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

Correspondence
Thomas J. Inzana
tinzana@vt.edu

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 (Wbtl_{S187Y} and Wbtl_{G191V}) were isolated that contained substitutions in conserved motifs in the sugar transaminase/perosamine synthetase (Wbtl) of the O-antigen locus, and the latter mutant was extensively tested and characterized. Wbtl_{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 Wbtl_{G191V} with the wild-type *wbtl* gene *in trans* restored normal LPS synthesis, phenotypic properties similar to the parent, and virulence in mice. Immunization with Wbtl_{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.

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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; tularaemia 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*: subsp. *tularensis* (type A), subsp. *holarctica* (type B), subsp. *mediasiatica* and subsp. *novicida* (Sjöstedt, 2005). However, the International Systematics Committee recognizes *Francisella novicida* as a separate species (<http://www.bacterio.cict.fr/aldl.html>). *F. tularensis* subsp. *tularensis* is the most virulent subspecies for humans, with an infectious dose

†These authors contributed equally to this work.

‡Present address: The Institute for Genomic Research, Rockville, MD, USA.

§Present address: College of Pharmacy, University of Southern Nevada, Henderson, NV, USA.

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.

of as few as 10 cells (Dennis *et al.*, 2001). Patients may present with several clinical forms of tularaemia, the most common being ulceroglandular tularaemia; other forms include oculoglandular, oropharyngeal, intestinal, pneumonic and typhoidal tularaemia. Pneumonic tularaemia 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 than 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 & Sjøstedt, 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 mannose 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.

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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* DH5 α 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 (BHIBC), and the cultures were incubated at 37°C in 5% CO_2 , 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*–Cm^R cassette that confers sucrose sensitivity and resistance to chloramphenicol on Gram-negative cells (Golovliov *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 $\times$ 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 *Sall* restriction site and the 3' primers incorporated either a *Bam*HI restriction site or a *Pst*I restriction site. The upstream and downstream PCR products were digested with *Sall*/*Bam*HI and *Sall*/*Pst*I, respectively, and separately cloned into pBluescriptKS+ (Stratagene). The two recombinant clones were digested with *Sall*/

Table 1. Bacterial strains, plasmids, and DNA primers used in this study

Strain, plasmid or primer	Characteristics	Reference or source
Strains		
DH5 α	F ⁻ Φ 80d <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) <i>U169 recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE44</i> λ ⁻ <i>thi-1 gyrA96 relA1</i>	Invitrogen
TOP10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS mcrBC</i>) Φ 80 <i>lac</i> Δ M15 Δ <i>lacX74 deoR recA1 araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Sm ^r) <i>endA1 nupG</i>	Invitrogen
Mach1 T1 ^R	F ⁻ Φ 80(<i>lacZ</i>) Δ M15 Δ <i>lacX74 hsdR</i> (r _K ⁻ m _K ⁺) Δ <i>recA1398 endA1 tonA</i>	Invitrogen
S17-1	<i>thi thr leu tonA lacy supE recA::RP4-2-Tc::Mu, Kn::Tn7</i>	Golovliov <i>et al.</i> (2003)
LVS	subsp. <i>holarctica</i> LVS	Dr May Chu
U112	<i>F. novicida</i> wild-type strain	Dr Karen Elkins
WbtI _{S187Y}	LVS mutant deficient in O-antigen biosynthesis, with mutation in amino acid 187 (S187Y) of WbtI	This study
WbtI _{G191V}	LVS mutant deficient in O-antigen biosynthesis, with mutation in amino acid 191 (G191V) of WbtI	This study
<i>A. pleuropneumoniae</i> J45-100	Isogenic non-encapsulated, serotype 5 mutant with deletion in <i>cps5ABC</i>	Ward <i>et al.</i> (1998)
Plasmids		
pBlueScript SK-	Cloning vector; Amp ^r	Stratagene
pUC19	Cloning vector; Amp ^r	Invitrogen
pCR 2.1-TOPO	Cloning vector for SSH PCR products; Amp ^r , Km ^r	Invitrogen
pPV	Knockout vector; Amp ^r , Cm ^r , <i>sacB</i> , <i>mob</i>	Golovliov <i>et al.</i> (2003)
pFNLTP6	Complementation shuttle vector; Km ^r , Amp ^r	Maier <i>et al.</i> (2004)
pTZ817	<i>EcoRI</i> - and <i>NheI</i> -digested 2.0 kb wild-type <i>wbtI</i> PCR fragment cloned into pFNLTP6 for complementation without <i>groE</i> promoter; Km ^r , Amp ^r	This study
pTZ819	pTZ817 containing <i>F. tularensis groE</i> promoter in <i>KpnI</i> site	This study
DNA primers		
FA6-7-NF	5'-TAGCACTGTCGACGAAGAAGTAACC-3'; <i>Sall</i> restriction site in bold type; PCR primer for <i>wbtB</i> knockout	This study
FA6-7-NR	5'-TAGAAAGGATCCTATTAGAGTCTATTATAT-3'; <i>BamHI</i> restriction site in bold type; PCR primer for <i>wbtB</i> knockout	This study
FA6-7-CF	5'-AAGAATCTTAGCTGCAGGTTTGAGTAG-3'; <i>PstI</i> restriction site in bold type; PCR primer for <i>wbtB</i> knockout	This study
FA6-7-CR	5'-TTTTTCGTCGACAAAACCAATAAATATCAAGAC-3'; <i>Sall</i> restriction site in bold type; PCR primer for <i>wbtB</i> knockout	This study
FA5-8NF1	5'-ATCAGGCAGCATATTCAAAGT-3'; PCR primer for confirming <i>wbtB</i> knockout	This study
FA5-8CR1	5'-CTTTGGATAGTTGCCTTTTGAGC-3'; PCR primer for confirming <i>wbtB</i> knockout	This study
F-wbtI-EcoRI	5'-ACGGGAATTCTGCTTATTCCTTACCAATTGAC-3'; <i>EcoRI</i> restriction site in bold type; forward PCR primer for wild-type <i>wbtI</i> cloning for <i>in trans</i> complementation	This study
R-wbtI-NheI	5'-ACGGGCTAGCTTCCCGCATTTGTACTATTT-3'; <i>NheI</i> restriction site in bold type; reverse PCR primer for wild-type <i>wbtI</i> cloning for <i>in trans</i> complementation	This study

BamHI and both the upstream and downstream insert fragments were ligated simultaneously into *Sall*-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 μ g polymyxin B ml⁻¹ (to inhibit the donor strain) and 10 μ g 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 l⁻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 *wbtI* complementation. A 2.0 kb DNA fragment containing *wbtI* from the LVS was amplified by PCR using primers F-wbtI-EcoRI and R-wbtI-NheI, as described above. The resulting fragment was cloned into pCR2.1-TOPO, digested with *EcoRI* and *NheI*, 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* WbtI_{G191V}. The bacteria were grown to a density of about 110 Klett units (correlating to about 10^9 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 WbtI_{S187Y} and WbtI_{G191V} 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/~3djigsaw/>). 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 A₂₈₀ 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 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 methanolysis (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 × 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 trihydroxyacetophenone ml⁻¹ in methanol, and 15 mg nitrocellulose ml⁻¹ in acetone: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 WbtI_{G191V} and complemented strain WbtI_{G191V}::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 5×10^5 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% precolostral calf serum (PCS, which contains no antibodies) for the LVS, mutant WbtI_{G191V} and WbtI_{G191V}::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 WbtI_{G191V} and the complemented mutant WbtI_{G191V}::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% CO₂, extracellular bacteria were removed by washing the cells with PBS, and the medium was replaced with 1 ml complete Dulbecco'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, 600 or 2000 c.f.u. per mouse), mutant WbtI_{G191V} (10^3 , 10^4 , 5×10^4 , 1.4×10^6 or 2.8×10^7 c.f.u. per mouse) or complemented mutant WbtI_{G191V}::pFNLTP/*wbtI* (10^4 c.f.u. per

mouse) in 100 µl PBS. IP inoculations were used to assess virulence because the LVS is most virulent to mice by this route (Fortier *et al.*, 1991), and therefore this route is the greatest test for attenuation. For tissue-clearance studies, 3–5 mice were inoculated intranasally (IN) with 10^7 c.f.u. per mouse of LVS, WbtI_{G191V} or WbtI_{G191V}::pFNLTP/*wbtI* in 20 µl PBS. IN challenge was also used because it is a natural but still severe challenge route (Conlan *et al.*, 2003), and is a measure of the capability of a strain to disseminate. Mice were anaesthetized with mixed oxygen/isoflurane gas prior to inoculation. All inoculation doses were confirmed by viable plate counting on BHIBC agar. Animals exposed to the LVS or mutant strains were maintained and cared for in an accredited ABSL-2 facility. In some cases, mice were euthanized with excess CO₂ at 2, 4, 8 or 16 days post-challenge, and bacterial numbers in the spleen, liver and lung were determined following tissue homogenization, serial dilution and viable plate counting.

Mice were immunized either intradermally (ID) or IP followed by IP challenge, which was used because the LD₅₀ 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 (10^4 c.f.u.), mutant WbtI_{G191V} (10^5 c.f.u.), complemented mutant WbtI_{G191V}::pFNLTP/*wbtI* (10^4 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 $25 \times \text{LD}_{50}$ (3×10^3 c.f.u.), $75 \times \text{LD}_{50}$ (9×10^3 c.f.u.) or $250 \times \text{LD}_{50}$ (3×10^4 c.f.u.) of the LVS parent strain (LD₅₀ ~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 $75 \times \text{LD}_{50}$ (groups immunized with 10^3 or 10^4 c.f.u. WbtI_{G191V}) or $250 \times \text{LD}_{50}$ (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 CO₂, 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→GTT 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 transaminase/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 WbtI_{G191V} 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_{G191V} 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_{G191V} 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_{G191V} LPS; 3, *F. novicida* LPS.

contain a *F. novicida*-reactive O antigen. The reactivity of low-molecular-mass LPS from mutant WbtI_{G191V} 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 WbtI_{G191V} 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*-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_{G191V} (17 ± 1, 33 ± 3, 5 ± 1, 15 ± 5 and 23 ± 3, respectively). However, the O-antigen-specific glycoside *N*-acetyl quinovosamine (QuiNAc) was not detected in WbtI_{G191V} 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_{G191V}, 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_{G191V}, a normal copy of *wbtI* was amplified and cloned into shuttle vector pFNLTP6 alone and behind the *groE* promoter, and each construct was electroporated into WbtI_{G191V}. 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 pFNLTP6 alone, and mutants containing pFNLTP6/*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_{G191V} 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 WbtI_{G191V}, the LVS parental strain and complemented mutant WbtI_{G191V}::pFNLTP/*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 WbtI_{G191V}::pFNLTP/*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 WbtI_{G191V} 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_{G191V} grew slowly but continuously during the first 48 h, but the growth rate increased dramatically after 48 h. Complemented mutant WbtI_{G191V}::pFNLTP/*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).

Fig. 2. Analysis of mutant WbtI_{G191V} LPS by MALDI-MS. The upper panel represents analysis of unmodified oligosaccharide core. The peak at *m/z* 1109 is consistent with a core containing a 2-keto-3-deoxyoctonic acid (3-deoxy-D-manno-2-octulosonic acid) (KDO) residue, four hexoses and one *N*-acetylhexosamine. There are also peaks at 14 amu below and 162 amu above this peak, which would be consistent with replacement of the acetyl group on hexosamine with a formyl group and addition of one hexose unit, respectively. The peaks at *m/z* 1012 and 1175 also represent a difference of one hexose unit and have corresponding peaks 18 amu lower than themselves, which would be consistent with loss of water. These may represent breakdown products of the core. The lower panel represents MALDI-MS of oligosaccharide core methylated twice. Methylation of the sample results in a shift from *m/z* 1109 to 1407. Breakdown products corresponding to loss of water, methanol and a 30 amu species were also observed in the KDO-containing oligosaccharides, as expected. Note that loss of KDO during MS eliminates these companion peaks from the *m/z* 926 species.

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 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

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 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

Fig. 3. Western blot of LPS from complemented mutant *Wbtl_{G191V}::pFNLTP/wbtl*. A normal copy of *wbtl* was amplified by PCR and its sequence confirmed, and it was then cloned into pFNLPT6 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, *Wbtl_{G191V}::pFNLTP6* alone; 3, *Wbtl_{G191V}::pFNLTP/wbtl* without the *groE* promoter; 4, *Wbtl_{G191V}::pFNLTP/wbtl* with the *groE* promoter; 5, molecular weight standards.

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 *Wbtl_{G191V}*; (◆) complemented mutant *Wbtl_{G191V}::pFNLTP/wbtl*; (▼) 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 *Wbtl_{G191V}* or complemented strain *Wbtl_{G191V}::pFNLTP/wbtl*, 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 *Wbtl_{G191V}*; (□) complemented mutant strain *Wbtl_{G191V}::pFNLTP/wbtl*.

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. 6. Susceptibility of *F. tularensis* parental and mutant strains to various concentrations of sodium deoxycholate. Bacteria were suspended in microtitre wells at 5×10^5 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 *Wbtl_{G191V}* was due to growth of the bacteria during the 45 min incubation period. SDS of less than 1 % are not visible. (■) LVS; (▲) mutant strain *Wbtl_{G191V}*; (●) complemented strain *Wbtl_{G191V}::pFNLTP/wbtl*.

Table 2. Virulence of LVS and WbtI_{G191V} in BALB/c mice

ND, Not determined.

Challenge dose IP (c.f.u)*	Dead/total number of mice†	
	LVS	WbtI _{G191V}
200	3/5	0/5
600	5/5	0/5
2×10^3 for LVS; 10^3 for WbtI _{G191V}	5/5	0/5
1×10^4	ND	0/5
5×10^4	ND	0/5
2.8×10^5	ND	0/5
1.4×10^6	ND	0/5
2.8×10^7	ND	0/5

*The LD₅₀ 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 1.4×10^6 c.f.u. and three mice challenged with 2.8×10^7 c.f.u. of mutant WbtI_{G191V} showed similar clinical symptoms, but completely recovered by 5 days PI.

WbtI_{G191V} 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 WbtI_{G191V}::pFNLTP/*wbtI* died or were euthanized by the fifth day PI, whereas by 8

days post-challenge all the mice challenged with WbtI_{G191V} appeared normal and had cleared the bacteria from all of their tissues (data not shown).

Immunoprotective capacity of WbtI_{G191V} for mice

Mice were immunized either ID or IP with mutant WbtI_{G191V} 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. WbtI_{G191V} without adjuvant twice, 2 weeks apart; control groups were inoculated with PBS alone or 10^4 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 $25 \times \text{LD}_{50}$ LVS died, whereas none of the mice immunized with LVS then challenged with $250 \times \text{LD}_{50}$ of the same strain died (not shown). None of the five mice immunized with WbtI_{G191V} and subsequently challenged with $25 \times \text{LD}_{50}$ LVS died. However, two and three out of five mice immunized with the mutant and later challenged with 75 or $250 \times \text{LD}_{50}$ 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 5×10^4 c.f.u. WbtI_{G191V} twice 2 weeks apart. Three weeks after the second immunization the mice were challenged IP with various doses of LVS, as

Fig. 7. Clearance of *F. tularensis* from the tissues of mice following IN inoculation. Surviving mice were euthanized at 48, 96 and 192 h PI (the last time is not shown). The lung, liver and spleen were aseptically removed and homogenized in PBS, and the c.f.u. (mg tissue)⁻¹ was determined. By 5 days PI all mice challenged with 10^5 c.f.u. LVS or complemented mutant WbtI_{G191V}::pFNLTP/*wbtI* died or needed to be euthanized. By 8 days PI all mice challenged with 10^5 c.f.u. WbtI_{G191V} were clinically normal and no bacteria were found in any of the tissues. Therefore, only results through to day 4 are shown. Filled bars, mutant WbtI_{G191V}; open bars, LVS; hatched bars, complemented mutant WbtI_{G191V}::pFNLTP/*wbtI*.

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 10^5 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 $25 \times \text{LD}_{50}$; (◆) immunized mice challenged with $25 \times \text{LD}_{50}$ [protection was highly significant ($P=0.004$)]; (■) immunized mice challenged with $75 \times \text{LD}_{50}$; (▲) immunized mice challenged with $250 \times \text{LD}_{50}$. (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 $25 \times \text{LD}_{50}$; (◆) mice immunized with 10^3 c.f.u. followed by challenge with $75 \times \text{LD}_{50}$; (■) mice immunized with 5×10^4 c.f.u. and challenged with $75 \times \text{LD}_{50}$ ($P=0.083$); (▲) 5×10^4 c.f.u.-immunized mice challenged with $250 \times \text{LD}_{50}$ ($P=0.083$). Following challenge mice were monitored for 12 days. Mice that appeared to be severely moribund were euthanized and counted as dead.

described in Methods. All five mice immunized with 10^3 c.f.u. WbtI_{G191V} and subsequently challenged with $75 \times \text{LD}_{50}$ LVS died. However, three of five mice immunized

with 10^4 c.f.u. mutant WbtI_{G191V} and later challenged with $75 \times \text{LD}_{50}$ LVS survived. Similarly, three of five mice immunized with 5×10^4 c.f.u. mutant and later challenged with $250 \times \text{LD}_{50}$ 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 cointegrand in the presence of sucrose apparently resulted in religation 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 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

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/perosamine 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, WbtI_{G191V} was completely killed by fresh 3% PCS, which lacks antibody, but is rich in complement. In contrast, the parent and complemented mutant were completely resistant to killing by a high concentration of PCS, even in the presence of hyperimmune rabbit serum. Thus, the LPS O antigen is essential to *F. tularensis* for resistance to the bactericidal activity of serum, and therefore virulence. However, WbtI_{G191V} 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 WbtI_{G191V} 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 WbtI_{G191V} 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 WbtI_{G191V} 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 WbtI_{G191V}. 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 WbtI_{G191V} 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 WbtI_{G191V} 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 WbtI_{G191V}::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 WbtI_{G191V} 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_{G191V} were not challenged with a type A strain, but similar studies with a type A mutant are in progress.

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