Francisella tularensis blue–gray phase variation involves structural modifications of lipopolysaccharide O-antigen, core and lipid A and affects intramacrophage survival and vaccine efficacy

Shilpa Soni[1], Robert K. Ernst[2], Artur Muszyński[3], Nrusingh P. Mohapatra[1], Malcolm B. Perry[4], Evgeny Vinogradov[4], Russell W. Carlson[3] and John S. Gunn[1]*

[1] Center for Microbial Interface Biology, Department of Molecular Virology, Immunology and Medical Genetics, and Department of Internal Medicine, Division of Infectious Diseases, The Ohio State University, Columbus, OH, USA
[2] Department of Microbial Pathogenesis, School of Dentistry, University of Maryland-Baltimore, Baltimore, MD, USA
[3] Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA
[4] Institute for Biological Sciences, National Research Council, Ottawa, ON, Canada

Francisella tularensis is a CDC Category A biological agent and a potential bioterrorist threat. There is no licensed vaccine against tularemia in the United States. A long-standing issue with potential *Francisella* vaccines is strain phase variation to a gray form that lacks protective capability in animal models. Comparisons of the parental strain (LVS) and a gray variant (LVSG) have identified lipopolysaccharide (LPS) alterations as a primary change. The LPS of the *F. tularensis* variant strain gains reactivity to *F. novicida* anti-LPS antibodies, suggesting structural alterations to the O-antigen. However, biochemical and structural analysis of the *F. tularensis* LVSG and LVS LPS demonstrated that LVSG has less O-antigen but no major O-antigen structural alterations. Additionally, LVSG possesses structural differences in both the core and lipid A regions, the latter being decreased galactosamine modification. Recent work has identified two genes important in adding galactosamine (*flmF2* and *flmK*) to the lipid A. Quantitative real-time PCR showed reduced transcripts of both of these genes in the gray variant when compared to LVS. Loss of *flmF2* or *flmK* caused less frequent phase conversion but did not alter intramacrophage survival or colony morphology. The LVSG strain demonstrated an intramacrophage survival defect in human and rat but not mouse macrophages. Consistent with this result, the LVSG variant demonstrated little change in LD_{50} in the mouse model of infection. Furthermore, the LVSG strain lacks the protective capacity of *F. tularensis* LVS against virulent Type A challenge. These data suggest that the LPS of the *F. tularensis* LVSG phase variant is dramatically altered. Understanding the mechanism of blue to gray phase variation may lead to a way to inhibit this variation, thus making future *F. tularensis* vaccines more stable and efficacious.

Keywords: *Francisella*, LPS, phase variation, tularemia, vaccine

INTRODUCTION

Francisella tularensis is a gram-negative, facultative intracellular pathogen that causes tularemia in humans and animals (Oyston et al., 2004; Keim et al., 2007; Sjostedt, 2007). The host can be infected by several routes including the lungs (inhalational), skin, or mucous membranes (cutaneous) or by ingestion of contaminated food or water (gastrointestinal) (Keim et al., 2007; Sjostedt, 2007). *F. tularensis* has been characterized as a category A bio-defense organism by the Centers for Disease Control and Prevention because of its high lethality and infectivity, particularly by the aerosol route. There are two major human virulent subspecies of *F. tularensis*: *F. tularensis* subspecies *tularensis* (Type A strain) found in North America and *F. tularensis* subspecies *holarctica* (Type B strain) found in Europe, Asia as well as North America (Ellis et al., 2002). The Type A strain is highly infectious and when inhaled, even low doses (<10 bacteria) can cause life-threatening disease in humans (Sjostedt, 2007). Type B strains are considered less virulent

but can still effectively cause diseases in humans. *F. tularensis* subspecies *novicida* (*F. novicida*) and *F. tularensis* subspecies *mediasiatica* are other known subspecies of *Francisella* that are considered relatively avirulent for immunocompetent humans but are capable of causing systemic infection in other mammals (Ellis et al., 2002; Keim et al., 2007).

There are no approved vaccines available to prevent or treat tularemia in the United States (Oyston, 2009). An attenuated live vaccine strain, *F. tularensis* LVS (*Ft* LVS), was derived from a Type B isolate of the pathogen (Oyston, 2009) and is used as a vaccine in Europe and is in clinical trials for potential approval in the US. It elicits diverse protection in humans, monkeys, guinea pigs, and mice depending on the route of vaccination against systemic challenge with virulent Type A *F. tularensis* (Eigelsbach and Downs, 1961). The molecular basis for the attenuation of *Ft* LVS still remains unknown, though candidate factors have been identified (Rohmer et al., 2006). Eigelsbach (Eigelsbach et al., 1951; Eigelsbach

and Downs, 1961) first reported colony variants of the prototypical virulent Type A SchuS4 strain and *Ft* LVS, which were identified on the basis of colony morphology (rough colonies and smooth colonies) and their appearance under a field microscope viewed with oblique light, where *Ft* LVS/SchuS4 appears blue and the variant as gray. Gray variants were reported to be less virulent with a lethal dose of $>10^7$ colony forming units (CFU) and were less immunogenic/protective in challenge studies, where they afforded minimal protection to Type A challenge (Eigelsbach et al., 1951). These variants also differentially reacted to acriflavine agglutination, and demonstrated variable stability of colony morphology upon subculturing (Eigelsbach et al., 1951). The observation of gray variants depended on growth conditions including culture media, size of inoculum, pH, and duration of culture growth. Hartley et al. (2006) also identified the spontaneous gray variants of three *F. tularensis* strains (LVS, SchuS4, and HN63), further suggesting that blue to gray variation is a frequent and perhaps common occurrence in wildtype strains in the environment.

Gray variants were first examined at the molecular level by Cowley et al. (1996). This variant (LVSG; *Ft* LVSG) demonstrated differential survival in certain macrophage types and the lipopolysaccharide (LPS) of this variant, which possessed a LPS O-antigen, was found to possess altered anti-LPS monoclonal antibody reactivity and stimulated increased nitric oxide (NO) production in macrophages. A rough gray variant (lacking an LPS O-antigen) was also recently characterized (Hartley et al., 2006). This variant was identified on the basis of size and opacity, grew slower, had reduced intramacrophage survival, and poorly protected against Type A *F. tularensis* challenge. These studies suggested that LPS played an important role in this phase variation phenomenon.

In the present study, we confirmed and extended the phenotypic characterization of the gray variants and further analyzed the LPS of one of these strains. Multiple LPS alterations were noted, including those in O-antigen, core and lipid A. The gray variant primarily characterized in this study, *Ft* LVSG, possessed a full length O-antigen (as opposed to previously characterized rough gray variants), survived less well in human and rat but not mouse macrophages and poorly protected against *F. tularensis* SchuS4 challenge in the mouse model. It is hoped that a greater understanding of the mechanism(s) behind phase variation will lead to phase locked strains that no longer vary, thus allowing the construction of safer, more immunogenic tularemia vaccines.

MATERIALS AND METHODS
STRAINS AND MEDIA
Francisella tularensis subsp. *holarctica* LVS (ATCC 29684) was obtained from Karen Elkins (Center for Biologics Research and Evaluation, U.S. Food and Drug Administration, Bethesda, MD, USA). *F. novicida* (U112) was obtained from ATCC, LVSG (a spontaneous gray phase variant) was provided by F. Nano (University of Victoria, Victoria, BC, Canada). *Ft* LVSGD (another spontaneous gray variant we found to lack O-antigen) was obtained from lot number 703-0102-080 produced at Cambrex BioSciences in Baltimore, MD, USA. The cell bank was produced from a lyophilized vial of *Ft* LVS lot NDBR-101, lot 4 (Salk produced). *F. tularensis* subsp. *tularensis* strain SchuS4, a Centers for Disease Control and Prevention clinical isolate, was provided by Rick Lyons

(University of New Mexico, Albuquerque, NM, USA). For most experiments, bacteria were grown overnight (~24 h) on Choc II agar (BD Biosciences, San Jose, CA, USA) at 37°C as the frequency of phase variation was minimal in these conditions. Liquid cultures were grown overnight (~16 h) in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI, USA) containing 0.1% cysteine HCl (Sigma-Aldrich, St. Louis, MO, USA) for specified times as described in the results or Figure legends.

GENETIC MANIPULATION
Constructs for the deletion of FTL1611 (*flmF2*) and FTL1609 (*flmK*) were made in pJC84 (Wehrly et al., 2009). The ~1 kb upstream region of *flmF2* was amplified using forward primer JG1823 (5′-aaacgagctcgGGGTTATGGTGACTTCTGCATC-3′) with *Sac*I restriction site at the 5′ end and reverse primer JG1824 (5′-cgcggatccCACAAATACAAAATATATTAACCTTAATTAATGCTATTATAACC-3′) with a 5′ *Bam*HI restriction site. The ~1 kb downstream region was amplified using forward primer JG1825 (5′-cgcggatccAATATTGTTTTAAGCTAATGAAT CAATACTTATTAAATTCTTAG-3′) and reverse primer JG1826 (5′-acgcgtcgacGTATTATATTTTTAGTAGCAGCTGTTGCTGTTAT-3′) with *Bam*HI and *Sal*I 5′ flanking restriction sites, respectively. Similarly, the *flmK* upstream region was amplified by JG2290 (5′-aaacgagctcgGATCTAATACTGGATACCACTCATTATC-3′) forward primer with a 5′ *Sac*I site and JG2291 (5′-cgcggatccCTTCTTTACCCTCAAATAGAAACTTATAC-3′) reverse primer with a 5′ *Bam*HI site, and the downstream region using the JG2292 (5′-cgcggatccGATTTATCAGCATTAATTACTTTGATAAGCTAAG-3′) forward primer with a 5′ *Bam*HI site and JG2293 (5′-acgcgtcgacCATAGATAAGCGTACAGTTGTTTCATG-3′) reverse primer with a 5′ *Sal*I site. Fragments were cloned in pJC84 sequentially and the construct was transformed into *Ft* LVS followed by chromosomal recombination using the procedure described by Wehrly et al. (2009). Mutants were confirmed by PCR amplification and sequencing of the deleted region.

MICROSCOPY
Choc II plates containing bacteria were visualized under oblique light settings as suggested by Robert Miller at Dynport Vaccine Company LLC, Frederick, MD, USA and as described by Eigelsbach (Eigelsbach et al., 1951). Briefly, a focused light source, concave mirror and dissecting microscope with 10× objective magnification and a transparent stage were used to visualize blue and gray variants. The concave mirror was placed horizontally tilted upward so that the light beam would hit the upper concave region and the distance between mirror and the microscope is adjusted so that the light beam would reflect on the plate sitting on the stage of the microscope. Blue and gray colonies were observed and counted using these conditions. Samples were prepared for electron microscopy from overnight (~16 h) grown cultures of *Ft* LVS or *Ft* LVSG in TSB containing 0.1% cysteine HCl using methods as described previously (Mohapatra et al., 2008). In brief, cells were pelleted by centrifugation, washed in PBS, and fixed with 2.5% warm glutaraldehyde for 15 min. followed by fixing with a combination of 2.5% glutaraldehyde and 1% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.3) for 15 min at 4°C. Staining of the cells was accomplished by using 0.25% uranyl acetate in

0.1 M sodium acetate buffer (pH 6.3) for 45 min, and viewed after further processing by transmission electron microscopy using an FEI Technai G2 Spirit microscope at 60 kV. Multiple fields (>50) were examined to determine the average size (diameter and length) and shape of bacteria.

SILVER STAINING AND WESTERN BLOTTING

Overnight (~24 h) grown bacteria from Choc II agar plates were suspended in PBS at a concentration of 3×10^{10} CFU/ml as determined previously by the optical density (OD_{600}) of diluted cultures and subsequent colony counts on solid agar. Bacteria equalized by optical density (OD_{600}) were then pelleted, frozen, and lyophilized overnight to obtain ~20 mg of dry cells. LPS was purified using hot phenol/water method using the standard protocol as described by Apicella et al. (1994).

Lipopolysaccharide was separated by 15% SDS-PAGE and silver stained as described (Clay et al., 2008). Briefly, after fixing overnight in 40% ethanol and 5% acetic acid, gels were incubated in 0.7% periodic acid in fixing solution for 7 min and subsequently washed with multiple exchanges of water. The staining solution (0.013% concentrated ammonium hydroxide, 0.02 N sodium hydroxide, and 0.67% silver nitrate (w/v) was applied with vigorous agitation for 10 min, followed by three washes (each 10 min) in water. Gels were developed using a solution containing 0.275% monohydrous citric acid (w/v) and 0.0025% formaldehyde. Upon completion, 5% acetic acid was used to stop the development.

Purified LPS samples (10 μg/well) were electrophoresed on a 15% SDS-PAGE gel and transferred on nitrocellulose membrane using the Bio-Rad semi-dry transfer system. Immunoblotting was performed using either anti-*F. tularensis* LVS or *F. novicida* polyclonal sera (from infected mice) or monoclonal sera specific to the LPS of *Ft* LVS or *F. novicida*. Polyclonal sera to *Ft* LVS or *F. novicida* (1:1000 dilution), commercial *F. tularensis* FB-11 (1:1000, Abcam, Cambridge, MA, USA), *F. tularensis* LPS specific monoclonal (1:10), or *F. novicida* LPS specific monoclonal (1:10) were used as primary antibodies with alkaline phosphatase conjugated goat anti-mouse IgG (1:4000) as the secondary antibody. The *F. tularensis*-specific and *F. novicida*-specific anti-LPS monoclonal antibodies were obtained from monoclonal hybridoma cell lines (ImmunoPrecise, Victoria, BC, Canada). Blots were developed using 5-bromo-4-chloro-3-indolyl phosphate/NBT (Sigma-Aldrich) as the substrate.

O-ANTIGEN AND CORE ANALYSIS

Lipopolysaccharide was isolated using the hot phenol/water method (Apicella et al., 1994). Crude LPS was enzymatically treated to remove contaminating nucleic acids and proteins and ultracentrifuged for 18 h. The LPS pellet was collected and the carbohydrate portion of LPS was released from lipid A via 2 h mild hydrolysis with 1% acetic acid at 100°C followed by centrifugation of the lipid A at 3500×*g*. The carbohydrate fraction in the supernatant was extracted threefold with chloroform to remove any contaminating lipid A, lyophilized, re-suspended in water, filtered through nylon filter 0.2 μm prior the HPLC separation, and lyophilized again. Carbohydrates from *Ft* LVS and *Ft* LVSG of were resolved on a Superdex Peptide HPLC column with ammonium acetate used as an eluent. The eluting fractions were pooled and salts removed by repeated evaporations from de-ionized water on a rotary evaporator. The elution profiles for the

Ft LVS and *Ft* LVSG carbohydrates were examined and Fraction 1 contained the O-polysaccharide (OPS), Fraction 2 contained slightly lower molecular weight OPS, and Fraction 3 contained the core oligosaccharides (OSs) with some possibly low molecular weight OPS repeat units. Fractions 1 and 3 were analyzed by NMR spectroscopy. Fraction 1 from both LVS and LVSG were compared to each other using 2D NMR. OSs found in Fraction 3 from LVS and LVSG were analyzed by 1D proton NMR spectroscopy.

LIPID A ANALYSIS

LPS purification and lipid A isolation

Lipopolysaccharide was isolated using the rapid small-scale isolation method for mass spectrometry analysis as described (Yi and Hackett, 2000). Briefly, 1.0 ml of Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) was added to a cell culture pellet (2–5 ml of an overnight culture), re-suspended, and incubated at room temperature for 15 min. Chloroform (200 μl) was added, vortexed, and incubated at room temperature for 15 min. Samples were centrifuged for 10 min at 12,000 rpm and the aqueous layer was removed. An aliquot of water (500 μl) was added to the lower layer and vortexed well. After 15–30 min, the sample was spun down and the aqueous layers were combined. The process was repeated two more times. The combined aqueous layers were lyophilized overnight. Lipid A was isolated after hydrolysis in 1% SDS at pH 4.5 (Caroff et al., 1988). Briefly, 500 μl of 1% SDS in 10 mM Na-acetate, pH 4.5 was added to a lyophilized sample. Samples were incubated at 100°C for 1 h and lyophilized. The dried pellets were resuspended in 100 μl of water and 1 ml of acidified ethanol (100 μl 4 N hydrochloric acid in 20 ml 95% ethanol). Samples were centrifuged at 5,000 rpm for 5 min. The lipid A pellet was further washed three times in 1 ml of 95% ethanol. The entire series of washes was repeated thrice. Finally, samples were re-suspended in 500 μl of water, frozen on dry ice, and lyophilized. Alternatively for harsher lipid A cleavage conditions, LPS samples were dissolved in water, and mixed with the same volume of 10% acetic acid to give final 5% acetic acid concentration. Samples were hydrolyzed with 5% acetic acid (100°C, 2 h, with constant stirring- the precipitate appeared after 1 h). The precipitate was collected by centrifugation at 14,000 rpm for 6 min, then re-suspended in water and lyophilized. The supernatant was stored for future chemical analyses.

MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometry analysis of lipid A was performed on a Voyager spectrometer. The samples were dissolved in $CH_3Cl–CH_3OH$ mixture (3:1) and 1 μl of each mixed with 1 μl of 0.5 M 2,5 dihydroxybenzoic acid in methanol matrix solution. Other MALDI-TOF experiments were performed using a Bruker Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Inc., Billerica, MA, USA). Each spectrum was an average of 200 shots. Calibration was performed with ES Tuning Mix (Agi-lent, Palo Alto, CA, USA). Spectra were recorded in both the negative-ion and positive-ion modes.

GC–MS fatty acid analysis

Fatty acids were analyzed as methyl esters. The lipid-containing fraction was dissolved in 0.5 ml of 2 M MeOH–HCl and the mixture was kept at 80°C for 18 h. After cooling down, the hydrolyzate

was mixed with 0.5 ml 50% NaCl solution and extracted with 1 ml of chloroform. The organic layer was collected and aqueous layer extracted two times more with chloroform. Combined organic layers were extracted again three times with water. Water traces were removed from collected chloroform phase by addition of anhydrous Na_2SO_4. The organic phase was then filtered through cotton filters prewashed with chloroform, concentrated under the stream of nitrogen and applied to GC–MS analyses.

Dephosphorylation of lipid A
To remove phosphate groups from lipid A, samples were treated with HF for 48 h at 4°C with constant stirring. HF was evaporated from samples under vacuum in a desiccator attached to NaOH trap for 1 h then removed with nitrogen.

Trimethylsilyl analysis of fatty acid methyl esters
To show the presence of hydroxyl groups in fatty acids of lipid A, the fatty acid methyl esters were treated with TriSil reagent for 30 min at 80°C. The samples were cooled and dried under a nitrogen stream. Derivatized samples were suspended in hexane and filtered through cotton filters. Filtrates were condensed under a nitrogen stream and analyzed by GC–MS.

Galactosamine quantification
Standards and samples were prepared using the established protocol (Kalhorn et al., 2009). Stock solutions of carbohydrate and internal standards were prepared in deionized water to a final concentration of 100 ng/ml. Serial dilution of carbohydrate standards were prepared to 0.125–25 ng/ml. Internal standards were prepared to a final concentration of 5 ng/ml. Individual samples containing a cocktail of carbohydrate standards ranging from 0.125 to 25 ng/ml in addition to the 5 ng/ml internal standard were prepared and lyophilized in glass screw top vials. After lyophilization, 100 ml water was added followed by 100 ml 2 M TFA. The standard samples were sealed with polytetrafluoroethylene (PTFE)-lined caps, vortexed briefly and heated at 90°C for 30 min for analysis, flash frozen and lyophilized. TFA-treated samples were reconstituted in 50 µl 0.2 M borate buffer, pH 8.8, and 50 µl 1.0 mg/ml derivatizing/labeling reagent (AccQ-Tag) in dry acetonitrile. The samples were vortexed and incubated at room temperature for 15–30 min after which they were dried under nitrogen stream at room temperature. Derivatized samples were reconstituted in 100 µl of distilled water, vortexed, and transferred to injection vials for analysis by GC–MS.

For analysis of lipid A samples, 10 mg of lipid A isolated from individual preparations was used. The vial was incubated at 90°C for 24 h for analysis of galactosamine. Aliquots were then frozen and lyophilized to dryness. They were then derivatized by the same procedure as the standards as described above (Kalhorn et al., 2009).

REAL-TIME PCR
RNA from log phase (0.4–0.5 optical density at 600 nm) cultures of *Ft* LVS and *Ft* LVSG was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA). The quality and quantity of RNA was determined using the Experion automated electrophoresis system (Bio-Rad, Hercules, CA, USA). One microgram of total RNA was reverse transcribed to cDNA using Superscript II RNase

H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA, USA). cDNA was then normalized according to the concentration and 2 ng of the converted cDNA was used for quantitative PCR with the SYBR green PCR master mixture in the Bio-Rad iCycler apparatus (Bio-Rad, Hercules, CA, USA). All primers were designed to give 200- to 220-nucleotide amplicons with melting temperatures of 48–52°C. Relative copy numbers and expression ratios of selected genes were normalized to the expression of the housekeeping gene (*dnaK*) and calculated as described by Mohapatra et al. (2007).

INTRAMACROPHAGE SURVIVAL ASSAYS
Human monocyte-derived macrophages (MDMs) were isolated using standard procedure as described elsewhere (Mohapatra et al., 2010) and obtained with informed consent from healthy donors by an OSU IRB approved protocol. Intramacrophage survival assays in human MDMs were performed using following procedure; 2×10^6 PBMCs/well (MDMs plus lymphocytes) were plated in a 24-well plate resulting in 2×10^5 MDMs/monolayer after adherence of MDMs and washing. *Francisella* spp. were opsonized with 0.1% serum for 30 min at 37°C. Macrophages were infected with *Ft* LVS, *Ft* LVSG and *F. novicida* at an MOI of 50 and incubated at 37°C in a CO_2 (5%) incubator for 2 h. Cells were washed and 50 µg/ml gentamicin was added to each well and incubated for 30 min. Cells were washed and replenished with fresh media containing 10 µg/ml gentamicin. At various time points cells were washed and lysed with 0.1% SDS and plated on Choc II plates to enumerate the colony forming units.

MICE VIRULENCE ASSAYS
Bacteria grown overnight (~24 h) on Choc II plates were scraped and suspended, washed twice and diluted in PBS. Four- to six-week-old BALB/c mice were anesthetized and infected with ~1000 bacteria in a 20-µl volume by the intranasal route and dilutions were plated on Choc II plates to enumerate the inoculum. Mice were anesthetized and challenged with 1000 CFU of overnight (~24 h) grown *F. tularensis* subsp. *tularensis* SchuS4 intranasally 4 weeks post vaccination and observed daily for survival. These procedures were performed as described in an OSU IACUC approved protocol in an inspected and approved biosafety level 3 laboratory.

RESULTS
BLUE–GRAY PHENOTYPIC VARIATION
Francisella tularensis LVS phase has been observed to vary from a blue (wild-type) colony to a gray colony variant (Eigelsbach et al., 1951; Cowley et al., 1996). Such gray variants have been both characterized with an extended LPS O-antigen (Cowley et al., 1996) as well as a truncated O-antigen (Hartley et al., 2006). Our work described here is with the *Ft* LVSG isolate (a variant with an extended O-antigen), but at times comparisons are made to *Ft* LVSGD (a variant with no O-antigen). We examined various media conditions and growth phases to determine the conditions that affected the degree of phase variation. We found that *Ft* LVSG grows slower than *Ft* LVS, forms smaller colonies on agar surfaces, and appeared gray by eye on Choc II agar plates under oblique lighting (**Figures 1A,B**). The frequency of blue to gray phase variation was higher (27–31%) in liquid cultures (TSB + 0.1% cysteine HCL) grown to stationary phase (typically 30–48 h) and plated on solid agar. The frequency of
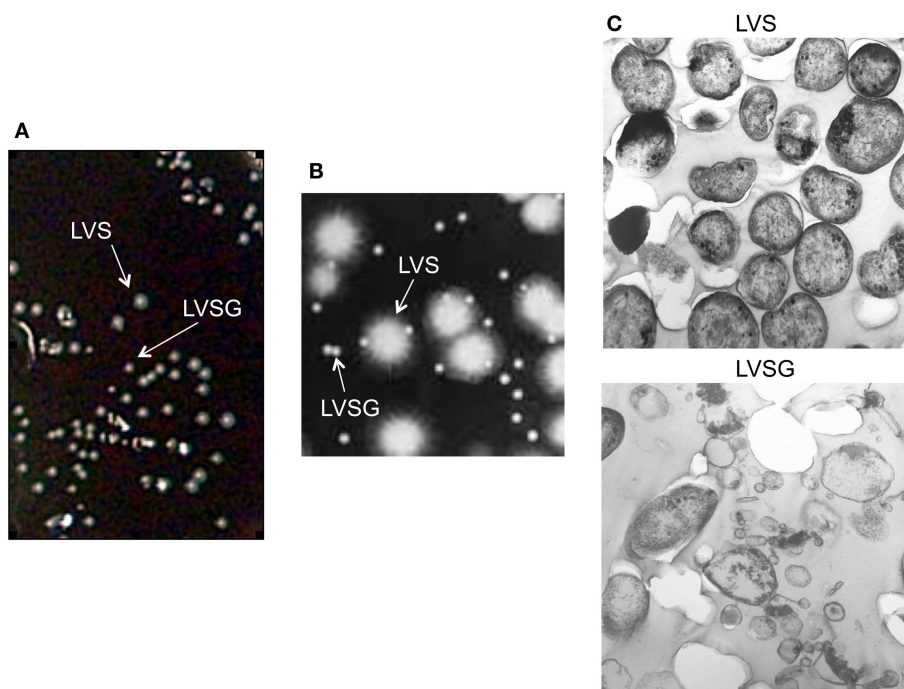
FIGURE 1 | *Ft* LVS blue–gray colony and bacterial morphology. (A) Mixed cultures of *Ft* LVS and phase variant (*Ft* LVSG) viewed on the surface of a Choc II agar plate. Note that *Ft* LVSG forms smaller colonies that *Ft* LVS. (B) Blue (LVS) and Gray (LVSG) colonies viewed under a compound light microscope by oblique lighting. The smaller colonies appeared gray. (C) Transmission electron microscopy of *Ft* LVS and *Ft* LVSG. While the bacterial dimensions were similar between these two strains from measuring >50 fields, the *Ft* LVSG strain formed a large amount of membrane vesicles.

phase variation was minimal (2–5%) for bacteria grown on plates 1–2 days and in log phase liquid cultures. We also observed that the frequency of blue to gray phase variation dramatically increased when *Ft* LVS was passed through macrophages (23–27%) or recovered from organs of infected animals (31–36%). We also observed that the frequency of forward phase variation in broth grown bacteria (blue to gray) was always higher (~30%) than frequency of reverse (gray to blue) phase variation (5–7%).

To more clearly compare *Ft* LVS to *Ft* LVSG bacteria, log phase cultures were examined by scanning electron microscopy. Comparisons of average cell size were not significantly different, but more membrane vesicles were observed in *Ft* LVSG cultures (**Figure 1C**). It is not clear what impact this increased vesiculation has on the subsequent phenotypes described for *Ft* LVSG.

FT LVSG LPS POSSESSES LESS O-ANTIGEN

It was shown previously that the LPS of *Ft* LVS and *Ft* LVSG had differential reactivity to monoclonal antibodies stated to be O-antigen specific (Cowley et al., 1996), suggesting an O-antigen antigenic switch. To further examine the LPS O-antigen and its antigenic properties, we purified LPS from *Ft* LVS, *Ft* LVSG, *F. novicida*, *Ft* LVSGD, *F. tularensis* SchuS4 and a *F. tularensis* SchuS4 small colony gray variant and performed silver staining on SDS-PAGE separated samples. Consistent with previously published results, the gray variant (*Ft* LVSG) possessed an O-antigen but *Ft* LVSGD was rough (lacked O-antigen) (**Figure 2A**). The *F. tularensis* SchuS4 small colony gray variant also appeared to produce an LPS with a repeating O-antigen.

Glycosyl composition analysis of the OSs released from the purified LPS preparations show that the *Ft* LVS and *Ft* LVSG OSs contain the same glycosyl residues, but there is a large quantitative difference, in that the *Ft* LVS OS contains much larger amounts of QuiN and Gal than what is found in the OS from the *Ft* LVSG strain (**Table 1**). The QuiN could be due to QuiNFo as NMR analysis (data not shown) shows a significant resonance at around 8 ppm, which is consistent with a formyl proton. During the preparation of trimethylsilyl (TMS) methyl glycoside, which is accompanied by N-acetylation, this formyl group would have been replaced by an acetyl group. The large difference in these components between the LPS of *Ft* LVS and *Ft* LVSG would indicate that the *Ft* LVS LPS contains much more of the QuiN/Gal-containing O-antigen chain polysaccharide than *Ft* LVSG. Thus, these data suggest that the *Ft* LVSG LPS has an O-antigen but the O-antigen contains fewer repeating units than seen in *Ft* LVS LPS or that LVSG lipid A-core is capped less frequently with O-antigen. Interestingly, even though the two variants (*Ft* LVSG and *Ft* LVSGD) have distinct LPS regarding the amount of O-antigen present (albeit both with amounts less than that of wildtype), they give rise to morphologically similar gray variants.

MONOCLONAL AND POLYCLONAL ANTIBODY REACTIVITY AND LPS STRUCTURAL ANALYSIS SUGGESTS BLUE/GRAY STRAIN LPS CORE ALTERATIONS

Western blot analysis was performed on LPS samples using commercially available anti-*F. tularensis* FB-11 (**Figure 2B**) antibodies stated to be specific to the O-antigen, as well as anti-*F. tularensis* (**Figure 2C**)
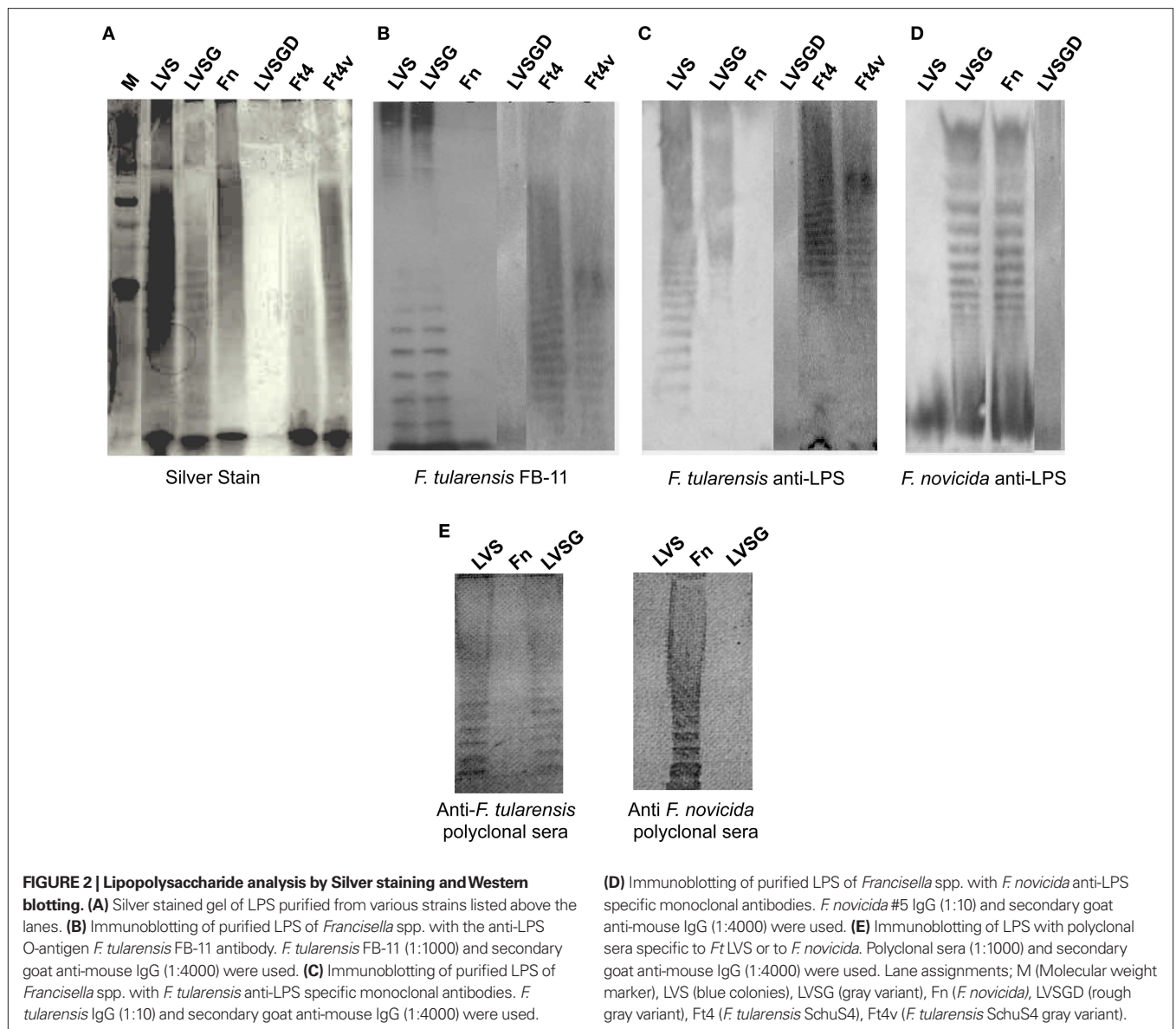
FIGURE 2 | Lipopolysaccharide analysis by Silver staining and Western blotting. (A) Silver stained gel of LPS purified from various strains listed above the lanes. (B) Immunoblotting of purified LPS of *Francisella* spp. with the anti-LPS O-antigen *F. tularensis* FB-11 antibody. *F. tularensis* FB-11 (1:1000) and secondary goat anti-mouse IgG (1:4000) were used. (C) Immunoblotting of purified LPS of *Francisella* spp. with *F. tularensis* anti-LPS specific monoclonal antibodies. *F. tularensis* IgG (1:10) and secondary goat anti-mouse IgG (1:4000) were used.

(D) Immunoblotting of purified LPS of *Francisella* spp. with *F. novicida* anti-LPS specific monoclonal antibodies. *F. novicida* #5 IgG (1:10) and secondary goat anti-mouse IgG (1:4000) were used. (E) Immunoblotting of LPS with polyclonal sera specific to *Ft* LVS or to *F. novicida*. Polyclonal sera (1:1000) and secondary goat anti-mouse IgG (1:4000) were used. Lane assignments; M (Molecular weight marker), LVS (blue colonies), LVSG (gray variant), Fn (*F. novicida*), LVSGD (rough gray variant), Ft4 (*F. tularensis* SchuS4), Ft4v (*F. tularensis* SchuS4 gray variant).

Table 1 | Trimethylsilyl methyl glycoside analysis of OSs purified from the LPS of *Ft* LVS LPS and the gray variant *Ft* LVSG LPS.

OS	Man	Gal	Glc	QuiNAc*	GalNAc	Kdo
LVS	13	18	28	36	3	3
LVSG	24	5	62	0.5	7	3

*The amount of the QuiNAc could not be precisely quantified since there is no original standard available and, therefore, quantification was based on using the response factor for GlcNAc.
Man, mannose; Gal, galactose; Glc, glucose; QuiNAc, 2-acetamino-2,6,dideoxy-ᴅ-glucose; GalNAc, N-acetyl galactosamine; Kdo, 3-deoxy-ᴅ-manno-octulosonic acid.

and anti-*F. novicida* LPS monoclonal antibodies (**Figure 2D**). The results showed that *Ft* LVS LPS reacted with both the *F. tularensis* monoclonal and FB-11 antibodies showing the typical LPS laddering,

and while the laddering was not observed on *Ft* LVS LPS Western blots with the *F. novicida* specific antibodies, reactivity was observed to a low molecular weight species that is typically lipid A plus core (**Figures 2B–D**). *F. novicida* LPS did not react at all to the anti-*F. tularensis* LPS or FB-11 antibody, but strongly to a low molecular weight species (plus typical laddering) with the *F. novicida* specific monoclonal antibody. *Ft* LVSG reacted with both *F. tularensis* and the *F. novicida* anti-LPS monoclonal antibodies. These results are consistent with those of Cowley et al. (1996) with regard to O-antigen ladder reactivity, but the lipid A core region is not clearly visible on their gels. As expected, LVSGD did not demonstrate the typical O-antigen ladder due to its lack of O-antigen (**Figures 2B–D**). The *F. tularensis* SchuS4 gray variant reacted with both anti-*F. tularensis* monoclonal antibodies and clearly possesses an O-antigen based on the observed laddering. However, the modal chain length or capping frequency of this O-antigen, like that of *Ft* LVSG, appears reduced versus *F. tularensis* SchuS4 (**Figures 2B–D**).

These LPS samples were reacted in a Western blot with anti-*F. tularensis* or anti-*F. novicida* polyclonal sera generated from infected mice (**Figure 2E**). *Ft* LVS LPS and *F. novicida* LPS only reacted with their respective antisera while *Ft* LVSG now reacted only with *F. tularensis* polyclonal sera. These results suggest that changes in the *Ft* LVSG LPS are specifically recognized by monoclonal but not polyclonal antibodies.

We next isolated and performed extensive NMR analyses on the *Ft* LVS and *Ft* LVSG OPSs to determine if any structural differences could be detected between these molecules. Crude LPS was enzymatically treated to remove contaminating nucleic acids and proteins and ultracentrifuged. The LPS pellet was collected and the carbohydrate portion of LPS was released from lipid A via mild hydrolysis. The carbohydrate fractions from *Ft* LVS and *Ft* LVSG of were resolved by HPLC. The carbohydrates eluted in three primary fractions. Fraction 1 contained the OPS, Fraction 2 contained slightly lower molecular weight OPS, and Fraction 3 contained the core OSs with some possibly low molecular weight OPS repeat units. From these results, the Fraction 1/3 ratio for *Ft* LVS LPS is 17, while it is 4.6 for LVSG (**Table 2**). These results are consistent with the above data showing that the *Ft* LVSG strain contains less OPS as reflected by the lower QuiN level during composition analysis. Fractions 1 and 3 were analyzed by NMR spectrometry. Fraction 1 from both *Ft* LVS and *Ft* LVSG were compared to each other using 2D NMR experiments – COSY, TOCSY, NOESY, and HSQC (data not shown). These results indicate that the OPS from *Ft* LVS and *Ft* LVSG have the same structures. In addition, the data are completely consistent with the structure reported for *F. tularensis* strain 15, strain SchuS4, and OSU10 (Vinogradov et al., 2002; Prior et al., 2003; Thirumalapura et al., 2005). The results clearly support the conclusion that *Ft* LVS and *Ft* LVSG have the following OPS structure as previously reported for the above *F. tularensis* strains:

-4)-α-D-Gal*p*NAcAN-(1 → 4)-α-D-Gal*p*NAcAN-(1 → 3)-β-D-Qui*p*NAc-(1 → 2)-β-D-Qui*p*4Fo-(1 →

The differential staining with the *F. tularensis* and *F. novicida* monoclonal antibodies coupled with the fact that the *F. novicida* monoclonal antibody binds to the LMW LPS and is, therefore, likely binding to the core OS, suggests that the true monoclonal antibody-tracked alteration between *Ft* LVS and *Ft* LVSG is related to the core region. Therefore, we analyzed OSs found in Fraction 3 from *Ft* LVS and *Ft* LVSG by 1D proton NMR spectroscopy. The results are shown in **Figure 3**. The spectrum of *Ft* LVS Fraction 3 indicates that a small amount of truncated OPS is still present.

We also see resonances that are consistent with the published core structure with the exception that we do not observe evidence for the core GalNAc residue. The *Ft* LVS and *Ft* LVSG proton spectra clearly differ from one another indicating that the *Ft* LVSG Fraction 3 contains different structures than found in the *Ft* LVS Fraction 3. Therefore, since the OSs in Fraction 3 would be those that would comprise the core region, as well as some possible truncated OPS, these data support the conclusion that the *Ft* LVSG has an altered core region compared to that of *Ft* LVS.

ANALYSIS OF *FT* LVSG LIPID A DEMONSTRATES A REDUCTION IN GALACTOSAMINE MODIFICATION

Cowley et al. (1996) demonstrated that *Ft* LVSG lipid A versus that of *Ft* LVS elicited increased NO induction in rat macrophages. We confirmed this finding (NO production by rat macrophages measured as nitrate by the Griess reagent system) by both *Ft* LVSG LPS and purified lipid A (data not shown). This suggested that the lipid A of *Ft* LVSG was different than that of *Ft* LVS. To explore these differences, we performed structural analyses on purified lipid A of *Ft* LVS and *Ft* LVSG. MALDI-TOF analysis in negative and positive ion mode was performed on replicate lipid A preparations.
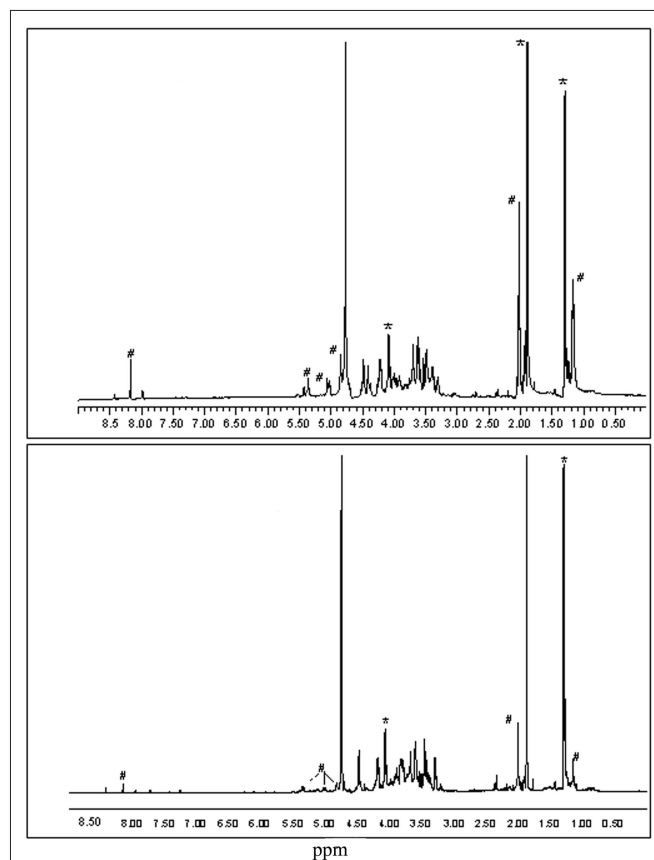

FIGURE 3 | The proton NMR spectra of the LPS core region oligosaccharides from *Ft* LVS and *Ft* LVSG. The proton NMR spectra of the oligosaccharides found in Fraction 3 from *Ft* LVS (top) and Fraction 3 from *Ft* LVSG (bottom) are shown. The resonances marked with # are likely due to OPS fragments. Those marked with * are due to contaminating acetate and lactate. Thus, non-marked resonances denote the LPS core region.
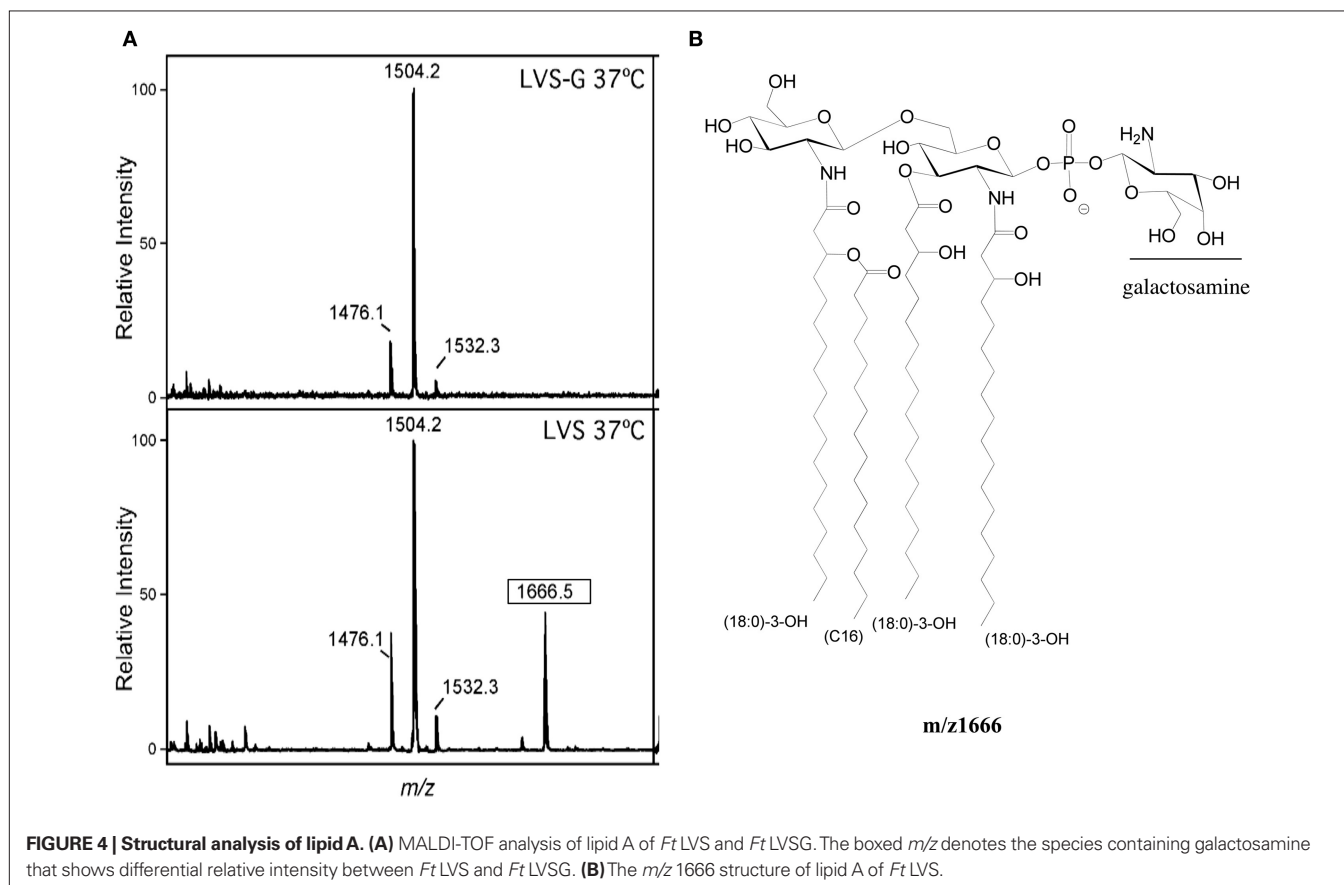
Table 2 | Main fraction and yields obtained in gel filtration using Superdex peptide column.

Oligosaccharide	HPLC Fr1 (mg) (tube 18–25)	HPLC Fr2 (mg) (tube 26–28)	HPLC Fr3 (mg) (tube 29–50)
Ft LVS	7.62*	0.3	0.46**
Ft LVSG	0.65*	0.02	0.14**

*Used in 1D and 2D NMR experiments.
**Used in 1D NMR experiments.

Both showed the absence of a peak at *m/z* 1666 in *Ft* LVSG that was present in *Ft* LVS. It is known that this peak represents the addition of galactosamine (161 Da) to the basic structure at *m/z* 1504 (2× GlcN, 3× C18:0 (3-OH), C16:0, P), though previous data suggested that this modification was not observed in the *Ft* LVS strain (Kanistanon et al., 2008; **Figures 4A,B**). This was the only structural alteration noted. Since the MALDI-TOF analysis is only semi-quantitative, we performed galactosamine quantitation assays to further demonstrate the reduced galactosamine modification of lipid A in *Ft* LVSG. The lipid A was derivatized and analyzed by GC–MS. *F. novicida* showed the highest degree of modification at 25% while *Ft* LVS was at 14%. Both *Ft* LVSG and *Ft* LVSGD showed reduced galactosamine modification, with 6 and 7%, respectively (**Figure 5A**).
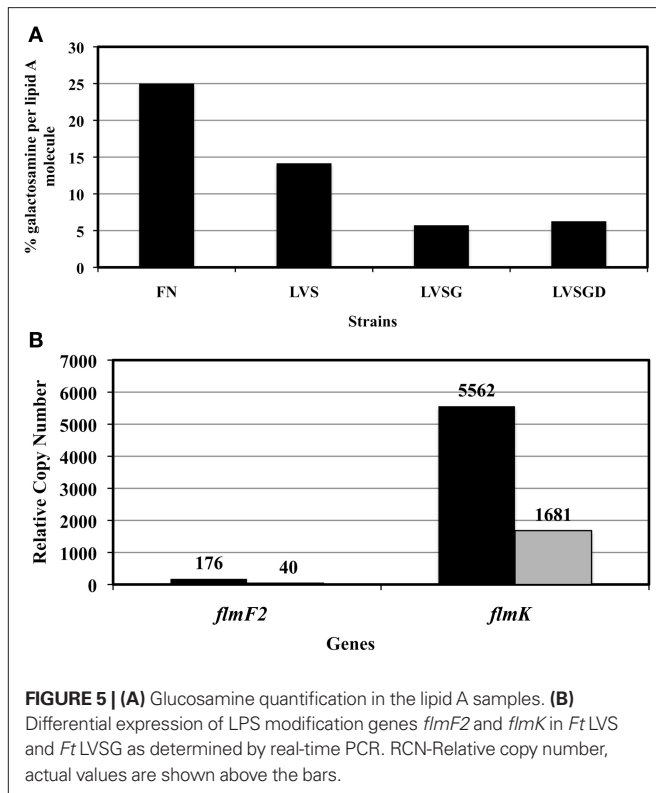
Three genes have been identified that are responsible for galactosamine or mannose lipid A modification (Gunn and Ernst, 2007; Kanistanon et al., 2008). The transferases FlmF1 and FlmF2 are required for adding mannose or glucosamine residues, respectively, to the lipid A. The glycosyltransferase FlmK can add both mannose and galactosamine to lipid A (Gunn and Ernst, 2007; Kanistanon et al., 2008). A real-time PCR assay was performed on the genes *flmF2* and *flmK* from *Ft* LVS and *Ft* LVSG to determine if their expression was altered and might be responsible for the observed lipid A galactosamine modification alteration. Expression of both genes was found to be significantly less in *Ft* LVSG (**Figure 5B**), correlating with the reduction in galactosamine modification. Mutation

of the *flmF2* gene in *Ft* LVS, which eliminates the galactosamine modification, reduced the frequency of phase variation from 30% to 5-7% in stationary phase liquid cultures. Though *F. novicida* strains carrying this mutation have been shown to affect mouse virulence and cytokine/chemokine induction in macrophages, the *Ft* LVS *flmF2* mutant demonstrated no defect in survival in macrophages of mouse (Raw, J774.1 and MH-S), rat (bone marrow derived, alveolar), or human (THP-1 and monocyte derived macrophages) origin (data not shown, see below section).

THE *FT* LVSG VARIANT HAS A RAT AND HUMAN BUT NOT MOUSE INTRAMACROPHAGE SURVIVAL DEFECT

It has been shown previously by Cowley et al. (1996) that *Ft* LVS and *Ft* LVSG intracellular growth/survival was similar in mouse macrophages, but that differences in growth/survival could be visualized in rat bone marrow-derived macrophages. We examined the survival of *Ft* LVS and *Ft* LSVG in various macrophages including J774.1 (a mouse macrophage cell line), MH-S (a mouse alveolar macrophage cell line) and mouse bone marrow-derived macrophages and did not find any significant differences in survival of *Ft* LVS and *Ft* LSVG (data not shown). However, we observed that *Ft* LVSG survived less well in rat bone marrow derived macrophages and a rat alveolar macrophage cell line (ATCC# CRL-2192) and that this inhibition of growth of *Ft* LVSG can be reversed by using the NO inhibitor NMMA (data not shown). These findings were consistent with the previous findings of Cowley et al. (1996). We
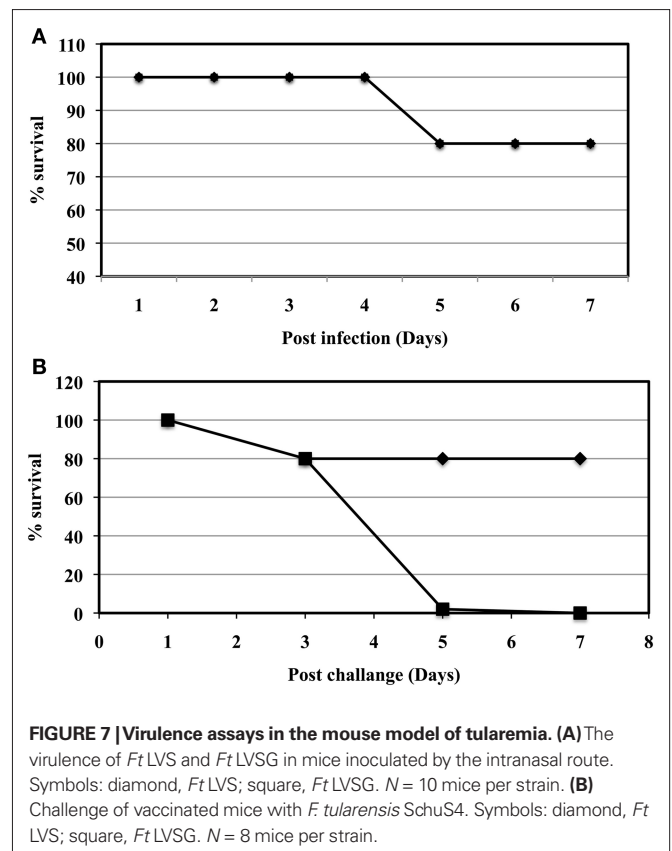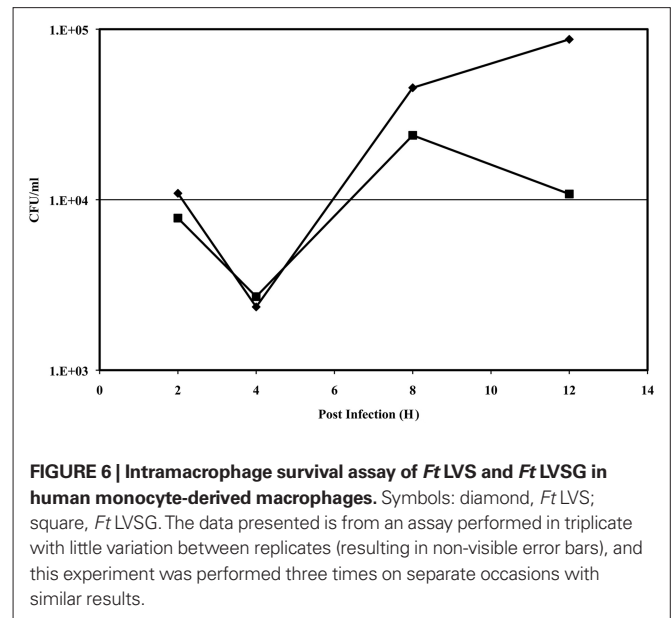

FIGURE 4 | Structural analysis of lipid A. (A) MALDI-TOF analysis of lipid A of *Ft* LVS and *Ft* LVSG. The boxed *m/z* denotes the species containing galactosamine that shows differential relative intensity between *Ft* LVS and *Ft* LVSG. **(B)** The *m/z* 1666 structure of lipid A of *Ft* LVS.

FIGURE 6 | Intramacrophage survival assay of *Ft* LVS and *Ft* LVSG in
human monocyte-derived macrophages. Symbols: diamond, *Ft* LVS;
square, *Ft* LVSG. The data presented is from an assay performed in triplicate
with little variation between replicates (resulting in non-visible error bars), and
this experiment was performed three times on separate occasions with
similar results.

FIGURE 5 | (A) Glucosamine quantification in the lipid A samples. (B)
Differential expression of LPS modification genes *flmF2* and *flmK* in *Ft* LVS
and *Ft* LVSG as determined by real-time PCR. RCN-Relative copy number,
actual values are shown above the bars.

then examined intramacrophage survival in human MDMs and
the THP-1 macrophage-like cell line. We observed that the *Ft* LVSG
strain survived less well over the first 12 h post-infection in both
cell types with macrophage cell death at later time points. The
defect was most prominent for the MDMs, where the *Ft* LVSG
strain demonstrated nearly a log defect in survival at 12 h post-
infection versus the *Ft* LVS strain (**Figure 6**). Thus, the *Ft* LVSG
strain has an intramacrophage survival defect in rat and human
but not mouse macrophages.

FT LVSG AND *FT* LVS ARE SIMILARLY VIRULENT IN THE MOUSE MODEL BUT DIFFERENTIALLY PROTECT AGAINST *F. TULARENSIS* SCHUS4 CHALLENGE

Gray variants have been shown to be less virulent (Eigelsbach and
Downs, 1961) and/or less protective as vaccines against *F. tularen-
sis* SchuS4 challenge (Eigelsbach and Downs, 1961; Cowley et al.,
1996; Hartley et al., 2006; Conlan and Oyston, 2007) However,
the specific virulence of the *Ft* LVSG strain in the mouse model
of tularemia has not been determined. To compare the virulence
of *Ft* LVS and *Ft* LVSG, BALB/c mice were infected with 100 CFU
of *Ft* LVS and *Ft* LVSG intranasally and observed for survival.
Both *Ft* LVS and *Ft* LVSG infected mice demonstrated 80% sur-
vival (*N* = 10 mice; **Figure 7A**). The surviving mice were chal-
lenged with 1000 CFU of *F. tularensis* SchuS4 (~100-fold above
the LD_{50}) intranasally 4 weeks post vaccination (**Figure 7B**). All
Ft LVS vaccinated mice (*N* = 8) survived the challenge whereas
Ft LVSG vaccinated mice (*N* = 8) could not survive the challenge
and succumbed to infection within 5 days. These results suggest
that *Ft* LVSG is as virulent in mice as *Ft* LVS but it does not protect
against Type A challenge.

FIGURE 7 | Virulence assays in the mouse model of tularemia. (A) The
virulence of *Ft* LVS and *Ft* LVSG in mice inoculated by the intranasal route.
Symbols: diamond, *Ft* LVS; square, *Ft* LVSG. *N* = 10 mice per strain. (B)
Challenge of vaccinated mice with *F. tularensis* SchuS4. Symbols: diamond, *Ft*
LVS; square, *Ft* LVSG. *N* = 8 mice per strain.

DISCUSSION

Francisella tularensis LVS has been known to phase vary from a
blue (i.e., wildtype) to a gray variant since the phenomenon was
first described by Eigelsbach in 1951 (Eigelsbach et al., 1951).
Such variation has proven to be an issue historically in vaccine
production runs of *Ft* LVS (Conlan and Oyston, 2007; Oyston,

2009). The *Ft* LVS gray variants are problematic because they are dramatically less efficacious than the blue colony morphotypes in protection studies against the virulent *F. tularensis* Type A strain. The phenotypes associated with the gray variants are quite variable, as they have been described to give rise to different colony sizes and opacity, but the only two that have been molecularly characterized share the characteristic of LPS alteration. While this characteristic is shared, this also presents yet another difference, as the gray variant described by Hartley et al. (2006), (similar to *Ft* LVSGD studied in this work) has been shown to lack O-antigen while *Ft* LVSG still possessed an O-antigen, albeit reduced in amount, which has altered *F. novicida*/*F. tularensis*-LPS specific monoclonal antibody reactivity patterns. Thus, gray variants are themselves variable, but an increased understanding of the mechanism(s) behind this variation would aid future tularemia vaccine production.

In addition to the presence/absence of the O-antigen, we demonstrate biochemically that the *Ft* LVSG O-antigen chain carbohydrates are reduced in *Ft* LVSG versus *Ft* LVS. It is unclear if this demonstrates that the O-antigen chain length of LVSG is shorter or if the lipid A plus core is capped less efficiently with O-antigen. A reduction in *Ft* LVSG O-antigen chain carbohydrates was previously suggested in a manuscript by Clay et al. (2008) based on evidence from silver staining patterns and Western blot analysis. Clay et al. (2008) also demonstrated that the *Ft* LVSG strain was bound by complement component C3 in higher amounts than *Ft* LVS and was dramatically more susceptible to complement-mediated killing. It is of interest that, while susceptible to complement, *Ft* LVSG is still as virulent as *Ft* LVS in the mouse model. It is likely that the effect of complement-mediated killing is quantitative rather than absolute and that the current mouse model is not sensitive enough to record an effect. Alternatively, the result in the mouse model may reflect a fundamental difference in complement function between mouse and man. For example, it is known that C3 in mouse serum is more labile than in human serum and this may result in different levels of C3 opsonization and regulation in mouse versus human serum.

Both *F. tularensis* and *F. novicida* anti-LPS monoclonal antibodies reacted with LVSG, which had been interpreted to demonstrate that the O-antigen of *Ft* LVSG was altered, creating an epitope(s) reactive to both antibodies. However, closer examination of the Western blots show *F. novicida* monoclonal antibody reactivity to *Ft* LVSG LPS with low molecular weight species, likely lipid A plus core, as well as higher species containing lipid A, plus core, plus O-antigen repeats. The *F. novicida* anti-LPS monoclonal antibody reacted only with the low molecular weight species to *Ft* LVS LPS. This suggested that the *F. novicida* anti-LPS monoclonal antibody recognized a core epitope and that core, and not the O-antigen regions of *Ft* LVS and *Ft* LVSG, may differ. This was confirmed by NMR analysis of purified LPS carbohydrates, which demonstrated identity between the *Ft* LVSG and *Ft* LVS O-antigen regions while the LPS core region of *Ft* LVSG was different from that of *Ft* LVS. If, as we suspect, the *F. novicida* anti-LPS monoclonal antibody epitope is within the core region and the *Ft* LVS anti-LPS monoclonal epitope is within the O-antigen, then because of observed Western blot reactivities, both *Ft* LVS and *Ft* LVSG contain an "*F. novicida*-like" core. Thus, it is possible that *Ft* LVS makes two

distinct core OSs (an *F. novicida* monoclonal antibody reactive and an *Ft* monoclonal antibody reactive), but cannot add O-antigen to the "*F. novicida*-like" core. *Ft* LVSG, on the other hand, can either produce both core types and ligate O-antigen to both, or can only make the *F. novicida* reactive core type and can add O-antigen to this core.

Regarding the mechanism of O-antigen chain length reduction/differential O-antigen ligation, these processes are typically mediated by an O-antigen ligase (e.g., RfaL), O-antigen polymerase (e.g., Wzy) and a chain length determinant (e.g., Cld). *Francisella* spp. appear to have multiple proteins with homology to RfaL, and their differential expression may account for the observed O-antigen phenotypes. BLAST searches of the *Ft* LVS genome with the RfaL locus of *Salmonella typhimurium* revealed three high scoring loci: FTL1122 (41% identity but 188 amino acids in *Ft* LVS versus 292 amino acids in *F. tularensis* SchuS4), FTL 0706 (37% identity), and FTL0598 (26% identity, called the *wzy* locus in *Ft* LVS; Prior et al., 2003). Surprisingly, BLAST searches with the *S. typhimurium* Wzy showed no strong identity to *Francisella* proteins. No Cld ortholog exists in *F. tularensis* (Prior et al., 2003; our recent BLAST searches). It is unclear if another unknown enzyme serves this function or is the O-antigen chain length is unregulated in this bacterium. Thus, it is possible that multiple or novel proteins mediate the O-antigen polymerase, ligase and length determination functions in *Francisella* and may be responsible for the core/O-antigen data described here. Further biochemical analysis is ongoing to determine the exact structural changes in the *Ft* LVSG LPS core region, as are genetic experiments to identify genes conferring *F. novicida* monoclonal antibody reactivity to *Ft* LVS.

The lipid A regions of *Ft* LVS and *Ft* LVSG were shown to be identical with the exception of a galactosamine modification, which was reduced/absent in the *Ft* LVSG variant. The galactosamine modification was not detected previously in *Ft* LVS, but was clearly evident in the *Ft* LVS strain analyzed here. Mannose is also observed as a modification of lipid A in *F. novicida* and Type A subspecies, but not Type B subspecies (the background of the *Ft* LVS strain). Consistent with these published data, mannose was not observed in our assays. While the *flmF2* (glucosamine addition) and *flmK* (both mannose and glucosamine addition) genes showed reduced transcription in the *Ft* LVSG strain consistent with the reduced lipid A modification, mutants in these genes in *Ft* LVS did not result in the small colony or gray phenotype on plates nor were the mutants defective in intramacrophage survival. Thus, these mutants did not exhibit obvious characteristics of the *Ft* LVSG gray variant. These mutants did, however, demonstrate a dramatically reduced phase variation rate to gray variants, suggesting that galactosamine modification may be involved in but is not sufficient for the gray phenotype. Further phenotypic testing (e.g., lipid A NO induction, mouse virulence) is ongoing, as is the construction of a double *flmF2 flmK* mutant. It is also possible that no expression versus reduced expression of these enzymes may have different effects, but titrated gene expression in *Francisella* is not yet a reality, so this concern will be addressed when the technology is available.

Experiments performed in this study confirmed those of Cowley et al. (1996) regarding increased NO induction in rat but not mouse phagocytes by the gray strain LPS. This correlated with decreased

survival of these gray variants in rat and human macrophages, which could be reversed in rat macrophages by the addition of a NO synthase inhibitor. However, experience suggests that stimulated mouse macrophages are better capable than, for e.g., human MDMs, of NO production. Thus, there is no direct correlation of strain intracellular survival with the inherent capabilities of the macrophages of the chosen animal model to produce NO, suggesting the involvement of additional factors.

Based on the results of Eigelsbach and colleagues (Eigelsbach et al., 1951; Eigelsbach and Downs, 1961) and Hartley et al. (2006), we were surprised that the *Ft* LVSG strain did not possess a virulence defect by the intranasal route. However, in the Hartley et al. (2006) study, mice were vaccinated and challenged by the subcutaneous route, and the Eigelsbach work (Eigelsbach et al., 1951; Eigelsbach and Downs, 1961) typically used intraperitoneal vaccination and subcutaneous challenge. Thus the route of administration may play a role in gray variant virulence. Consistent in all gray variant mouse model vaccination experiments is their reduced capacity to protect against challenge by the Type A *F. tularensis* subspecies. The mechanism behind the lack of protective capacity is not known. *Ft* LVS *flmF2* and *flmK* mutants were avirulent in the mouse model, thus the observed reduction in galactosamine modification may play a role in early clearance and the lack of development of a protective immune response (Kanistanon et al., 2008). While 1-D gel electrophoresis of whole cell lysates and fractions showed no obvious protein differences between *Ft* LVSG and *Ft* LVS, it is possible that these bacteria may possess alterations other than those observed in the LPS. Ongoing assays include microarray analysis, 2-D gel electrophoresis and other more sophisticated proteomic analysis. The continued study of gray variants of *F. tularensis* will provide important mechanistic details behind these phenotypically distinct bacteria, which will move the field closer to the ability to phase lock a wildtype strain for the development of effective and safe tularemia vaccines.

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