

## The Immunologically Distinct O Antigens from *Francisella tularensis* Subspecies *tularensis* and *Francisella novicida* Are both Virulence Determinants and Protective Antigens<sup>∇</sup>

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**We have determined the sequence of the gene cluster encoding the O antigen in *Francisella novicida* and compared it to the previously reported O-antigen cluster in *Francisella tularensis* subsp. *tularensis*. Immunization with purified lipopolysaccharide (LPS) from *F. tularensis* subsp. *tularensis* or *F. novicida* protected against challenge with *Francisella tularensis* subsp. *holarctica* and *F. novicida*, respectively. The LPS from *F. tularensis* subsp. *tularensis* did not confer protection against challenge with *F. novicida*, and the LPS from *F. novicida* did not confer protection against challenge with *F. tularensis* subsp. *holarctica*. Allelic replacement mutants of *F. tularensis* subsp. *tularensis* or *F. novicida* which failed to produce O antigen were attenuated, but exposure to these mutants did not induce a protective immune response. The O antigen of *F. tularensis* subsp. *tularensis* appeared to be important for intracellular survival whereas the O antigen of *F. novicida* appeared to be critical for serum resistance and less important for intracellular survival.**

*Francisella tularensis* is the causative agent of tularemia, a disease that affects many mammals including humans and rodents. The bacterium is a small (0.2 to 0.5 μm by 0.7 to 1.0 μm) gram-negative intracellular pathogen, and the natural reservoir is thought to be rodents, with ticks being the primary vector (11).

*F. tularensis* has been divided into three subspecies: *F. tularensis* subsp. *tularensis* (also known as type A) is the most virulent and was thought to be confined to North America but has recently been isolated in Europe (16, 26). *F. tularensis* subsp. *holarctica* (also known as type B) is less virulent and is found mainly in Europe and in Asia (27). *F. tularensis* subsp. *mediasiatica* has only been isolated from locations in Central Asia (27, 32) and is considered to be of relatively low virulence (10, 26). In addition, *Francisella novicida*, which is considered by some investigators to be another subspecies of *F. tularensis*, is reported to be pathogenic only in immunocompromised humans (10).

The sequences of O-antigen gene clusters and the structures of the O antigens from strain SchuS4 (*F. tularensis* subsp. *tularensis*) and from the LVS strain (*F. tularensis* subsp. *holarctica*) have previously been shown to be identical (31, 39, 40). In contrast, although the *F. novicida* (strain U112) O antigen has many sugars in common with the *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* O antigens, it is struc-

turally distinct (39). This suggests that the O-antigen gene cluster is also distinct in *F. novicida*. To date, the sequences of the genes that encode the O antigen of the LPS of *F. novicida* have not been reported.

Several studies have shown that antibody to the lipopolysaccharide (LPS) of *F. tularensis* can play a role in protective immunity to tularemia (12, 31). It is not clear whether the *F. novicida* O antigen is immunologically distinct from the O antigen produced by *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*. However, Shen et al. showed that immunization of mice with *F. novicida* induces only marginal protection against a subsequent challenge with *F. tularensis* subsp. *tularensis* or *F. tularensis* subsp. *holarctica* strains (35). This suggests that the differences in the structures of the O antigens from *F. novicida* and *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* structure are too great to confer cross-protection.

There is some evidence from studies with the LVS strain that the O antigen plays a role in the virulence of *F. tularensis* and *F. novicida* (17). The LVS strain forms two colony types or opacity variants, referred to as blue and gray variants, when grown on solid medium (9). The gray variants are devoid of O antigen and are less virulent than the blue variants. In addition, they do not induce protective immunity in mice (17). However, the genetic events which accompany this phenotypic change are not known, and it is possible that there are differences additional to the loss of O-antigen expression (17). Also, it is known that the pathogenesis of tularemia in mice caused by the LVS strain is not identical to the pathogenesis of the disease caused by high-virulence strains (5, 7). Therefore, these

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics or description <sup>a</sup>	Reference or source <sup>b</sup>
<b>Strains</b>		
<i>E. coli</i> S17-1	<i>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Kn::Tn7</i>	36
<i>F. novicida</i> strain U112	Avirulent strain	Water, 1950, Utah (16, 19)
<i>F. tularensis</i> subsp. <i>tularensis</i> SchuS4	Virulent type A	Human ulcer, 1941, Ohio; FSC237
<i>F. tularensis</i> subsp. <i>holarctica</i> HN63	Virulent type B	Hare, 1963, Telemark, Norway; FSC092
<b>Plasmids</b>		
pSMP22	Suicide vector for allelic replacements in <i>F. tularensis</i> , constructed based on pPV2 (a gift from I. Golovliov) (15). CHL resistance gene was removed by digestion with HindIII, and the plasmid backbone was religated. A <i>F. tularensis</i> <i>groES</i> promoter with <i>sacB</i> fusion cassette was cloned into the Sall/MluI sites.	This study
pSMP22-RT1	Suicide plasmid used to inactivate O-antigen cluster of <i>F. tularensis</i> subsp. <i>tularensis</i> ; 2.5-kb fragment cloned into pSMP22; CHL resistance cassette inserted between amplified fragments of <i>F. tularensis</i> subsp. <i>tularensis</i> <i>wbtD</i> and <i>wbtF</i> genes.	This study
pSMP22-RT2	Suicide plasmid used to inactivate O-antigen cluster of <i>F. novicida</i> ; 2.5-kb fragment cloned into pSMP22; CHL resistance cassette inserted between amplified fragments of <i>F. novicida</i> <i>wbtD</i> <sub>Fn</sub> and <i>wbtF</i> <sub>Fn</sub> genes.	This study

<sup>a</sup> CHL, chloramphenicol.

<sup>b</sup> Identifiers are from the *Francisella* Strain Collection (FSC) in Umeå, Sweden.

studies may not indicate the roles of the O antigen in highly virulent strains of *F. tularensis*.

The aim of this study was to compare and contrast the roles of the O antigens in virulence of *F. tularensis* subsp. *tularensis* and *F. novicida* and to determine the extent of immunological cross-reactivity between these antigens.

#### MATERIALS AND METHODS

##### Bacteria, plasmids, growth conditions, and general enzymes and chemicals.

The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise stated, enzymes for the manipulation of DNA, nucleotides, and reagents for the detection of the digoxigenin (DIG)-labeled probe for Southern blotting were obtained from Roche Diagnostics Limited (Lewes, United Kingdom); chemicals were obtained from Sigma Chemical Co. (Poole, United Kingdom), and culture medium was obtained from Oxoid Limited (Basingstoke, United Kingdom).

Strains of *F. tularensis* subsp. *tularensis* and *F. novicida* (19, 22) were cultured on blood cysteine glucose agar (BCGA) supplemented with 10 ml of 10% (wt/vol) histidine per liter or on modified Thayer Martin agar (BBL GC agar base [Becton Dickinson, United Kingdom] supplemented with 1% [wt/vol] hemoglobin and 1% [vol/vol] BBL Isovitalax [Becton Dickinson]) or in liquid culture in Chamberlain's Defined Medium (CDM) (4). *Escherichia coli* was cultured on Luria-Bertani (LB) plates or broth (3). Chloramphenicol was added to growth medium at 25 µg/ml for *E. coli* and 10 µg/ml for *F. novicida* and *F. tularensis* subsp. *tularensis*.

##### Identification and sequencing of the O-antigen gene cluster of *F. novicida*.

Presence of SchuS4-like O-antigen genes in *F. novicida* strain U112 was determined by PCR. Primers were designed from the SchuS4 sequence. Primers IsFtu2 F, 2F, 2R, 3F, wbtF F, wbtG R, wbtG F, wbtH F, wbtH R, and wbtMR (Table 2) were used in combination to sequence the O-antigen gene cluster using the Expand Long Template polymerase (Roche), according to the manufacturer's instructions. PCR products were sequenced by primer walking to publication quality by MWG (Ebersberg, Germany). Genes amplified from *F. novicida* encoding proteins with high homologies to O-antigen proteins of *F. tularensis* subsp. *tularensis* were denoted with the subscript Fn. Those genes which were found to have no homology to O-antigen genes of *F. tularensis* subsp. *tularensis* were instead given a new gene name.

**Construction of the O-antigen disruption cassettes.** A region of the *F. novicida* and *F. tularensis* subsp. *tularensis* O-antigen gene clusters (*wbtDEF*<sub>Fn</sub> and *wbtDEF*, respectively) was targeted for deletion and the insertion of an antibiotic

marker to disrupt the entire cluster's function. An internal region of genes *wbtD*<sub>Fn</sub> and *wbtD* (the upstream region) and an internal region of genes *wbtF*<sub>Fn</sub> and *wbtF* (the downstream region) were amplified by the PCR. The disruption cassette was then constructed by performing splice overhang extension PCR (6) using a PCR-amplified chloramphenicol acetyltransferase gene (*cat*). The 2.5-kb DNA fragments were ligated into pSMP22 and transformed into *E. coli* S17-1 (36). The appropriate plasmids were transferred to *F. tularensis* subsp. *tularensis* (pSMP22-RT1) and *F. novicida* (pSMP22-RT2) by conjugation.

The use of chloramphenicol as a marker has been approved for this specific study. All work undertaken in the United Kingdom is reviewed locally by a genetic manipulation safety committee on a project-by-project basis and is also approved nationally by our health and safety authorities.

**Conjugal transfer of pSMP22-RT1 and pSMP22-RT2.** Clones pSMP22-RT1 and pSMP22-RT2 were introduced into *F. tularensis* subsp. *tularensis* strain SchuS4 *F. novicida* by conjugation using essentially the same method as Golovliov et al. (15). Conjugation mixtures were incubated on BCGA plates at 25°C before selection of transconjugants. The transconjugants were selected on modified Thayer Martin plates containing polymyxin B (100 µg/ml for counter-selection of the *E. coli* strain) and 10 µg/ml chloramphenicol. The plates were incubated at 37°C for up to 5 days.

**Analysis of transconjugants.** Southern blotting and PCR were used to confirm the deletion/insertion in the *wbtDEF* or *wbtDEF*<sub>Fn</sub> gene region. The *F. tularensis* subsp. *tularensis* and the *F. novicida* double recombinant strains were designated  $\Delta wbtDEF_{Fn}::Cm$  or  $\Delta wbtDEF_{Fn}::Cm$ , respectively.

**LPS purification, gel electrophoresis and immunodetection.** The hot phenol-water extraction method was used to purify LPS from 1.16 g of freeze-dried *F. novicida* (31). An identical extraction method was used to isolate LPS from 2.2 g of *F. tularensis* subspecies *tularensis*. Glycine gel electrophoresis was performed using a 12.5% separating gel with a 4.5% stacking gel (21). Colonies were boiled in solubilization buffer prior to incubation with 3.3 mg/ml proteinase K for 1 h at 60°C before being loaded onto gels. For immunodetection a murine monoclonal antibody to *F. tularensis* LVS LPS O side chain (18) and an antibody to *F. novicida* LPS (a kind gift from F. Nano) (8) were used. An antisppecies horseradish peroxidase-labeled antibody (Amersham, United Kingdom) was used as a secondary antibody. The ECL Plus substrate (GE Healthcare, United Kingdom) was used to detect antibody binding according to the manufacturer's instructions.

**Structural analysis of the polysaccharide O antigen: NMR spectroscopy.** The water soluble O-antigen polysaccharide component was isolated from the LPS by acid hydrolysis as outlined by Phillips et al. (29). The lyophilized crude polysaccharide was purified using size exclusion chromatography on a Sephadex G-50 column (15 by 5 by 500 mm), with 0.05 M pyridinium acetate buffer as eluant (pH

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5'-3') <sup>a</sup>	Purpose	Location relative to SchuS4 genome (bp)
wbtD F	<b>ACGCGT<b>CGACT</b>CCAAATGTAACAGGCTTAG</b>	Disruption cassette cloning; used to make Southern blot probe	1512972–1512953
wbtD R	<u>GTGAAAGTTGGAACCTCTTACAGATCTACGTGCAT</u> <u>TTTGCTGTAAGTATGCATTGAC</u>	Disruption cassette cloning; used to make Southern blot probe	1512219–1512242
wbtF F	<u>GATGAGTGGCAGGGCGGGGCGTAAAGATCTTGCA</u> <u>CGGCTTACGATAATGTTAAATTTTC</u>	Disruption cassette cloning	1510886–1510864
wbtF R	<b>ACGCGT<b>CGAC</b>ATTAATTAATACCACTCAACAGC</b>	Disruption cassette cloning	1509920–1509943
wbtE F	TAGTAGTAGACGAGGAG	Used to make Southern blot probe	1511621–1511604
wbtE R	AGCTGCCTTGTACGTTAG	Used to make Southern blot probe	1511365–1511382
Cam F	GCACGTAGATCTGTAAGAGGTTCCAACCTTTCAC	Used to make Southern blot probe	
Cam R	CGTGCAAGATCTTTACGCCCGCCCTGCCACTCATC	Used to make Southern blot probe	
wbtF F	CTAATGAAGAGTGGGCGAGAC	PCR	1510407–1510387
wbtF R	TATCACCCGCTCTATCTGGAC	PCR	1510030–1510050
wbtG F	CCTTAACCAAGGTGGTGTCTGAA	PCR	1508645–1508624
wbtG R	ACTCCCCTACCCATAAGTGA	PCR	1508544–1508563
wbtH F	CTGAGCATGCAAGAGCAGTAG	PCR	1506655–1546635
wbtH R	ATCACCAGCGTCACCTGATAG	PCR	1506450–1506470
wbtM R	GCATAACGCCAATCATGTCC	PCR	1500120–1500074
2F	TGTCTTAGATATGGGGCAACC	PCR	1515021–1515001
2R	ACAAATATCAAATCCTAACACATC	PCR	1512073–1512096
3F	TAGAAGCAGCTGCGATAGGTAGAC	PCR	1512463–1512440
ISFtu2 F	ATGAATTATCATATAAAAAGAAGTATTC	PCR	1517296–1517250
GroES prom F	GTACGTGCACAATAAACATCGCAAAAGGTGTA	Construction of pSMP22	1763632–1763653
GroES prom R	ACTTGATCATCGGTACCCATAACAATCTTACTCCT	Construction of pSMP22	1763983–1763966
SacB F	TACCGTACGATGAACATCAAAAAGTTTGCA	Construction of pSMP22; amplification of <i>sacB</i> from pRL271 (GenBank accession no. L05081)	
SacB R	TGCCGTACGTTATTTGTTAACTGTTAATTG	Construction of pSMP22; amplification of <i>sacB</i> from pRL271 (GenBank accession L05081)	

<sup>a</sup> SalI sites are in boldface; *cat* sequences are underlined.

4.5), at a flow rate of 16 ml/h. Fractions were assessed for carbohydrate content by charring on thin-layer chromatography plates (H<sub>2</sub>SO<sub>4</sub> and ethanol); those containing carbohydrate were analyzed by nuclear magnetic resonance (NMR) spectroscopy.

Purified polysaccharide was dissolved in deuterium oxide (D<sub>2</sub>O) (99.998% purity; Cambridge Isotope Laboratories) and cycled through three steps of lyophilization/dissolution to remove exchangeable protons. <sup>1</sup>H and <sup>13</sup>C NMR experiments were performed at 600 MHz and 150 MHz, respectively, at 298 K in D<sub>2</sub>O using a Bruker Avance spectrometer. Spectral assignment was verified by the recording of one-dimensional (1D) <sup>1</sup>H, correlated spectroscopy, total correlated spectroscopy (120 ms mixing time), and <sup>1</sup>H-<sup>13</sup>C-heteronuclear single-quantum coherence spectra. A sample was also prepared in dimethyl sulfoxide-d<sub>6</sub> (99.9% purity; Cambridge Isotope Laboratories) and nuclear Overhauser effect spectroscopy (200 ms) was acquired at 323 K. All spectra were acquired using unmodified pulse sequences from the Bruker pulse sequence library.

**Isotyping enzyme-linked immunosorbent assay.** Plates were coated with heat-killed whole bacteria. Anti-mouse Fab immunoglobulin G (IgG) and an anti-mouse μ-chain with specific affinity for the IgM subtypes were used. The antibodies were serially diluted across the plate, and naïve mouse serum was included as a control. The appropriate secondary conjugate was diluted and used, and the reaction was developed with TMB (3,3',5,5'-tetramethyl benzidine) substrate. The plates were then incubated at room temperature, and the reaction was stopped by adding 2 M H<sub>2</sub>SO<sub>4</sub>. The absorbance was read at 450 nm. The revelations program (version 4.22) was used to calculate the standard curve and antibody concentration values.

**Immunization with LPS and protection studies.** Groups of 6 adult female BALB/c mice (Charles River Laboratories, Kent, United Kingdom) aged 6 to 8 weeks were immunized via the intraperitoneal route with 50 μg of purified LPS in 100 μl phosphate-buffered saline, from either *F. tularensis* subsp. *tularensis* or *F. novicida*. Mice were given three doses of LPS at 14-day intervals. Serum samples were taken from mice 14 days after the final immunization, and the level of antibody against immobilized bacteria was determined. Twenty-one days after immunization the mice were challenged with either 100 times the minimum lethal dose (100 MLD) of *F. novicida* strain U112 by the intraperitoneal route or

100 MLD of *F. tularensis* subsp. *holarctica* strain HN63 by the subcutaneous route. All procedures were carried out in accordance with Home Office guidelines. Animals were observed for signs to indicate humane end points, and these were used to direct culling.

**Immunization with Δ*wbtDEF*<sub>FCm</sub> or Δ*wbtDEF*<sub>FCm</sub>:Cm and challenge of mice.** Groups of 5 adult female BALB/c mice aged 6 to 8 weeks were immunized subcutaneously with 10<sup>5</sup> CFU of Δ*wbtDEF*<sub>FCm</sub>:Cm. Groups of 6 female BALB/c mice aged 6 to 8 weeks were immunized with 10<sup>5</sup> CFU of Δ*wbtDEF*<sub>FCm</sub>:Cm via the subcutaneous route. The bacteria were harvested after 18 h of growth at 37°C on BCGA agar. Mice were challenged on day 49 postvaccination with either 100 MLD of *F. novicida* strain U112 via the intraperitoneal route or 100 MLD of *F. tularensis* subsp. *tularensis* strain SchuS4 via the subcutaneous route. All procedures were carried out in accordance with Home Office guidelines. Animals were observed for signs to indicate humane end points, and these were used to direct culling.

**Macrophage infection and survival assays.** The murine macrophage cell line J774A.1 (ECCAC, Salisbury, United Kingdom) was cultivated at 37°C with 5% CO<sub>2</sub> in Dulbecco's Modified Essential Medium (Gibco Invitrogen, Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (Gibco Invitrogen) and 4 mM L-glutamine. The assay was carried out essentially as described by Hartley et al. with a multiplicity of infection of bacteria to macrophage of 10:1 (17). Values for all time points were determined in duplicate, and each experiment was done in triplicate. Results were plotted as number of CFU/ml.

**Serum sensitivity assays.** Bacteria were grown overnight in CDM. Cultures were diluted 1/100 in fresh CDM to determine the MIC using guinea pig serum at final concentrations of 50 to 0.02% (vol/vol) in 96-well polypropylene microtiter plates. The MIC<sub>50</sub> was determined as the concentration of the compound that gave a 50% optical density reading compared to wells with no substrate after incubation overnight for *F. novicida* and after incubation for 48 h for *F. tularensis* subsp. *tularensis* at 37°C. These assays were performed in triplicate.

**Nucleotide sequence accession number.** The nucleotide sequence of the *F. novicida* O-antigen gene cluster was determined and deposited in the EMBL/GenBank/DBJ databases under accession number EF059983.

TABLE 3. O-antigen genes and gene products of *F. novicida*

Gene or gene product	Length (aa) <sup>a</sup>	Function	Similar protein(s)	% Identity with <i>F. tularensis</i> SchuS4 <sup>c</sup>	% Positive <sup>d</sup>	Organism	Accession no.
IstuF2	181 <sup>b</sup>	Transposase	ISFtu2	95 (173/181)	96 (175/181)	<i>F. tularensis</i>	YP_170453
<i>wbtA</i> <sub>Fn</sub>	368	dTDP-glucose 4,6-dehydratase	WbtA	98 (99/101)	99 (100/101)	<i>F. novicida</i>	YP_170401
<i>wbtN</i>	371	Aminotransferase	Unnamed	64 (235/366)	80 (293/366)	<i>Bacillus cereus</i>	NP_981686
			DegT, DnrJ, EryC1, StrS	63 (231/365)	78 (287/365)	<i>Clostridium thermocellum</i> ATCC 27405	ZP_00510645
<i>wbtO</i>	209	UDP-galactose phosphate transferase	Unnamed	62 (128/204)	80 (165/204)	<i>Oceanobacillus iheyensis</i>	NP_693814
			PglC	62 (126/201)	76 (154/201)	<i>C. jejuni</i>	ZP_01099808
<i>wbtP</i>	220	Acetyltransferase	Unnamed	41 (84/201)	61 (123/201)	<i>Oceanobacillus iheyensis</i>	NP_693813
			PglB	38 (76/199)	55 (111/199)	<i>N. meningitidis</i>	AAC25979
<i>wbtD</i> <sub>Fn</sub>	363	Galacturonosyl transferase	WbtD	86 (310/359)	93 (334/359)	<i>F. novicida</i>	YP_170398
<i>wbtE</i> <sub>Fn</sub>	401	UDP-glucose/GDP-mannose dehydrogenase	WbtE	98 (393/401)	98 (396/401)	<i>F. novicida</i>	YP_170397
<i>wbtF</i> <sub>Fn</sub>	328	NAD-dependent epimerase; UDP-glucose 4 epimerase	WbtF	98 (315/320)	99 (318/320)	<i>F. novicida</i>	YP_170396
<i>wzy</i>	430	Capsule biosynthesis	CapE	22 (94/427)	40 (172/427)	<i>Staphylococcus aureus</i>	AAA64644
		O-Antigen polymerase	Wzy	20 (64/314)	44 (140/314)	<i>E. coli</i>	AAT77177
<i>wbtG</i> <sub>Fn</sub>	371	Glycosyl transferase	WbtG	89 (324/361)	95 (345/361)	<i>F. novicida</i>	YP_170394
<i>wbtQ</i>	378	Glycosyl transferase	WbnK	26 (92/345)	47 (163/345)	<i>S. dysenteriae</i>	AAO39702
			WbqH			<i>Escherichia coli</i>	AAR97066
<i>wbtH</i> <sub>Fn</sub>	630	Asparagine synthase; glutamine amidotransferase	WbtH	97 (611/628)	98 (618/628)	<i>F. novicida</i>	YP_170393
<i>wzx</i>	415	O-Antigen flippase	Wzx	25 (102/398)	54 (217/398)	<i>E. coli</i>	AAK60454
Transposase	156	Transposase	Transposase	55 (79/142)	72 (103/142)	<i>N. meningitidis</i>	AAP44503

<sup>a</sup> aa, amino acids.

<sup>b</sup> Partial sequence data.

<sup>c</sup> Values in parentheses indicate number of invariant residues/total number of residues.

<sup>d</sup> Values in parentheses indicate number of invariant and/or conserved residues/total number of residues.

## RESULTS

The *F. novicida* strain U112 O-antigen biosynthetic gene cluster shows homology with the gene cluster from *F. tularensis* subsp. *tularensis* strain SchuS4. We first sequenced the O-antigen gene cluster in *F. novicida* strain U112. The cluster was approximately 16 kb in length, contained 12 genes (Table 3), and was flanked by transposases (Fig. 1). The overall G+C

content of this region (30.45%) was lower than that reported for *F. tularensis* subsp. *tularensis* strain SchuS4 (31) and lower than the reported overall G+C content of the *F. novicida* genome of 32% (<http://www.genome.washington.edu/UWGC/Projects/index.cfm?PID=168&ST=4>). Based on homologies with gene sequences deposited in the GenBank database, a function for each gene product was assigned. Genes from *F.*

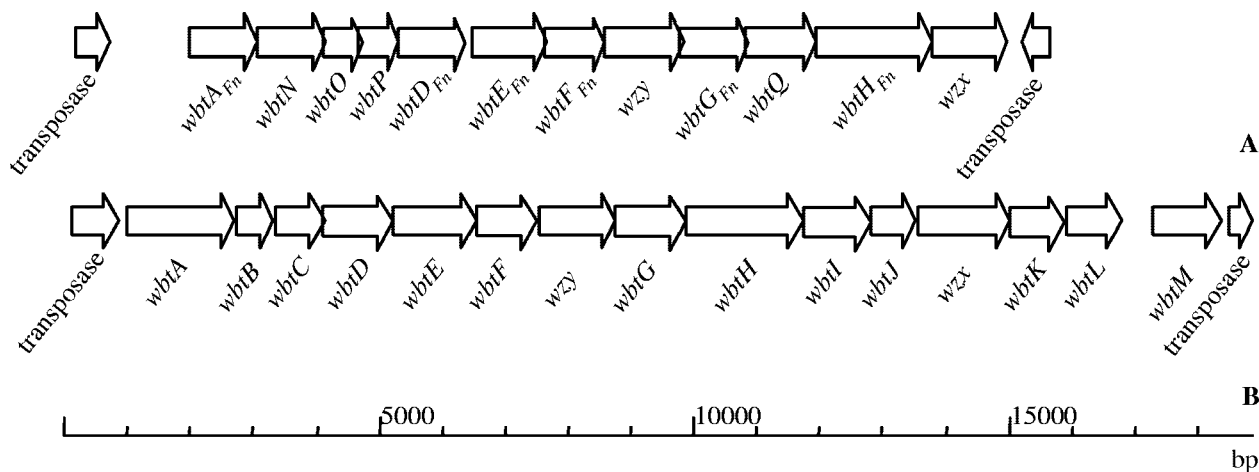


FIG. 1. Genetic organization of the *F. tularensis* subsp. *tularensis* and *F. novicida* O-antigen gene clusters. (A) *F. novicida* strain U112. Genes with homologies greater than 90% to those of *F. tularensis* subsp. *tularensis* strain SchuS4 have the subscript <sub>Fn</sub> added to the gene name. Genes unique to *F. novicida* have no subscript. (B) *F. tularensis* subsp. *tularensis* strain SchuS4.

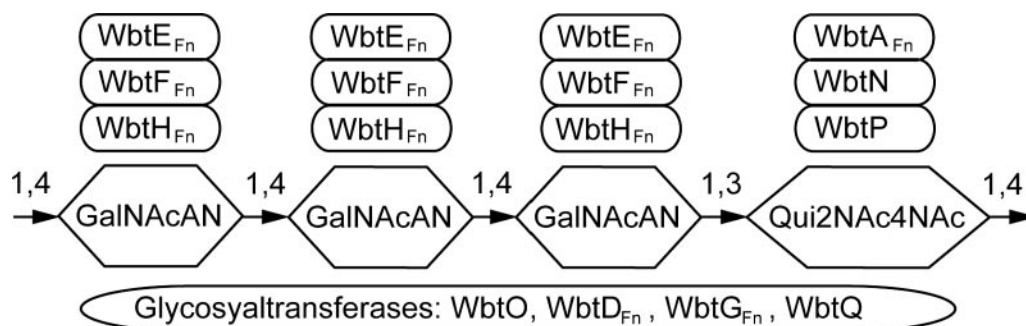


FIG. 2. Schematic structure of an O-antigen subunit of *F. novicida* and the putative functions of the gene products of the O-antigen gene cluster. A single unit is shown, with sugar residues and glycosidic linkages indicated (see text).

*novicida* encoding proteins with high homologies to corresponding proteins of *F. tularensis* subsp. *tularensis* were denoted with the subscript  $_{Fn}$ . The O-antigen repeat unit of *F. novicida* is shown in Fig. 2, with the putative role of each gene of the O-antigen biosynthetic gene cluster annotated. The O-antigen unit *F. novicida* contains four sugar residues: three 2-acetamido-2-deoxygalacturonamide (GalNAcAN) residues and one 4,6-diacetamido-2,4,6-dideoxyglucose (Qui2NAc4NAc) residue (39). The biosynthesis of GalNAcAN is likely to involve WbtE $_{Fn}$  and WbtF $_{Fn}$  as they share 99% homology to proteins WbtF and WbtE (present in the *F. tularensis* subsp. *tularensis* O-antigen cluster) and are assigned as a putative UDP-glucose dehydrogenase and NAD-dependent epimerase, respectively (31). WbtH $_{Fn}$  shows identity to an amidotransferase from *F. tularensis* subsp. *tularensis* and may be involved in the formation of the GalNAcAN amido group. Biosynthesis of the remaining sugar, Qui2NAc4NAc, probably requires WbtA $_{Fn}$  and WbtN. Sequence similarity suggests that WbtA $_{Fn}$  may function as a TDP-glucose 4,6-dehydratase having 99% homology to WbtA (31). WbtN, a predicted aminotransferase, may be involved in Qui2NAc4NAc amination, probably at position 4. Finally, WbtP is a putative acetyltransferase, showing 61% sequence homology to *Neisseria meningitidis* PglB known to be involved in *N*-acetyl sugar biosynthesis (30).

The gene cluster revealed four potential glycosyltransferases necessary for the biosynthesis of the tetrasaccharide O-antigen repeat unit. One of the first glycosyltransferases is WbtO having identity to PglC of *Campylobacter jejuni*, which has been demonstrated to add the first sugar residue onto the lipid acceptor undecaprenyl pyrophosphate (13). WbtD $_{Fn}$  is another putative glycosyltransferase and possibly transfers GalNAcAN onto Qui2NAc4NAc, based on its homology to WbtD from *F. tularensis* subsp. *tularensis*. Elongation to form the GalNAcAN trisaccharide is likely to involve WbtG $_{Fn}$  and WbtQ, based on their both showing sequence homology to glycosyltransferases. WbtG $_{Fn}$  has homology to WbtG from *F. tularensis* subsp. *tularensis*, while WbtQ is proposed to be involved in the formation of GalNAcAN1-4GalNAcAN based on homology to WbnK of *Shigella dysenteriae*, which also contains the disaccharide as a component of its O-antigen repeat (20). It was noted that WbtP has sequence similarity to *N. meningitidis* PglB, previously reported to undertake both glycosyltransferase and acetyltransferase functions. It is possible that the WbtP homolog may be bifunctional in *F. novicida*, although the pre-

dicted activity of *N. meningitidis* PglB glycosyltransferase involves the transfer of a sugar residue onto undecaprenyl pyrophosphate (30), a role similar to that assigned to *F. tularensis* subsp. *tularensis* WbtO.

There are two main O-antigen synthesis modes, O-antigen polymerase-dependent and O-antigen polymerase-independent (which can be referred to as *wzy* dependent and *wzy* independent) modes. Two genes in the *F. novicida* O-antigen gene cluster would encode proteins with a high degree of sequence similarity to Wzy and Wzx, suggesting that transportation and polymerization of the O antigen is via a *wzy*-dependent pathway. Similarly, *F. tularensis* subsp. *tularensis* O-antigen biosynthesis is also predicted to proceed via the *wzy*-dependent pathway (31).

**Confirmation of O-antigen structure.** Structural identification of the O antigen of *F. novicida* strain U112 was achieved using 1D and 2D NMR analysis (results not shown). The 1D spectrum of the lipopolysaccharide O antigen of *F. novicida* was consistent with the reported structure of *F. novicida* O-specific polysaccharide (39). It should be noted that the methods used to isolate the O antigen are different from those reported by Vinogradov et al. (39). However, we have also isolated the same material. LPS was isolated from *F. novicida* and *F. tularensis* subsp. *tularensis* with yields of 1.8% and 0.3%, respectively.

**Immunization with LPS does not protect against a heterologous challenge.** Immunization with the LPS of strain SchuS4 does not confer full protection against a subsequent challenge with strain SchuS4 (31) but does confer complete protection against a subsequent challenge with *F. tularensis* subsp. *holarctica* (12). Therefore, *F. tularensis* subsp. *holarctica* was used as the challenge strain in this study.

Mice were immunized with three doses of 50  $\mu$ g of LPS in 100  $\mu$ l of phosphate-buffered saline at 14-day intervals. The sera from mice immunized with the LPS of *F. novicida* had a mean IgM concentration of 4,604 ng/ml and a mean IgG3 concentration of 1,433 ng/ml. In each case these titers were higher than those achieved by immunization with the same level of *F. tularensis* subsp. *tularensis* LPS, which were 3,716 ng/ml of IgM antibody and 244 ng/ml of IgG3 antibody. LPS-immunized mice were challenged with 100 MLD of *F. tularensis* subspecies *holarctica* or *F. novicida*.

Mice immunized with LPS from *F. novicida* were protected against the *F. novicida* challenge but not against a heterologous *F. tularensis* subsp. *holarctica* challenge. Conversely, mice im-

TABLE 4. Ability for LPS to protect against challenge with *F. tularensis* or *F. novicida*

Immunization	Survival (no. of mice surviving/no. of mice in group) after challenge with: <sup>a</sup>	
	<i>F. tularensis</i> subsp. <i>holarctica</i>	<i>F. novicida</i>
<i>F. tularensis</i> subsp. <i>tularensis</i> LPS	6/6	0/6
<i>F. novicida</i> LPS	0/6	4/6
Naïve	0/6	0/6

<sup>a</sup> Survival of mice following immunization with *F. tularensis* subsp. *tularensis* or *F. novicida* LPS and subsequent challenge 21 days postimmunization. No cross-protection was afforded.

munized with the *F. tularensis* subsp. *tularensis* LPS were not protected from the heterologous *F. novicida* challenge but were protected from *F. tularensis* subsp. *holarctica* challenge (Table 4).

**Production of defined O-antigen mutants.** Deletion/insertion mutants in genes *wbtDEF* were made by conjugation of the appropriate modified genes into *F. tularensis* subsp. *tularensis* or *F. novicida*. Southern blotting (Fig. 3) and PCR (results not shown) were used to identify double crossover mutants which were referred to as  $\Delta wbtDEF_{Ft}::Cm$  or  $\Delta wbtDEF_{Fn}::Cm$  for the *F. tularensis* subsp. *tularensis* and *F. novicida* O-antigen mutants, respectively. The deletion/insertion within the O-antigen gene cluster was confirmed by the

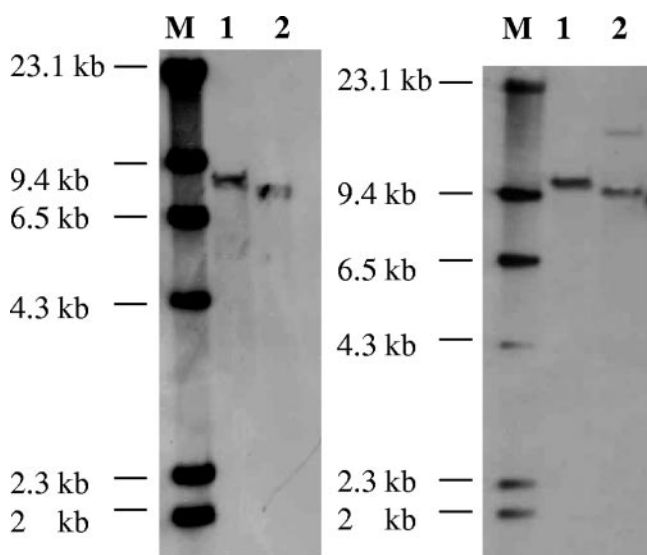


FIG. 3. Southern blot analysis of DNA from *F. tularensis* subsp. *tularensis* and  $\Delta wbtDEF_{Ft}::Cm$  and *F. novicida* and  $\Delta wbtDEF_{Fn}::Cm$  showing deletion/insertion in the O-antigen clusters. DNA samples were digested with NdeI prior to separation by agarose gel electrophoresis, Southern blotting, and hybridization. The blots were probed with a DIG-labeled probe specific to the upstream flank of *wbtDEF\_{Fn/Ft}*. Lane M, DNA standards of DIG-labeled HindIII-digested  $\lambda$  (molecular size marker II; Roche Diagnostics). (Left) Lane 1, *F. tularensis* subsp. *tularensis* SchuS4 DNA; lane 2,  $\Delta wbtDEF_{Ft}::Cm$  DNA with probe-reactive band smaller than that of lane 1. (Right) Lane 1, *F. novicida* U112 DNA; lane 2,  $\Delta wbtDEF_{Fn}::Cm$  DNA with probe-reactive band smaller than that of lane 1. The faint upper band seen in lane 2 is due to incomplete digestion genomic DNA.

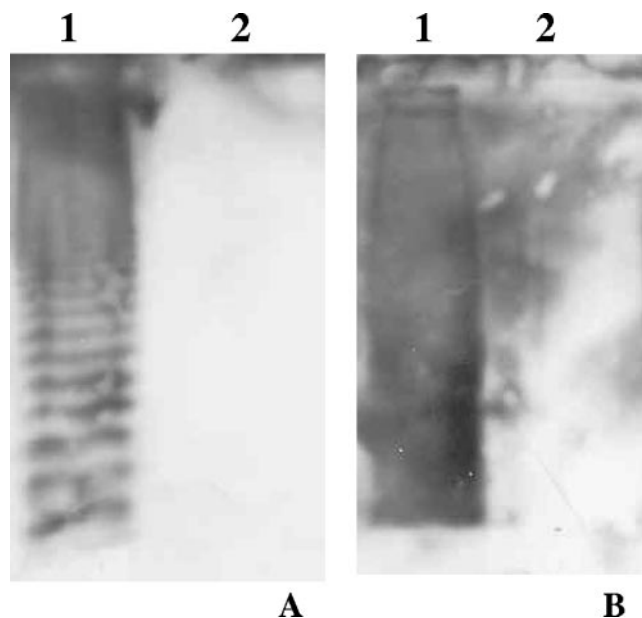


FIG. 4. LPS analysis of *F. tularensis* subsp. *tularensis* and  $\Delta wbtDEF_{Ft}::Cm$  and *F. novicida* and  $\Delta wbtDEF_{Fn}::Cm$ . Membranes were probed with monoclonal antibody reactive with *F. tularensis* subsp. *tularensis* LPS (A) and monoclonal antibody reactive with *F. novicida* (B). (A) Lane 1, wild-type *F. tularensis* subsp. *tularensis*; lane 2,  $\Delta wbtDEF_{Ft}::Cm$ . The  $\Delta wbtDEF_{Ft}::Cm$  strain has lost all reactivity with the antibody. (B) Lane 1, wild-type *F. novicida*; lane 2,  $\Delta wbtDEF_{Fn}::Cm$ . The  $\Delta wbtDEF_{Fn}::Cm$  strain has lost all reactivity with the antibody.

observation that the probe-reactive band specific to the upstream region was smaller than that of the wild type. This size difference corresponds to 3,052 bp of the *wbtDEF* region in the SchuS4 sequence compared to only 2,480 bp of the deletion/insertion cassette.

The LPS profiles of  $\Delta wbtDEF_{Ft}::Cm$  and  $\Delta wbtDEF_{Fn}::Cm$  were analyzed by Western blotting using monoclonal antibodies to *F. tularensis* subsp. *tularensis* or *F. novicida* LPS. The monoclonal antibody to *F. novicida* LPS failed to react with the LPS extract from  $\Delta wbtDEF_{Fn}::Cm$ . Similarly, the monoclonal antibody to *F. tularensis* subsp. *tularensis* LPS failed to react with the LPS extract from  $\Delta wbtDEF_{Ft}::Cm$  (Fig. 4).

**The O antigen of *F. novicida* is critical for serum resistance.** A serum concentration of 0.02% (vol/vol) was sufficient to inhibit 50% of the growth (the MIC<sub>50</sub>) of the  $\Delta wbtDEF_{Fn}::Cm$  strain. For the wild-type strain U112, the MIC<sub>50</sub> was produced with an excess of 50% serum, with the bacteria growing more quickly at the highest concentrations of serum. For the  $\Delta wbtDEF_{Ft}::Cm$  strain the MIC<sub>50</sub> of serum was 6.25%, whereas the MIC<sub>50</sub> for the wild type was 25% serum.

**The O antigen of *F. tularensis* subsp. *tularensis* is important for intracellular survival.** To investigate the role of O antigen in macrophage survival and growth, wild-type or O-antigen mutants of *F. tularensis* were incubated with J774 cells, and the number of intracellular bacteria was determined at intervals. In our macrophage model the  $\Delta wbtDEF_{Ft}::Cm$  and  $\Delta wbtDEF_{Fn}::Cm$  strains behaved differently. Both mutants were taken up at a similar rate to that of their respective wild-type strains.  $\Delta wbtDEF_{Fn}::Cm$  was able to replicate within

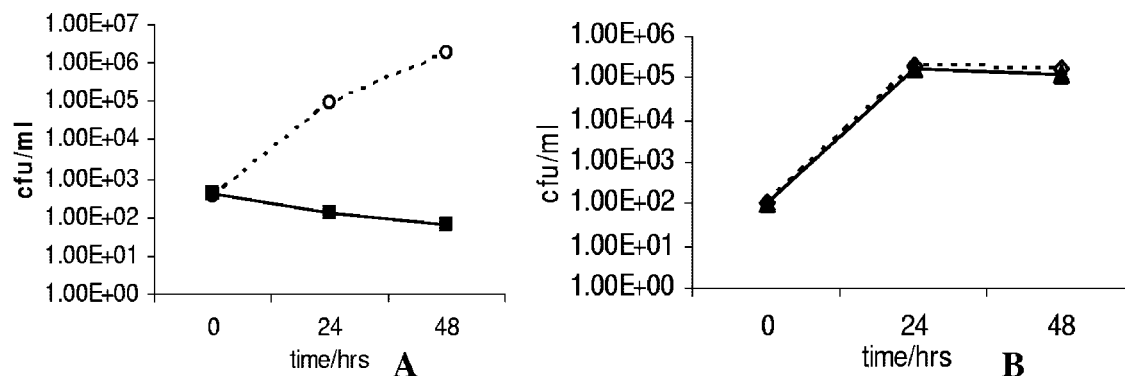


FIG. 5. Macrophage uptake and survival assay. The uptake and survival of  $\Delta wbtDEF_{Ft}$  ■ compared to SchuS4 ○ (A) or of  $\Delta wbtDEF_{Fn}$  ▲ compared to strain U112 ◇ (B) in J774 macrophages. Intracellular bacteria were counted as the number of CFU per ml of lysed macrophages at 0, 24, and 48 h postinfection. The assays were completed in triplicate with similar results observed each time.

macrophages. However, although  $\Delta wbtDEF_{Ft}::Cm$  survived in macrophages, it did not replicate over the course of the experiment (Fig. 5).

**O-antigen mutants of *F. tularensis* and *F. novicida* are attenuated but not protective.** The degree of attenuation of the O-antigen mutants of *F. tularensis* and *F. novicida* was determined by subcutaneous administration of  $10^5$  CFU. For wild-type strains the MLD, by the subcutaneous route, is less than 10 CFU for *F. tularensis* subsp. *tularensis* and less than 1,000 CFU for *F. novicida* (10, 23). Both  $\Delta wbtDEF_{Ft}::Cm$  and  $\Delta wbtDEF_{Fn}::Cm$  were attenuated at the given doses. When  $\Delta wbtDEF_{Ft}::Cm$ -immunized mice were challenged by the subcutaneous route with 100 MLD of SchuS4 49 days postimmunization, there was no protection afforded. Mice immunized with  $\Delta wbtDEF_{Fn}::Cm$  and challenged intraperitoneally with 100 MLD of strain U112 were not protected.

## DISCUSSION

Our study has found that *F. novicida* has an O-antigen biosynthetic gene cluster which contains genes also found in the O-antigen gene cluster of *F. tularensis* subsp. *tularensis* strain SchuS4. This is consistent with the similarity of the sugars within the O-antigen repeat subunits. *F. novicida* is proposed to be the progenitor of *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *mediasiatica*, and *F. tularensis* subsp. *holarctica* (38). The evolution of high-virulence strains of *F. tularensis* may have involved the acquisition of genes and or the rearrangement of the *F. novicida* O-antigen gene cluster.

Because of the similar compositions of the O antigens from *F. tularensis* subsp. *tularensis* and *F. novicida*, we sought to investigate whether immunization with the LPS from one species could provide heterologous cross-protection. During the intracellular life of *F. tularensis* and *F. novicida*, the bacterium escapes the late endosome/phagosome of phagocytic cells to reside in the cytoplasm (reviewed in reference 33). The mechanism by which this happens is unclear. However, it has been proposed that the LPS, or the proposed capsule, is partly shed, and this aids degradation of the phagosomal membrane, permitting the bacterium to “escape” from the phagosome (14). This process could reveal the new or alternative epitopes which may be the same as those exposed by *F. novicida*. There has

previously been a report in the literature of “phase variation” of the *F. tularensis* subsp. *tularensis* LPS, with *F. novicida*-like LPS being expressed (8). This phenomenon could be explained by the shared polysaccharide structures in the O-antigen repeating subunits. Although Cowley et al. have previously reported that some epitopes are common to both LPS types (8), our experiments indicate that at least in mice this is insufficient to afford any cross-protection.

We have also compared the roles of the O antigens of *F. tularensis* subsp. *tularensis* and *F. novicida* in virulence. A previous study by Hartley et al. (17) characterized an O-antigen-deficient (gray) variant of the LVS, but the genetic basis of this phenotypic change is not known, and it is possible that pleiotrophic changes existed. Furthermore, the pathogenesis of disease caused by the attenuated LVS in mice may not fully reflect the pathogenesis of disease caused by virulent strains of *F. tularensis* and *F. novicida*. This is the first report of genetically defined O-antigen mutants of *F. tularensis* and *F. novicida*.

For some bacteria the O antigen is important for protecting the bacterial cell from complement-mediated killing by serum (1, 24, 25). Our findings indicate that the O antigens of both *F. tularensis* subsp. *tularensis* and *F. novicida* play roles in protection from serum killing. However, the role of the O antigen from *F. tularensis* subsp. *tularensis* appears to be relatively minor compared to the role of the *F. novicida* O antigen. This difference may indicate that in *F. tularensis* subsp. *tularensis* other surface structures play a role in resistance to serum killing. In *Yersinia pestis* surface adhesins (such as the Ail-like proteins) may be involved in serum resistance and compensate for the lack of O antigen (28).

The data presented in this study indicate that, as in many gram-negative bacteria (2, 34, 37), the O antigens of both *F. tularensis* subsp. *tularensis* and *F. novicida* are virulence determinants. However, the functions of the O antigens differ in these two species. Both O-antigen mutants were taken up by macrophages at similar rates to the wild-type strains, but only the  $\Delta wbtDEF_{Fn}::Cm$  strain was able to replicate within the macrophage. The role of the O antigen in promoting the growth of *F. tularensis* subsp. *tularensis* in macrophages merits further attention.

The data from this study suggest that the *F. tularensis* subsp. *tularensis* O antigen is important for intracellular survival and

replication. In contrast, the O antigen of *F. novicida* is less important in the intracellular niche but more important for the viability of the bacterium before it enters a host cell. Overall, our findings may provide insight into the reasons for the different levels of virulence of *F. novicida* and *F. tularensis* subsp. *tularensis* in humans.

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