 was highly conserved in all 25 enzymes (data not shown). Therefore, the phenotype of mutant WbtIG191V and its virulence in mice were further characterized.

Computational structural modelling

The locations of residues Ser187 and Gly191 in WbtI were computationally investigated, as were the effects of S187Y and G191V mutations on enzyme structure (data not shown). Both residues were in the core of the WbtI sugar transamine/perosamine synthetase. The alteration of S187Y or G191V changed the number of \(\alpha\)-helices, \(\beta\)-sheets and turns of the enzyme, which would result in substantial conformational changes, suggesting that WbtIS187Y and WbtIG191V had lost biological activity.

Physical and chemical characterization of LPS

Since the mutation in WbtIG191V was predicted to be in the O-antigen region, purified LPS samples from WbtIG191V and the parent were separated by electrophoresis and immunoblotted with rabbit polyclonal antiserum to LVS (Fig. 1a) or murine mAb to LPS O antigen (Fig. 1b). A characteristic ladder-like pattern was observed with LVS LPS reacted with both LVS antiserum and mAb, but this pattern was absent from WbtIG191V LPS incubated with either antibody. However, immunoreactive low-molecular-mass material was present in mutant LPS blotted with LVS antiserum, but not with mAb to O antigen, suggesting that the core, but not the O antigen, was present in the mutant. Furthermore, unlike the parental LPS, there was no ladder-like pattern in the LPS of WbtIG191V on polyacrylamide gels stained with Emerald Q fluorescent stain (data not shown). LVS LPS O antigen has been shown to undergo antigenic phase variation to an F. novicida O antigen, resulting in a switch from reactivity to LVS O-antigen antibodies to reactivity with F. novicida O-antigen antibodies (Cowley et al., 1996). However, neither LVS LPS nor WbtIG191V O antigen reacted with antiserum to F. novicida strain U112 in a Western blot, indicating that the mutant did not
contain a *F. novicida*-reactive O antigen. The reactivity of low-molecular-mass LPS from mutant WbtI<sub>G191V</sub> with *F. novicida* antiserum indicated conservation of core LPS epitopes between the mutant and *F. novicida*. The lack of reactivity of low-molecular-mass LPS from WbtI<sub>G191V</sub> reacted with antisera to both LVS and *F. novicida* whole cells, indicating conservation of core epitopes. The lack of any reactivity of LVS LPS with antiserum to *F. novicida* suggested that O-antigen epitopes blocked binding to the conserved LPS core. Lanes: 1, LVS LPS; 2, WbtI<sub>G191V</sub> LPS; 3, *F. novicida* LPS.

**Fig. 1.** Western blots of LPS from the LVS and WbtI<sub>G191V</sub> mutant. Purified LPS (20 μg) was separated by SDS-PAGE, transferred to nitrocellulose, and blotted with (a) a 1:1000 dilution of rabbit polyclonal antiserum to LVS, (b) 1:10 mouse mAb to LVS O antigen, or (c) 1:1000 dilution of antiserum to *F. novicida*. Production of antiserum and blot development were as described in Methods. The ladder-like pattern of LPS from mutant WbtI<sub>G191V</sub> was absent in all blots, indicating a loss of O antigen and not phase variation. LVS LPS reacted with antiserum to whole LVS cells and mAb to O antigen. Only low-molecular-mass LPS from WbtI<sub>G191V</sub> reacted with antisera to both LVS and *F. novicida* whole cells, indicating conservation of core epitopes. The lack of any reactivity of LVS LPS with antiserum to *F. novicida* suggested that O-antigen epitopes blocked binding to the conserved LPS core. Lanes: 1, LVS LPS; 2, WbtI<sub>G191V</sub> LPS; 3, *F. novicida* LPS.

The glycosyl composition and structure of purified LPS was analysed by combined GC/MS and MALDI-MS, respectively. The mol% of total carbohydrate for mannose, glucose, N-acetylgalactosamine, N-acetylglucosamine and 3-deoxy-D-manno-2-octulosonic acid, which are components of the core oligosaccharide, were comparable in the LPS of the LVS parent and WbtI<sub>G191V</sub> (17±1, 33±3, 5±1, 15±5 and 23±3, respectively). However, the O-antigen-specific glycosyl N-acetyl quinovosamine (QuiNAc) was not detected in WbtI<sub>G191V</sub> LPS, confirming that the O antigen was not present in the mutant. The presence of a complete core structure (Vinogradov et al., 2002) in WbtI<sub>G191V</sub>, but no O antigen, was confirmed by MALDI-MS (Fig. 2; details in the legend).

**Complementation**

To confirm that the mutation in *wbtI* was solely responsible for the loss of O antigen in WbtI<sub>G191V</sub>, a normal copy of *wbtI* was amplified and cloned into shuttle vector pFNLT6 alone and behind the *groE* promoter, and each construct was electroporated into WbtI<sub>G191V</sub>. The presence of the plasmid in recombinant clones was confirmed by gel electrophoresis and PCR. Western blots of extracted LPS from the parent strain, the mutant containing pFNLT6 alone, and mutants containing pFNLT6/*wbtI*, showed that the *wbtI* gene in trans with or without the *groE* promoter complemented the mutation and restored normal LPS O-antigen production (Fig. 3).

**Growth rate**

To assess whether the mutation in WbtI<sub>G191V</sub> affected its growth rate, the mutant, parental and complemented strains were inoculated in BHIC broth at a cell density of 55 Klett units, and the OD<sub>600</sub> was monitored for 8 h, by which time all strains had reached stationary phase. The growth rates of WbtI<sub>G191V</sub>, the LVS parental strain and complemented mutant WbtI<sub>G191V::pFNLT6/wbtI</sub> were highly similar, with a growth-rate slope of about 0.6 for each (data not shown).

**Serum bactericidal assay**

The parental LVS and complemented mutant WbtI<sub>G191V::pFNLT6/wbtI</sub> were completely resistant to the bactericidal action of fresh PCS up to at least 40 % (v/v). The LVS (as well as our type A strains Schu S4 and TI0902) and the complemented mutant were also resistant to at least 40 % PCS supplemented with 40 % hyperimmune rabbit serum made to irradiated LVS (data not shown). However, mutant strain WbtI<sub>G191V</sub> was completely killed by <3 % fresh PCS only, and was much more sensitive to PCS than a non-encapsulated *A. pleuropneumoniae* control strain. Of interest was that serum susceptibility increased sharply from essentially no killing in 0.5 % PCS to >80 % killing in 1 % PCS (Fig. 4). Similar results were obtained when mouse, canine or human sera were used (data not shown).

**Intracellular survival in J774A.1 cells**

Following infection of the J774A.1 macrophage-like cell line with LVS there was continuous exponential growth over the study period of 72 h. Mutant WbtI<sub>G191V</sub> grew slowly but continuously during the first 48 h, but the growth rate increased dramatically after 48 h. Complemented mutant WbtI<sub>G191V::pFNLT6/wbtI</sub> grew somewhat faster and in parallel to that of the mutant throughout the time period, but not as rapidly as the parental strain after the initial 24 h (Fig. 5).
Sensitivity to deoxycholate

F. novicida mutants defective in LPS biosynthesis may have decreased or increased sensitivity to the detergent sodium deoxycholate (Cowley et al., 2000). Therefore, the sensitivity of WbtIG191V incubated with various concentrations of sodium deoxycholate in PBS for 45 min was examined (Fig. 6). On average, in the presence of 0.1 % deoxycholate, more than 100 % of WbtIG191V cells survived (indicating growth of the bacteria during the incubation period), whereas fewer than 60 % of the LVS parental strain cells survived (P<0.02). When the sodium deoxycholate concentration was increased to 1 %, essentially all LVS cells were killed, but 12.4 % of WbtIG191V cells remained viable. Moreover, up to 4.4 % of WbtIG191V mutant cells were able to tolerate up to 10 % deoxycholate. Complemented mutant WbtIG191V::pFNLTTP/wbtI was intermediate between the LVS and the mutant in sensitivity to 0.01 % and 0.1 % deoxycholate, but was as susceptible as the parent at concentrations of deoxycholate of 1 % or more.

Virulence of WbtIG191V to mice

The mouse IP LD₅₀ for our strain of LVS was previously determined to be about 120 c.f.u. (Inzana et al., 2004). For this study, mice were inoculated IP with doses of LVS, mutant WbtIG191V or complemented mutant WbtIG191V::pFNLTTP/wbtI that ranged from 200 to 2.8 × 10⁷ c.f.u. (Table 2). Clinical symptoms were not detected in any of the mice until about 48 h PI, by which time mice inoculated with 2000 c.f.u. LVS had ruffled fur, were inactive and appeared lethargic. All the mice inoculated with this dose of the parental strain died between 4 and 5 days PI. Mice inoculated with lower doses of the parental strain had ruffled fur and were lethargic at 72 h PI. By 6 days PI, all mice inoculated with 600 c.f.u. LVS had died, and three of five mice inoculated with 200 c.f.u. LVS died by 7 days PI. However, by 14 days PI all mice inoculated IP with up to 2.8 × 10⁵ c.f.u. mutant WbtIG191V remained clinically normal. Mice infected with the two highest doses of the mutant (1.4 × 10⁶ and 2.8 × 10⁷ c.f.u.) showed some early clinical symptoms, such as ruffled fur, stressed breathing and hunched gait, but recovered completely. Mice challenged with 10⁴ c.f.u. complemented mutant WbtIG191V::pFNLTTP/wbtI became moribund within 2 days and died by 4 days PI.

Clearance of the LVS, mutant WbtIG191V and complemented mutant from the tissues of challenged mice was evaluated following IN inoculation with 10⁷ c.f.u. of each strain. At 48 h PI there were about 2–3 logs fewer cells of mutant WbtIG191V in the tissues of mice than cells of the parental LVS, although none of the mice appeared ill. Thus, IN challenge was less severe than IP challenge for these mice. The number of complemented mutant cells in the lungs was similar to that of the mutant, probably because the challenge route was IN, but the numbers of the...
complemented mutant strain were more similar to those of the parental strain in the liver and spleen. From 2 days post-challenge to 4 days post-challenge the number of...
WbtIG191V cells had dropped by about 1 log in all tissues, whereas the numbers of the parental strain had increased by about 1 log in the lungs and by more than 3 logs in the spleen. The numbers of the complemented mutant increased about 1 log in all tissues; these animals were now moribund (Fig. 7). Mice challenged with the parental and complemented mutant WbtIG191V::pFNLTP/wbtI died or were euthanized by the fifth day PI, whereas by 8 days post-challenge all the mice challenged with WbtIG191V appeared normal and had cleared the bacteria from all of their tissues (data not shown).

Immunoprotective capacity of WbtIG191V for mice

Mice were immunized either ID or IP with mutant WbtIG191V to assess whether the mutant could confer protection against subsequent IP challenge with LVS. For ID immunization, three groups of five mice each were vaccinated with $10^5$ c.f.u. WbtIG191V without adjuvant twice, 2 weeks apart; control groups were inoculated with PBS alone or $10^5$ c.f.u. LVS. Three weeks after the second immunization mice in each group were challenged IP with various doses of LVS, as described in Methods, and monitored for up to 21 days. The mice immunized with the mutant made a strong antibody response to LVS whole cells, but a relatively weak response to purified LVS LPS (data not shown), which was expected given that the mutant lacked O antigen. All five mice in the PBS control group challenged with $2 \times 10^6$ LD$_{50}$ LVS died, whereas none of the mice immunized with LVS then challenged with $25 \times 10^6$ LD$_{50}$ of the same strain died (not shown). None of the five mice immunized with WbtIG191V and subsequently challenged with $25 \times 10^6$ LD$_{50}$ LVS died. However, two and three out of five mice immunized with the mutant and later challenged with 75 or $250 \times 10^6$ LD$_{50}$ LVS, respectively, died (Fig. 8a). About 3 days post-challenge, the surviving animals developed some clinical symptoms, but later recovered.

For IP immunization, three groups of five mice each were vaccinated with $10^3$, $10^4$ or $5 \times 10^4$ c.f.u. WbtIG191V twice 2 weeks apart. Three weeks after the second immunization the mice were challenged IP with various doses of LVS, as

Table 2. Virulence of LVS and WbtIG191V in BALB/c mice

<table>
<thead>
<tr>
<th>Challenge dose IP (c.f.u)*</th>
<th>Dead/total number of mice†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LVS</td>
</tr>
<tr>
<td>200</td>
<td>3/5</td>
</tr>
<tr>
<td>600</td>
<td>5/5</td>
</tr>
<tr>
<td>$2 \times 10^4$ for LVS; $10^3$ for WbtIG191V</td>
<td>5/5</td>
</tr>
<tr>
<td>$1 \times 10^4$</td>
<td>ND</td>
</tr>
<tr>
<td>$5 \times 10^4$</td>
<td>ND</td>
</tr>
<tr>
<td>$2.8 \times 10^5$</td>
<td>ND</td>
</tr>
<tr>
<td>$1.4 \times 10^6$</td>
<td>ND</td>
</tr>
<tr>
<td>$2.8 \times 10^7$</td>
<td>ND</td>
</tr>
</tbody>
</table>

*The LD$_{50}$ was determined to be ~120 c.f.u. for the LVS challenge strain IP.
†Challenged mice were carefully observed for 21 days PI. All mice challenged with LVS showed clinical symptoms (ruffled fur, inactivity, refusal to eat) by 48–72 h PI. Only two mice challenged with $1.4 \times 10^6$ c.f.u. and three mice challenged with $2.8 \times 10^7$ c.f.u. of mutant WbtIG191V showed similar clinical symptoms, but completely recovered by 5 days PI.

http://mic.sgmjournals.org 3149
with 10^4 c.f.u. mutant WbtI_{G191V} and later challenged with 75 × LD_{50} LVS survived. Similarly, three of five mice immunized with 5 × 10^4 c.f.u. mutant and later challenged with 250 × LD_{50} LVS survived (Fig. 8b). The surviving mice in this group developed clinical symptoms by day 2 post-challenge, but later recovered. Controls were not immunized IP with LVS because the lethal dose of LVS for mice is low by this route (some mice die from as few as 10 cells).

**DISCUSSION**

The virulence factors and biology of *F. tularensis* are not well characterized. One approach to identify such virulence factors is to identify genes present in virulent *F. tularensis* that are absent or have low homology to genes in closely related *F. novicida*. SSH has been used to successfully identify virulence genes present in pathogens that are absent in closely related species (Bernier & Sokol, 2005; DeShazer *et al.*, 2001; Harakava & Gabriel, 2003; Liu *et al.*, 2003; Newton *et al.*, 2006; Parsons *et al.*, 2003; Winstanley, 2002), and was therefore used to identify genes uniquely expressed by the more virulent type A and B strains. Seventy-six LVS-specific genes with hypothetical or known functions were identified in eight genomic regions in multiple SSH clones (Ahmed & Inzana, 2004). A putative glycosyl transferase in region VII was chosen for deletion due to its homology to a polysaccharide-biosynthesis gene. This gene was later identified as wbrB, and genomic region VII as the O-antigen locus.

Attempts to knock out wbrB using the sacB–Cm^R_ cassette in vector pPV resulted in the cotointegration of the sacB–Cm^R_ gene, as determined by PCR amplification of plasmid DNA from the genome. However, excision of the cotointegrant in the presence of sucrose apparently resulted in relaxation of the original sequence without deletion of wbrB, as PCR and Southern blotting indicated a single, normal copy of wbrB in the genome, but no evidence of plasmid DNA in the genome. Nonetheless, screening of sucrose-resistant strains for lack of iridescence and a dark-red phenotype on Congo red agar (characteristic of *F. novicida*, but not type A or B strains) resulted in the identification of two LVS mutant strains both containing single-residue (S187Y and G191V) changes in WbtI, as determined by sequencing the O-antigen locus. The G191V mutation in wbtI was confirmed by complementation *in trans* with a normal copy of the gene, which restored O-antigen synthesis, resistance to the bactericidal action of serum, enhanced susceptibility to sodium deoxycholate, and virulence in mice. Of interest was that complementation occurred in the presence and absence of the groE promoter upstream of wbtI, indicating that a promoter in the plasmid was also likely driving expression of wbtI.

A single amino acid substitution in MglA has been reported to cause the loss of intramacrophage survival and growth of *F. novicida* (Baron & Nano, 1998). A similar residue alteration appeared to affect the enzymic function described in Methods. All five mice immunized with 10^3 c.f.u. WbtI_{G191V} and subsequently challenged with 75 × LD_{50} LVS died. However, three of five mice immunized

---

**Fig. 8.** Immunoprotection of BALB/c mice against LVS IP challenge following immunization with WbtI_{G191V}. (a) Groups of five mice each were immunized ID with 10^3 c.f.u. WbtI_{G191V} or buffer twice, 2 weeks apart, followed by IP challenge with the indicated doses of LVS 3 weeks after the second immunization. (○) Control mice inoculated with PBS only and challenged with 25 × LD_{50}; (■) immunized mice challenged with 25 × LD_{50} [protection was highly significant (P=0.004)]; (▲) immunized mice challenged with 75 × LD_{50}; (▲) immunized mice challenged with 250 × LD_{50}. (b) Groups of five mice each were immunized IP with 10^3, 10^4 or 5 × 10^4 c.f.u. WbtI_{G191V} twice, 2 weeks apart, followed by IP challenge with LVS 3 weeks after the second immunization. (○) Control mice inoculated with PBS only and challenged with 25 × LD_{50}; (■) mice immunized with 10^3 c.f.u. followed by challenge with 75 × LD_{50}; (■) mice immunized with 5 × 10^4 c.f.u. and challenged with 75 × LD_{50} (P=0.083); (▲) 5 × 10^4 c.f.u.-immunized mice challenged with 250 × LD_{50} (P=0.083). Following challenge mice were monitored for 12 days. Mice that appeared to be severely moribund were euthanized and counted as dead.
of WbtI. Comparative computational modelling showed that Ser197 and Gly191 were both in the core of the wild-type enzyme, and that the side atoms of these residues extended into the milieu. Gly is the smallest and the most flexible amino acid residue. Substitutions or deletions of Gly residues can cause steric hindrance, block the necessary conformational changes in bacterial enzymes, and affect enzyme activity (Li & Rosen, 1998; Li et al., 2000). The wbtI gene is proposed to be a sugar transaminase/persaminase synthetase, required for biosynthesis of 4,6-dideoxy-4-formamido-D-glucose (Prior et al., 2003). Therefore, mutagenesis of this gene should result in complete loss of O antigen, which was confirmed by MALDI-MS analysis. It is not clear why attempts to mutate wbtB resulted in mutations in wbtI. However, the 5′ and 3′ ends of the O-antigen locus were found to be bordered by the transposase and pseudotransposase IS sequences isftu2 and isftu1, respectively. The presence of these IS sequences may cause the O-antigen locus to be a hypermutable region, resulting in loss of O antigen (the grey colony variant; Hartley et al., 2005), or phase variation to a F. novicida-type O antigen (Cowley et al., 1996).

The loss of polysaccharide capsule or O antigen commonly enhances the susceptibility of Gram-negative bacteria to the bactericidal action of serum (Joiner, 1988). As expected, WbtIG191V was completely killed by fresh 3% the bactericidal action of serum (Joiner, 1988). As expected, WbtIG191V was completely killed by fresh 3% normal serum, even in the presence of hyperimmune rabbit serum. However, WbtIG191V was still able to grow in the macrophage-like cell line J774A.1, albeit initially at a slower rate than the parental strain. Therefore, the LPS O antigen does not appear to be required for survival inside macrophages, although early intracellular growth was impaired. The susceptibility to normal serum, but resistance to intracellular killing, of WbtIG191V was similar to that of a non-encapsulated mutant of LVS described by Sandström et al. (1988). However, the genetic and biochemical nature of that mutant was not determined. One concern was that mutant WbtIG191V was an LPS phase variant that converted to a F. novicida-like LPS (Cowley et al., 1996). However, the LPS O antigen from this mutant did not react with antiserum to F. novicida or to the parent strain. Another concern was that mutant WbtIG191V was a ‘grey’ colony variant, which has also been reported to lack O antigen (Hartley et al., 2005). There were many phenotypic similarities between the LVS grey-colony variant and WbtIG191V. However, sequence analysis of the wbtI gene of a grey-colony variant that we isolated showed no base change or deletion from that of the parental strain or published LVS genome sequence. Therefore, it appears that any complete loss of O antigen may result in the ‘grey’ phenotype, which as mentioned above, may in part be due to the presence of IS sequences isftu2 and isftu1 upstream and downstream of the O-antigen locus.

Mutant WbtIG191V was more resistant to killing by sodium deoxycholate than the parental strain, which contrasted with results obtained by Cowley et al. (2000), who found that most O-antigen-defective mutants were more susceptible to killing by deoxycholate than the parental strain. However, the mutants tested by Cowley et al. (2000), were derived from F. novicida, which contains an O antigen distinct from that of the LVS. The ability of Gram-negative bacteria to exclude hydrophobic detergents such as deoxycholate depends largely upon the LPS maintaining the stability of the outer membrane through interaction with outer-membrane proteins, and maintaining divalent cations and a hydrophilic cell surface (Cowley et al., 2000). However, the LPS O antigen of type A and B strains is composed entirely of dideoxyglycoses, and contains the sugar 4,6-dideoxy-4-formamido-D-glucose, which is not present in F. novicida (Vinogradov et al., 2004). Furthermore, it was determined that a large amount of the LPS from type A and B strains was extracted into the phenol phase following hot aqueous phenol extraction (unpublished data). Therefore, it is probable that the unusual LPS glycosyl composition of type A and B strains makes the bacterial surface hydrophobic, resulting in enhanced interaction with detergents such as sodium deoxycholate and reduced binding of Congo red.

An important feature of mutant WbtIG191V is that it was highly attenuated in mice following IN challenge, again indicating that the O antigen is required for virulence. The IN route was used to evaluate the capability of each strain to disseminate from the lungs to other tissues, and hence is a better measure of invasiveness than the IP route. Even within 2 days post IN challenge there were more than 2 logs fewer cells of the bacterial mutant present in the tissues than the parental strain, whereas the parental strain and complemented mutant WbtIG191V::pFNLTP/wbtI continued to multiply in the tissues until the death of the animal. In contrast, the mutant strain continued to diminish in numbers until at sometime after 4 days post-challenge it was completely cleared from all tissues. Since the mutant was not quickly cleared from the tissues, it would be expected that a protective immune response would develop. Although WbtIG191V did induce protection against a relatively low IP challenge dose with the parental strain (25×LD50), less protection was provided against a higher challenge dose (75–250×LD50). However, increasing the immunization dose did increase the resistance to higher challenge doses. The IP route was used for challenge because although not natural, it is the most invasive route and therefore a more sensitive indicator of adaptive immunity, as this route bypasses many aspects of innate immunity. Nonetheless, these results indicate that a complete O antigen does contribute to maximum induction of a protective immune response. Since the LVS strain itself provides only route-dependent protection against challenge with type A F. tularensis (Chen et al., 2003;
Conlan et al., 2005; Shen et al., 2004), mice immunized with WbtLGWV were not challenged with a type A strain, but similar studies with a type A mutant are in progress.

Acknowledgements

This work was supported by grant U54-AI57168 from NIAID/NIH to the mid-Atlantic Regional Center for Excellence, by grant DAMD17-03-1-0008 from the US Army Medical Research and Material Command, and in part by the Department of Energy-funded (DE-FG09-93ER-20097) Center for Plant and Microbial Complex Carbohydrates. We thank Dr. May Chu, Centers for Disease Control, for supplying F. tularensis subsp. holarctica strain LVS, Dr. Karen Elkins, US Food and Drug Administration, for providing F. novicida strain U112 and advice on macrophage phagocytosis of F. tularensis. Anna Champion and Gretchen Berg for technical assistance, Jane Duncan for advice, and the animal care staff at the Center for Molecular Medicine and Infectious Diseases for assistance with handling and monitoring animals. We gratefully acknowledge Tina Guina, Mitchell Brittnacher and Rajinder Kaul at the University of Washington for sharing with us the DNA sequence of the F. novicida strain U112 O-antigen gene cluster. We also thank Anders Sjöstedt, Umeå University, for kindly providing vector pPV, Daphne Rainey for bioinformatics advice, and Alan Cross for review of the manuscript.

References


**Francisella tularensis** isolated from a cat. *J Vet Diagn Invest* 16, 374–381.


Edited by: N. High