Attenuation and protective efficacy of an O-antigen-deficient mutant of \textit{Francisella tularensis} LVS

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\textit{Francisella tularensis} is a zoonotic, Gram-negative cocccobacillus that causes tularemia in humans and animals. \textit{F. tularensis} subspecies \textit{tularensis} (type A) and \textit{F. tularensis} subspecies \textit{holarctica} (type B) are antigenically similar and more virulent than \textit{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 \textit{F. novicida}. One LVS-specific gene with homology to a galactosyl transferase was selected for allelic replacement using a \textit{SacB}–chloramphenicol expression suicide plasmid, and recombinants were screened for colony morphology on Congo red agar that matched that of \textit{F. novicida}. Two mutants (WbtI \textit{S187Y} and WbtI \textit{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 \textit{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 \textit{G191V} with the wild-type \textit{wbtI} gene \textit{in trans} restored normal LPS synthesis, phenotypic properties similar to the parent, and virulence in mice. Immunization with WbtI \textit{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 \textit{F. tularensis}, but also compromises the induction of full protective immunity against \textit{F. tularensis} infection in mice.

INTRODUCTION

\textit{Francisella tularensis} is a zoonotic, facultative intracellular cocccobacillus and the aetiologic agent of tularemia for a wide variety of vertebrate and invertebrate animal species; tularoëmia is a zoonotic disease for humans (Hopla & Hopla, 1994; Penn, 2005; Sjostedt, 2005; Timoney \textit{et al.}, 1988; Titball & Sjostedt, 2002). \textit{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 & Sjostedt, 2002). There are four subspecies of \textit{F. tularensis}: \textit{subsp. tularensis} (type A), \textit{subsp. holarctica} (type B), \textit{subsp. mediasiatica} and \textit{subsp. novicida} (Sjostedt, 2005). However, the International Systematics Committee recognizes \textit{Francisella novicida} as a separate species (http://www.bacterio.cict.fr/aldl.html). \textit{F. tularensis} \textit{subsp. tularensis} is the most virulent subspecies for humans, with an infectious dose

Abbreviations: Amp, ampicillin; Cm, chloramphenicol; ID, intradermal(ly); IN, intranasal(ly); IP, intraperitoneal(ly); Kan, kanamycin; LVS, live vaccine strain; PCS, precolostral calf serum; PI, post-inoculation; QuiNAc, N-acetyl quinovosamine; SSH, subtractive suppression hybridization.

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of as few as 10 cells (Dennis et al., 2001). Patients may present with several clinical forms of tularoëmia, the most common being ulceroglandular tularoëmia; other forms include oculoglandular, oropharyngeal, intestinal, pneumonic and typhoidal tularoëmia. Pneumonic tularoëmia 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 dideoxyglycoses, the core oligosaccharide contains man- nose 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 intraperitoneal (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) (BHIc) at 37 °C. For culture on agar plates, 5% (v/v) sheep blood was added to BHIc agar (BHIcB), 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 chloramphenicol on Gram-negative cells (Golovlev 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 SacI restriction site and the 3′ primers incorporated either a BamHI restriction site or a PsiI restriction site. The upstream and downstream PCR products were digested with SacI/BamHI and SacI/PsiI, respectively, and separately cloned into pBluescriptKS+ (Stratagene). The two recombinant clones were digested with SacI/
the plasmid from the genome and presumably also the wild-type Cm-sensitive colonies were isolated to identify clones that had deleted sucrose to select for a second recombination event. Sucrose-resistant, resistant colonies were subcultured onto medium containing 5% genome, since the plasmid cannot replicate in the LVS. The Cm-iridescence (indicating a loss of surface carbohydrate) under gene. Sucrose-resistant colonies were screened for diminished ligated simultaneously into SalBamI-digested pPV, resulting in plasmid ara-leu lacX74 galU galK rpsL lacZ80d lacZ(D80), and subcloned into the corresponding

### 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>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td>DH5x</td>
<td>F- φ80d lacZAM15A (lacZYA-argF) U169 recA1 endA1 hsdR17(rK m+); phoA supE44; recA1 gyrA96 relA1</td>
<td>Invitrogen</td>
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<tr>
<td>TOP10</td>
<td>F- mcrA (Δmr-hsdRMS merBC) φ80 lacAM15 ΔlacX74 deor recA1 araD139 Δ ara-leu) 7697 galU galK rpsL(5m') endA1 nupG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Mach1 TH</td>
<td>F′ (lacZΔM) ΔM15 ΔlacX74 hsdR(rK m+) 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. holarctica LVS</td>
<td>Dr Karen Elkins</td>
</tr>
<tr>
<td>U112</td>
<td>F. novicida wild-type strain</td>
<td></td>
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<tr>
<td>WbtI_S187Y</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>WbtI_G191Y</td>
<td>LVS mutant deficient in O-antigen biosynthesis, with mutation in amino acid 191 (G191Y) of WbtI</td>
<td>This study</td>
</tr>
<tr>
<td>A. pleuropneumonia J45-100</td>
<td>Isogenic non-encapsulated, serotype 5 mutant with deletion in cps5ABC</td>
<td>Ward et al. (1998)</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<td>pBlueScript SK– pUC19</td>
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<td>Stratagene</td>
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<tr>
<td>pCR 2.1-TOPO</td>
<td>Cloning vector; Amp'</td>
<td>Invitrogen</td>
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<tr>
<td>pPV</td>
<td>Knockout vector; Amp', Cm', sacB, mob</td>
<td>Invitrogen</td>
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<tr>
<td>pFNLTP6</td>
<td>Complementation shuttle vector; Km', Amp'</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 pFNLTP6 for complementation without groE promoter; Km', Amp'</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>
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<tr>
<td>FA6-7-NF</td>
<td>5′- TAGCAGCTGTCGACAAGAAGTAAACC-3′; SalI restriction site in bold type; PCR primer for wbtB knockout</td>
<td>This study</td>
</tr>
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<td>FA6-7-NR</td>
<td>5′- TAAAAGGACCTTATTAGAGTCTATTAT-3′; BamHI restriction site in bold type; PCR primer for wbtB knockout</td>
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<td>FA6-7-CF</td>
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<tr>
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<td>5′- ATCAGGCCGCACTATTACAAAGT-3′; PCR primer for confirming wbtB knockout</td>
<td>This study</td>
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<td>FA5-8CR1</td>
<td>5′- CTTTGGATAGTGGCTTTTGGAGGC-3′; PCR primer for confirming wbtB knockout</td>
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<td>5′- ACGGGAATTTGCTGATTTACCTACATTACGGAC-3′; EcoRI restriction site in bold type; forward PCR primer for wild-type wbtI cloning for in trans complementation</td>
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<tr>
<td>R-wbtl-Nhel</td>
<td>5′- ACGGCTAGTTCCCGAGATTGTTACATT-3′; Nhel restriction site in bold type; reverse PCR primer for wild-type wbtI cloning for in trans complementation</td>
<td>This study</td>
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BamHI and both the upstream and downstream insert fragments were ligated simultaneously into SalI-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 BHIC agar containing 100 μg polymyxin B ml⁻¹ (to inhibit the donor strain) and 10 μg Cm ml⁻¹. Cm-resistant and polymyxin B-resistant colonies were screened for the presence of the pPV-encoded Amp-resistance gene, which was confirmed by PCR, indicating that pFA501 had integrated into the genome, since the plasmid cannot replicate in the LVS. The Cm-resistant colonies were subcultured onto medium containing 5% sucrose to select for a second recombination event. Sucrose-resistant, Cm-sensitive colonies were isolated to identify clones that had deleted the plasmid from the genome and presumably also the wild-type wbtB gene. Sucrose-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 1⁻¹. 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 wbtB from the LVS was amplified by PCR using primers F-wbtl-EcoRI and R-wbtl-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 pFNLTP6 (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 10^6 c.f.u. ml^-1), washed three times with 0.5 M sucrose, and suspended in 10 ml 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 mutant proteins WbtIS192 and WbtIS192 were modelled as previously described (Li *et al.*, 2005) to predict the conformational changes caused by residue substitution. *Helicobacter pylori* aminotransferase Psec (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/~3djigsw). 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-Aldrich) 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). Proteinase K (Sigma-Aldrich) 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 A260 and A325 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) and Emerald Q fluorescence staining (Molecular Probes), 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 *F. tularensis* LVS (Inzana *et al.*, 2004), rabbit polyclonal antiserum to *F. novicida* U112 (1:1000 dilution each) or murine mAb (Chemicon International) to with 4-chloro-1-naphthol (Bio-Rad). Rabbit polyclonal antiserum to GroEL (Abcam) was used as secondary conjugates at 1 : 2000 dilution, and colour was developed peroxidase (HRP; Jackson ImmunoResearch Laboratories) were used. Anti-rabbit IgG or anti-murine IgG coupled to horseradish antiserum to developed with rabbit polyclonal antiserum to antibacterial activity of 0–40% pre-colonial calf serum (PCS, which contains no antibodies) for the LVS, mutant WbtIS192 and WbtIS192; pFNLT/pwbtI 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.

**Sensitivity to sodium deoxycholate.** The bactericidal activity of 0–40% pre-colonial calf serum (PCS, which contains no antibodies) for the LVS, mutant WbtIS192 and WbtIS192; pFNLT/pwbtI 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% CO₂, extracellular bacteria were removed by washing the cells with PBS, and the medium was replaced with 1 ml complete Dulbeco’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% CO₂ 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 lysate 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, 6000 or 2000 c.f.u. per mouse), mutant WbtIS192 (10^5, 10^6, 5 x 10^6, 1.4 x 10^7 or 2.8 x 10^7 c.f.u. per mouse) or complemented mutant WbtIS192; pFNLT/pwbtI (10^8 c.f.u. per...
Mice were immunized either intradermally (ID) or IP followed by IP challenge, which was used because the LD$_{50}$ of LVS for mice is lowest by the IP route (Fortier et al., 1991). For ID immunization, groups of five BALB/c mice each were inoculated with 100 μl LVS (10$^5$ c.f.u.), mutant WbtI$_{G191V}$ (10$^5$ c.f.u.), complemented mutant WbtI$_{G191V}$::pFNLT/pwbtl (10$^5$ c.f.u.) or PBS alone. Fourteen days post-inoculation (PI), the mice were reimmunized with the same doses of the same strains, and 21 days later the mice were challenged IP with 25 × $\text{LD}_{50}$ (3 × 10$^3$ c.f.u.), 75 × $\text{LD}_{50}$ (9 × 10$^3$ c.f.u.) or 250 × $\text{LD}_{50}$ (3 × 10$^4$ c.f.u.) of the LVS parent strain (LD$_{50}$ 120 c.f.u.). For IP immunization, groups of five BALB/c mice each were inoculated with 10$^3$, 10$^4$ or 5 × 10$^4$ c.f.u. of the mutant in 100 μl of PBS twice 2 weeks apart. Twenty-one days after the second immunization the mice were challenged IP with 75 × $\text{LD}_{50}$ (groups immunized with 10$^5$ or 10$^6$ c.f.u. WbtI$_{G191V}$ or 250 × $\text{LD}_{50}$ (group immunized with 5 × 10$^4$ c.f.u. WbtI$_{G191V}$) of LVS. Each challenged animal was monitored for 21 days, and severely moribund mice were euthanized. Surviving mice were humanely euthanized using excess CO$_2$, and select tissues cultured for the presence of bacteria, as described above.

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 × 2 contingency tables. All statistical analyses were carried out using InStat software (Graphpad).

RESULTS

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 wbtI, 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 WbtI$_{S187Y}$ caused a codon change of TCT→TAT, resulting in the change of Ser to Tyr in residue 187. A G→T transversion in mutant WbtI$_{G191V}$ causing a codon change of GGT→GGT resulted in residue 191 changing from Gly to Val. The wbtI 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 WbtI$_{G191V}$ 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 α-helices, β-sheets and turns of the enzyme, which would result in substantial conformational changes, suggesting that WbtI$_{S187Y}$ and WbtI$_{G191V}$ had lost biological activity.

Physical and chemical characterization of LPS

Since the mutation in WbtI$_{G191V}$ was predicted to be in the O-antigen region, purified LPS samples from WbtI$_{G191V}$ 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 WbtI$_{G191V}$ 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 WbtI$_{G191V}$ 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 WbtI$_{G191V}$ O antigen reacted with antiserum to F. novicida strain U112 in a Western blot, indicating that the mutant did not
Fig. 1. Western blots of LPS from the LVS and WbtIG191V 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 WbtIG191V 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 WbtIG191V 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, WbtIG191V LPS; 3, F. novicida LPS.

contain a F. novicida-reactive O antigen. The reactivity of low-molecular-mass LPS from mutant WbtIG191V with F. novicida antiserum indicated conservation of core LPS epitopes between the mutant and F. novicida. The lack of reactivity of low-molecular-mass LVS LPS with F. novicida antiserum was probably due to steric interference of core epitopes by O antigen, further supporting the suggestion that the LPS of WbtIG191V lacked O antigen (Fig. 1c).

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-acetylglicosamine 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 WbtIG191V (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 WbtIG191V LPS, confirming that the O antigen was not present in the mutant. The presence of a complete core structure (Vinogradov et al., 2002) in WbtIG191V, 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 WbtIG191V, 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 WbtIG191V. 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 WbtIG191V 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 OD600 was monitored for 8 h, by which time all strains had reached stationary phase. The growth rates of WbtIG191V, the LVS parental strain and complemented mutant WbtIG191V::pFNLT6/wbtI 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 WbtIG191V::pFNLT6/wbtI 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 WbtIG191V 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 WbtIG191V grew slowly but continuously during the first 48 h, but the growth rate increased dramatically after 48 h. Complemented mutant WbtIG191V::pFNLT6/wbtI 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

by Cowley et al., 2000). Therefore, the sens-
itivity of WbtI G191V 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 WbtI G191V 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 WbtI G191V cells remained
viable. Moreover, up to 4.4 % of WbtI G191V mutant cells
were able to tolerate up to 10 % deoxycholate. Complemented mutant WbtI G191V::pFNLTp/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 WbtI G191V to mice

For this study, mice were inoculated IP with doses of LVS, mutant WbtI G191V or complemented mutant WbtI G191V::pFNLTp/wbtI that ranged from 200 to 2.8×10^7 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 2.8×10^5 c.f.u. mutant WbtI G191V remained clinically normal. Mice infected with the two highest doses of the mutant (1.4×10^6 and 2.8×10^7 c.f.u.) showed some early clinical symptoms, such as ruffled fur, stressed breathing and hunched gait, but recovered completely. Mice challenged with 10^4 c.f.u. complemented mutant WbtI G191V::pFNLTp/wbtI became moribund within 2 days and died by 4 days PI.

Clearance of the LVS, mutant WbtI G191V and complemented mutant from the tissues of challenged mice was evaluated following IN inoculation with 10^5 c.f.u. of each strain. At 48 h PI there were about 2–3 logs fewer cells of mutant WbtI G191V 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

Fig. 4. Complement-mediated killing of *F. tularensis* in PCS. Values shown are percentage killing of *F. tularensis* or non-encapsulated control strain *A. pleuropneumoniae* J45-100 in various dilutions of PCS after 60 min incubation at 37 °C. (■) LVS parent strain; (●) mutant strain WbtI<sub>G191V</sub>; (○) complemented mutant WbtI<sub>G191V</sub>:pFNLT6/wbtI; (△) serum-susceptible control strain *A. pleuropneumoniae* J45-100. Each point represents the mean of three experiments. Where error bars are not visible, the SEM was less than 1%.

Fig. 5. Intracellular growth of *F. tularensis* in J774A.1 cells. The J774A.1 monolayer was infected with LVS, mutant strain WbtI<sub>G191V</sub> or complemented strain WbtI<sub>G191V</sub>:pFNLT6/wbtI, and intracellular bacterial growth was monitored up to 72 h post-infection, as described in Methods. Data are shown on a log scale as the average number of bacteria recovered from dilutions of lysates of J774A.1 cells. The results shown were from a single experiment with samples tested in triplicate at each time point. Identical experiments were repeated on at least two other days with similar results. (◎) LVS; (●) mutant strain WbtI<sub>G191V</sub>; (□) complemented mutant strain WbtI<sub>G191V</sub>:pFNLT6/wbtI.

Fig. 6. Susceptibility of *F. tularensis* parental and mutant strains to various concentrations of sodium deoxycholate. Bacteria were suspended in microtitre wells at 5×10<sup>5</sup> c.f.u. in 100 µl and an equal volume of sodium deoxycholate was added to the final concentrations indicated. Aliquots were plated at 0 min and again after 45 min incubation at 37 °C. Results shown are from a single experiment with each strain tested in triplicate, but identical experiments with similar results were done on three other separate occasions. The 120% survival of WbtI<sub>G191V</sub> was due to growth of the bacteria during the 45 min incubation period. Sds of less than 1% are not visible. (■) LVS; (●) mutant strain WbtI<sub>G191V</sub>; (○) complemented strain WbtI<sub>G191V</sub>:pFNLT6/wbtI.

Fig. 3. Western blot of LPS from complemented mutant WbtI<sub>G191V</sub>:pFNLT6/wbtI. A normal copy of wbtI was amplified by PCR and its sequence confirmed, and it was then cloned into pFNLT6 with and without the groE promoter. Each strain was grown on BHIBC and the LPS extracted by a micro-phenol/water procedure for SDS-PAGE analysis (Inzana, 1983). Samples (5 µl) of LPS extract were applied to each lane, followed by electrophoresis and Western blotting with rabbit antiserum to LVS. Lanes: 1, LVS; 2, WbtI<sub>G191V</sub>:pFNLT6 alone; 3, WbtI<sub>G191V</sub>:pFNLT6/wbtI without the groE promoter; 4, WbtI<sub>G191V</sub>:pFNLT6/wbtI with the groE promoter; 5, molecular weight standards.

Fig. 2. Western blot of LPS from parental strain LVS. A normal copy of wbtI was amplified by PCR and its sequence confirmed, and it was then cloned into pFNLT6 with and without the groE promoter. Each strain was grown on BHIBC and the LPS extracted by a micro-phenol/water procedure for SDS-PAGE analysis (Inzana, 1983). LPS from each strain was separated by SDS-PAGE and Western blotting with rabbit antiserum to LVS. Lanes: 1, LVS; 2, parental strain LVS; 3, complemented strain WbtI<sub>G191V</sub>:pFNLT6/wbtI; 4, parental strain LVS; 5, molecular weight standards.
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 2.5 \times 10^6 LVS died, whereas none of the mice immunized with LVS then challenged with 2.5 \times 10^5 of the same strain died (not shown). None of the five mice immunized with WbtIG191V and subsequently challenged with 2.5 \times 10^5 LVS died. However, two and three out of five mice immunized with the mutant and later challenged with 75 or 250 \times 10^5 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

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

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with 10^4 c.f.u. mutant WbtI_{G191V} and later challenged with 75 \times LD_{50} LVS survived. Similarly, three of five mice immunized with 5 \times 10^3 c.f.u. mutant and later challenged with 250 \times 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 *wbtB*, and genomic region VII as the O-antigen locus.

Attempts to knock out *wbtB* using the *sacB–Cm^R* cassette in vector pPV resulted in the cointegration of the *sacB–Cm^R* gene, as determined by PCR amplification of plasmid DNA from the genome. However, excision of the cointegrant in the presence of sucrose apparently resulted in relocation of the original sequence without deletion of *wbtB*, as PCR and Southern blotting indicated a single, normal copy of *wbtB* 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 PCR and Southern blotting indicated a single, normal copy of WbtI 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 enzymatic function...
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

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::pFNLTIP/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 WbtI<sub>G191V</sub> were not challenged with a type A strain, but similar studies with a type A mutant are in progress.

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