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The Structure and Function of *Francisella* Lipopolysaccharide

John S. Gunn[a] and **Robert K. Ernst**[b]

[a]The 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 43210, USA

[b]Department of Medicine, Division of Allergy and Infectious Diseases, University of Washington, HSB T-293, Box 357710, 1959 NE Pacific Street, Seattle, WA 98195, USA

Abstract

A key factor in the biology of *Francisella* spp. is lipopolysac-charide (LPS). *Francisella* LPS has many unique structural properties and poorly activates proinflammatory responses due to its lack of interaction with toll-like receptor 4 (TLR4). The LPS of this organism can be modified by various carbohydrates including glucose, mannose and galactosamine, which affect various aspects of virulence. Spontaneously occurring colony variants of *F. tularensis* have altered LPS. This altered LPS may account for the novel phenotypes of these variants that include effects on susceptibility to killing by normal human serum, intracellular survival and animal virulence. Mutants devoid of O-antigen (directed mutants in O-antigen biosynthetic genes) show reduced intracellular survival and mouse virulence. Thus, this surface component is important in *F. tularensis* pathogenesis, and the inability of the LPS to alarm the immune system coupled with its frequent modification/alteration likely aid the success of this pathogen during human infection

Keywords

LPS; lipopolysaccharide; gray variant; phase variation; LPS modification; LPS structure; lipid A; O-antigen

Introduction

Lipopolysaccharide (LPS, endotoxin) is the primary constituent of the outer leaflet of the outer membrane of Gram-negative bacteria. The structure of LPS includes a lipid portion (lipid A) that anchors it into the membrane, a polysaccharide core and an oligo- or polysaccharide extending from the core beyond the bacterial surface. LPS has been shown to be essential in most Gram-negative organisms likely due to its role in membrane integrity, and is a key factor in immune stimulation via its detection by host pattern recognition receptors.[1]

In many respects, the LPS of *Francisella* spp. is unique. Of critical importance is that the *Francisella* LPS lacks free phosphate moieties and exhibits very low endotoxicity. Because of the current advances in *Francisella* genetics and genomics, considerable new information regarding the LPS of this organism has been recently discovered. This new information relates to LPS structural variation between species and subspecies, intra-strain phase/antigenic variation and its role in virulence and immune recognition. This chapter will provide an up-to-

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Address for correspondence: John S. Gunn, The Ohio State University, Biomedical Research Tower, Rm. 1006, 460 W. 12th Ave., Columbus, OH 43210-1214, USA. Voice: 614-292-6036; fax: 614-292-5495. gunn.43@osu.edu.

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date as well as historical perspective on these aspects of LPS biology in the Francisellae. Much of the information is currently unpublished but important to incorporate here, as it will form the basis for future studies in this field.

Structure of *Francisella* LPS (LIPID A, CORE, and O-ANTIGEN)

Lipid A

The genus *Francisella tularensis* consists of four subspecies: *tularensis* (Type A), *holartica* (Type B), *mediasiatica*, and *novicida*. The structure of lipid A isolated from two type B *Francisella tularensis* subspecies *holartica* strains (1547–57 and LVS) and *F. novicida* (U112) after growth at 37°C were elucidated using mass spectrometry (MS), gas chromatography, nuclear magnetic resolution (NMR) and chemical methods.[2–4] In these studies, only the primary structures from the dominant molecular ions were elucidated. The base lipid A structure for all strains was similar and consisted of a β-([1,6])-linked diglucosamine disaccharide with amide-linked fatty acids at the 2 (3-OH C18) and 2′ (3-OH C18-O-C16) positions, and ester-linked fatty acids at the 3 (3-OH C18), but not the 3′ positions (FIG. 1). Additions to the base lipid A structure in *F. holartica* (1547–57) and *F novicida* included a single phosphate moiety at the 1 position of the reducing glucosamine residue (absent in the LVS strains and suggests a potential role in virulence). Interestingly, the 1-phosphate moiety was further substituted with the positively charged sugar, galactosamine [2] that was recently shown to be α-linked in *F novicida* by Wang *et al*.[4] The functional outcome of galactosamine modification of lipid A, previously described as a component of total lipid A preparations from the oral pathogen, *Selenomonas sputigena*, is unknown.[5]

F. novicida lipid A was further modified by a mannose residue predicted to be at the 4′ position of the non-reducing glucosamine or by the addition of an α-linked glucose moiety at the 6′ position.[4] Interestingly, Wang *et al*. demonstrated this unique modification in free lipid A, i.e extracted without hydrolysis, which results in the lack the usual Kdo, core, and O-antigen sugars.[4] Furthermore, they suggest that over 95% of the lipid A recovered after Bligh-Dyer lipid extraction was present in the “free” form and suggests the presence of a novel system of LPS remodeling enzymes in the Francisellae. Finally, matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry of lipid A isolated from clinical and environmental strains of *F. tularensis* subspecies *tularensis, holartica*, and *mediasiatica* after growth on rich medium at 37°C were similar and suggests a common lipid A structure for all *F. tularensis* subspecies.[6]

Core

The structure of the core region isolated from the type B *F holartica* strain (LVS)[3] and *F. novicida* (U112)[7] after growth at 37°C was elucidated using MS and NMR techniques (FIG. 1). The core region is linked to lipid A through the eight-carbon sugar, 3-deoxy-D-manno-octulosonic acid (Kdo). The core structure from *F. novicida* is nearly identical to that of *F. holartica*, differing by the addition of an additional α-glucose residue attached to the β-Glc attached to the central inner core α-Man residue. (Note error in core structure[3] – the α-Glc residue is shown on the α-Man instead of the β-Man residue). The core region of both strain backgrounds was shown to lack phosphate modifications and contain a single Kdo residue, suggesting the presence of a Kdo-specific hydrolase similar to enzymatic activity recently identified in membrane extracts from *Helicobacter pylori*.[8]

O-antigen

Since the original NMR structural characterization of the O-antigen region of LPS isolated from the *F. holartica* vaccine strain, strain 15 by Vinogradov *et al*. in 1991,[9] the O-antigen structures of two additional type A *F. tularensis* strains (Schu S4 and OSU10),[10,11] a second

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type B *F. holartica* strain (LVS),[10,12] and *F. novicida* (U112)[13] after growth at 37°C have been elucidated (FIG. 1). These data clearly show that the O-antigen from the Type A and B strains are identical in structure, whereas the structure of *F. novicida* O-antigen shares the same internal two carbohydrate residues (α-D-GalNAcAN- α-D-GalNAcAN), though differs for the outside two residues (α-D-GalNAcAN and β-DQui2NAc4NAc for *F. novicida* versus β-D-Qui4NFm and β-D-QuiNAc for Type A and B). Analysis of the O-antigen gene clusters from various Francisellae backgrounds confirms the structural analysis results. The O-antigen gene clusters for Schu4 and LVS are identical, as compared to that from *F. novicida*, which has fewer genes in the cluster and a lower G+C ratio.[10,14]

Identification and Regulation of Genes Involved in Lipid A Biosynthesis

Recently, six genes have been identified in *F. novicida* that encode enzymes required for the synthesis of the base lipid A structure[1,15,16] (FIG. 2). These enzymes include two phosphatases (LpxE and LpxF, not present in *E. coli*), two dolichyl phosphate-mannose synthase-like enzymes (PmrF1 and PmrF2), one glycosyltransferase (PmrK), and a second early acyltransferases (LpxD2). All enzymes are highly conserved (>98% identity, at the amino acid level) in the four currently sequenced *Francisella* background strains: Schu S4 (Type A), LVS and OSU200 (Type B), and *F. novicida* (U112), thereby suggesting a conserved role for these enzyme homologs in overall Francisellae lipid A synthesis.

The phosphatases, LpxE (FTN0416)[17,18] and LpxF (FTN0295)[19] remove the terminal phosphate moieties from either the 1- or 4′ position, respectively (www.Francisella.org). Expression cloning in *E. coli* identified LpxE enzymatic activity, however in *F novicida*, this activity is either inactive or rapid translocation of newly synthesized lipid A doesn't allow for the removal of the 1-positon phosphate and galactosamine residue[17] Recently, LpxF enzymatic activity was shown to be involved in the removal of the 4′-phosphate moiety from *F. novicida* lipid A. Interestingly, mutants lacking LpxF activity while retaining the 4′-phosphate moiety also retained the previously deacylated 3-hydroxyacyl fatty acid suggesting that the 4′-phosphate group must be removed before the 3′-hydroxyacyl fatty acid[20]

The dolichyl phosphate-mannose synthase-like enzymes, PmrF1 (FTN1403) and PmrF2 (FTN0545), are required for the transfer of mannose or galactosamine residues to bactoprenol phosphate on the cytoplasmic side of the inner membrane.[1] Individual genes were identified in *F. novicida* that were required for addition of galactosamine (*pmrF2*) or a second hexose predicted to be mannose (*pmrF1*) (R.K. Ernst, unpublished observations). This is in contrast to *Salmonella typhimurium*, which only has a single PmrF enzyme required for the addition of the positively charged sugar, aminoarabinose (~30% identical to the either of the *F. novicida* PmrF orthologs).[21] The addition of galactosamine to *F. novicida* lipid A was through the 1-position phosphate and a mutant in *pmrF2* was shown to be attenuated in mice either by intradermal or aerosol routes of infection (R.K. Ernst, unpublished observations).

Mannose modification of *F. novicida* is predicted to be attached to the 4′-position of the non-reducing glucosamine of the lipid A backbone disaccha-ride and has been hypothesized to function as a replacement for the phosphate residue removed by LpxF. This modification has previously been described for lipid A isolated from the obligate predatory bacterium *Bdellovibrio bacte-riovorus, Rhodomicrobium vannielii*, and various purple sulfur phototrophic bacteria.[22–25] Analysis *of pmrF1* mutants showed this strain to be as virulent as wild type bacteria in mice by either route of infection described above. Finally, modification of lipid A by mannose was also observed in a temperature-dependent manner (i.e., increased mannose addition after growth at environmental temperatures <25°C) (R.K. Ernst, unpublished observation) in a subset of *F. tularensis* Type A clinical isolates but not *F. holartica* or *F. mediasiatica* isolates suggesting a potential role in environmental bacterial adaptation or survival.

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The glycosyltransferase enzyme, PmrK (FTN0546) is required for the addition of both mannose and galactosamine to *F. novicida* lipid A on the outer leaflet of the inner membrane. In contrast to enzymatic activity from *S. ty-phimurium* or *E. coli* PmrK (~24% identical to the *F. novicida* PmrK or-tholog),[26] which transfers a single carbohydrate species (aminoarabinose) to the lipid A molecule, *F. novicida* PmrK interestingly has less substrate specificity. The *F. novicida* PmrK enzyme surprisingly has the ability to attach two different carbohydrate residues at either end of the base lipid A structure. A mutant in *pmrK* was shown to lack both lipid A modifications and had similar structure as lipid A isolated from the LVS vaccine strain. Finally, mutants in *pmrK* were shown to be attenuated in mice either by intradermal or aerosol routes of infection and provided protection against wild type *F. novicida* infection (R.K. Ernst, unpublished observation).

In addition to the essential LpxD1 (FTN1480) acyltransferase enzyme present as part of a four-gene operon (*lpxB/lpxA/fabZ/lpxD*),[1,15] a second early acyltransferase; LpxD2 (FTN0200) was identified in F. *novicida* with homol-ogy to LpxD enzymes present in *H. pylori* and *Campylobacter jejuni*. This acyltransferase activity was shown to play a role in generating lipid A with shorter N-linked fatty acids normally present after growth at 25°C as compared to growth at 37°C (3-OH C16 versus 3-OH C18, respectively). Using negative electrospray ionization with a linear ion trap Fourier transform ion cyclotron resonance hybrid mass spectrometer, the Ernst laboratory has recently identified a high degree of structural heterogeneity in *F. novicida* lipid A that suggests a role for multiple LpxD enzymes in temperature-dependent membrane remodeling. Finally, though normally essential and required for membrane integrity, no growth defect was observed for LpxD2 deficient enzyme mutants after growth in rich medium either at 25°C or 37°C (R.K. Ernst, unpublished observation).

Blue Versus Gray: Lipopolysaccharide Phase/Antigenic Variation

Phenotypic diversity in *F. tularensis* colony morphology has been known for some time. First reported in 1951 by Eigelsbach,[27] spontaneously appearing colony variants of *F. tularensis* Schu S4 were identified on the basis of appearance under oblique light (blue or gray color to the colony), reaction to acriflavine agglutination (positive or negative), and colony morphology (rough or smooth). Many different colony types were identified. The characteristics of these variants were relatively stable when sub-cultured, but could be shown to vary back to the original colony morphotype. Changes in virulence and immunogenicity were associated with colony variation. In general, rough and/or gray colony variants were less virulent and less immunogenic ($LD_{100} > 10^7$), while blue/smooth variants were more virulent and produced a robust immunogenic response. In addition, the gray variants often exhibit a small colony morphology and grow slightly slower than the blue “wild type” strain. The appearance of variants was dependent on the age of the culture, pH, inoculum size, and one or more as-of-yet unknown factors present in spent culture filtrates. This information established a set of culturing methods that could be used to minimize or increase the appearance of *F. tularensis* variants. In general, the longer the time of culture, the greater the frequency of blue to gray transition. Thus, blue/smooth colonies represent the “wild type” *F. tularensis* strain while the gray/rough represents the variant. While this phenomenon in *Francisella* has been termed phase variation (the off/on of a phenotype), this may more accurately be labeled antigenic variation with regard to some of the specific alterations described in these strains below.

With regard to the LVS strain, only two distinct colony variants (blue and gray, FIG. 3) were described by Eigelsbach when this vaccine strain was grown on agar and observed with obliquely transmitted light.[28] These variants were purified, maintained and tested in animals to determine the relative virulence and immunogenicity. As with the Schu S4 strain described above, the LVS blue variant was more virulent and immunogenic than the gray variant. The mouse sub-cutaneous LD_{50} for the blue variant was determined to be 10^5 colony-forming units

(CFU) while the gray was 10^9 CFU. These variants were evaluated in mice and guinea pigs as a vaccine. Approximately ninety percent of mice inoculated subcutaneously with 100–1000 cfu's of the blue variant survived vaccination. Sixty-seven to eighty-three percent of that population survived at least 60 days post challenge with 100–1000 LD_{50}'s of *F. tularensis* Schu S4. One hundred percent of mice immunized with 10^8 gray variants survived vaccination, but none were protected when challenged with Schu S4. Guinea pigs were less susceptible to LVS blue variants, and all animals inoculated with 10^8 organisms survived. One hundred percent of guinea pigs challenged with >10 LD_{50}'s of Schu S4 survived for 15 days, but the percentage dropped to between 17 and 33 at 60 days. A dose of 10^9 of the gray variant did not result in death in the guinea pigs and did not protect any animals against >10 LD_{50}'s of Schu S4 challenge.

Many techniques have been employed since the 1950s to examine such colony variation with none being embraced as foolproof and/or simple to perform. Methods have included viewing the bacteria on chocolate agar plates or on cysteine heart agar (CHA) with or without 5% fresh sheep blood by eye or with a dissecting microscope set up with a specific mirror and oblique light source ala Eigelsbach. Most recently, a fluorescent activated cell sorter (FACS) approach has been described which will separate gray variants without O-antigens but not all gray variants. No matter the approach, it is not simple to see such variants, and takes patience and experience to become skilled at doing so.

Although it is not obvious what the entire repertoire of alterations are in a gray variant, it is clear that LPS plays a role. From the outset, it should be made clear that there is not a single LPS structure associated with the gray variant. Thus, as will be apparent from the results discussed below, gray variants are not uniform in genetic structure or in their phenotypes (TABLE 1).

In 1996, Cowley *et al*. identified LVS variants, LVSG (gray) and LVSR (rough).[31] These variants were identified via colony opacity and morphology, with the LVSG strain being further characterized. Revertants of these pheno-types were identified. The blue to gray transition occurred with a dramatically increased frequency in stationary phase bacteria as well as after passage through animals or *in vitro* through macrophages. The LPS of this mutant was examined with monoclonal antibodies to the *F. novicida* LPS O-antigen, which recognizes only this subspecies. The antibody recognized the LVSG strain, suggesting an alteration of the O-antigen in the gray variant LVSG. The LVSG LPS and lipid A was also shown to be more bioactive, as they induced more nitric oxide (NO) activity from rat peritoneal macrophages. Because the LVSG lipid A itself demonstrated the ability to induce more NO, this implied that this gray variant also had an altered lipid A structure. Studies have also clearly shown that this strain is more sensitive to killing by normal human serum and binds larger amounts of the complement component C3 to its surface compared to wild type *F. novicida* (J.S. Gunn, unpublished observation). Studies are underway to define the LPS structure of the LVSG variant as well as more global approaches (DNA microarray, 2-D mass spectrometry fingerprinting) to ascertain other possible phenotypic alterations in this strain and to identify the putative genetic basis of the variation. Initial analysis of the LVSG structure demonstrates no major structural alterations in the O-antigen and lipid A that would be predicted from the data of Cowley *et al*. (J.S. Gunn, unpublished observation). Subtle alterations in the LPS (e.g., sugar modifications to the lipid A, acetylation of the O-antigen) may be present and are the current focus of attention in these studies.

In 2005, a different gray variant was characterized in the Titball laboratory.[32] This *F. tularensis* LVS variant had an altered LPS, but in this case the defect was clear: the O-antigen was not present. Such gray variants appear identical to those recovered from DynPort Vaccine Company LLC cultures of LVS that have been characterized by the Gunn laboratory, and will

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henceforth be called $LVSG^D$ (DynPort). Thus, it is clear that gray variants can have different LPS structures, demonstrating that the LPS structure is not the determining factor in the blue to gray variation. Like the LVSG strain, the $LVSG^D$ strain grew slower than the LVS and appeared at an increased rate in stationary phase cells. Compared to the wild type LVS "blue variant", these $LVSG^D$ gray variants showed a diminished ability to survive in macrophages and had reduced virulence in the mouse. As has been shown by Eigelsbach with gray variants, immunization of mice with $LVSG^D$ bacteria did not induce protective immunity towards fully virulent *F. tularensis*.

A third type of *F. tularensis* LVS variant has been described by the opacity of the colonies on solid agar (transparent as opposed to the opaque "wild type" strain, J. Cannon, unpublished observation). Such variants have been demonstrated to possess a truncated LPS O-antigen, thus an intermediate LPS phenotype between the LVSG and $LVSG^D$. Henceforth this strain will be called $LVSG^T$. This variant was more sensitive to killing by human sera and survived less well in human macrophage-like THP-1 cells. Surprisingly, these variants demonstrated increased resistance to H_20_2 and polymyxin B, suggesting that they may have a selective advantage in certain microenvironments in the host.

The above description of the gray variants points to the fact that these variations occur likely in all *F. tularensis* subspecies and affect intracellular survival, resistance to innate and possibly adaptive immune components, and the protective capacity of vaccine preparations. Thus, this variation is an important consideration in live, whole cell vaccine preparations. Unfortunately, the phe-notypes of the variants cannot yet be directly attributed to the observed LPS alterations until the genetic/cellular basis of variation is discovered and characterized. If the basis of the variation is genetic, it may be possible to lock *F. tularensis* in the "blue" form and eliminate the concerns over this variation.

LPS-Mediated Inflammatory Responses to *Francisella* LPS

All animals have innate immune mechanisms for recognizing prokary-otic pathogens without the delays inherent in *de novo* antigen-dependent re-sponses.[33,34] These mechanisms are induced in response to bacterial molecules that the host recognizes as foreign, such as those signaling pathogen colonization and invasion of host tissues. The surface of Gram-negative bacteria is composed of numerous Toll-like receptor (TLR) ligands, including flagella, lipoproteins, peptidoglycan, and LPS. Among the best characterized and most important of these molecules is LPS, the major component of the Gram-negative bacterial envelope. Lipid A, or endotoxin, is the sole portion of LPS recognized by the innate immune system.

Recognition of LPS occurs largely through the mammalian LPS receptor, the TLR4-MD2-CD14 complex, which is present on many cell types including macrophages and dendrite cells,[35–40] whereas, epithelial cells do not express CD14; hence respond only to much higher concentrations of LPS (via CD14-independent pathways). Recognition of lipid A also requires an accessory protein, LPS binding protein (LBP), which converts oligomeric micelles of LPS to a monomer for delivery to CD14, a glycosylphosphatidylinositol (GPI) anchored, high affinity membrane protein which can also circulate in a soluble form. CD14 alone does not stimulate innate microbial immune responses, because its GPI anchor does not span the plasma membrane. Instead, TLRs which contain leucine rich repeat (LLR) domains, bind CD14-associated bacterial lig-ands and transduce the microbial recognition event across the membrane. In humans, TLR4 (with accessory protein termed MD-2) is primarily responsible for recognition to Gram-negative bacteria and more specifically for responses to lipid A. How this complex recognizes lipid A and signals across the plasma membrane is still not completely understood. Finally, association of the TLR cytoplasmic tail with MyD88 and IRAK triggers a phosphorylation cascade (via TRAF6, NIK and IKK) that results in the phosphorylation (and

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subsequent degradation) of IkB and translocation of NFkB to the nucleus. There, NFkB initiates transcription of various gene products important in inflammation including; synthesis and secretion of cytokines and chemokines, induction of adhesion molecules, migration of host cells, phagocytosis and killing of microbes within membrane-bound vacuoles and synthesis and secretion of cationic antimicrobial peptides.

LPS structure and stimulatory potential varies between bacterial species. Lipid A from the enteric commensal and occasionally pathogenic *E. coli* is highly immune stimulatory, even at low doses. However, in several *in vivo* and *in vitro* assays, LPS isolated from *F. tularensis* subspecies remarkably displayed little to no endotoxic properties: specifically it was neither a lymphocyte stimulus nor a lymphocyte mitogen. In addition, *F. tularensis* subspecies LPS also displayed little to no endotoxic properties in either galactosamine-treated mice, by limulus assay (a standard for determining LPS endotoxin potential), or after aerosolization in mice, and do not stimulate mononuclear cells to release cytokines of nitric oxide.[6,41–44]

Several properties of *F. tularensis* subspecies lipid A are uncommon among Gram-negative bacteria.[35] First, the absence of a phosphate at the 4′ position on the non-reducing glucosamine backbone dimer likely contributes to the lack of stimulatory activity, as demonstrated for monophosphoryl lipid A (MPL).[45–47] MPL is a chemically dephosphorylated *Salmonella* lipid A molecule that is of low endotoxic activity, and has recently been used as a vaccine adjuvant.[46,48] Second, Francisellae lipid A is hypo-acylated (tetra-acylated) and contains longer acyl side chains of 16–18 carbons in length,[2,3,19]as compared highly inflammatory lipid A from enteric bacteria that is normally hexa-acylated containing acyl side chains of 12 to 14 carbons in length. Third, *F. tularensis* LPS or lipid A does not act as an antagonist for either human or mouse TLR4-mediated innate immune responses and was recently shown by Barker *et al*. that *F. holartica* LVS LPS did not bind LBP or the closely related polymorpho-nuclear granule protein, bactericidal/permeability-increasing protein (BPI). Taken together, these unique *F. novicida* lipid A structural modifications result in the inability to bind to TLR4 or other components of the LPS receptor complex and result in the failure to initiate a downstream innate immune response.

Role Of O-Antigen and Lipid A Modifications in Virulence

Alterations in the structure of LPS has been shown to affect virulence/virulence phenotypes of various pathogens. For example, loss of the O-antigen generally results in increased susceptibility to killing by membrane active compounds such as serum and antimicrobial peptides, reduced intracel-lular survival and decreased virulence,[1] The above-mentioned work relating to the loss of virulence of gray variants suggests that O-antigen plays a role in *Francisella* pathogenesis. Recent studies have specifically addressed this topic.

Directed mutations within the O-antigen gene cluster have been performed in *F. tularensis* Schu 4, *F. novicida* and *F. tularensis* LVS. These mutations (most in the first gene [*wbtA*] of the O-antigen biosynthetic cluster) all resulted in the loss of O-antigen on the LPS and dramatically reduced serum resistance and mouse virulence.[14,49] While the *F. tularensis* Schu S4 and LVS O-antigen mutations also reduced survival in murine macrophage cell lines, no effect on intracellular survival was observed for *F. novicida*. Studies with human cells have yet to be reported and could possibly yield different results. This work has also shed some light on the protective capacity of LPS in vaccine approaches, but this work will be reviewed in Chapter 12 of this book.

Conclusions And Outlook

The intense study of *Francisella* LPS, particularly the genetic aspects of LPS biosynthesis and modification, has occurred only recently. The very recent availability of genome sequences of

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the various *Francisella* subspecies has greatly aided this analysis. Such analysis has lead to the identification of *Francisella* orthologs of lipid A, core and O-antigen biosynthetic genes and the comparisons of these genes between the subspecies. Also aiding this work has been the seminal work over the years that has defined the structure of the LPS in various strains and subspecies. Thus, the unique marriage of structural biology and biochemistry with genomics has provided a windfall of information regarding the *Francisella* LPS.

It is clear from the studies described above that LPS is a key virulence factor of *Francisella* spp., as several O-antigen and lipid A modification mutants are attenuated in the mouse model. In addition, lipid A biosynthetic enzymes are essential in *Francisella* as is found in most Gram-negative bacteria. Colony variants that have reduced virulence and diminished protective capacity as vaccines also possess altered LPS structure, which further suggests a role for LPS in pathogenesis. Thus, this knowledge suggests several targets for therapeutic intervention of *Francisella* pathogenesis. This approach has been used with success, at least in the laboratory, in other Gram-negative pathogens.[50] Such therapeutics might provide an advantage over vaccine strategies as they might be used for a more targeted population and may provide treatment post-exposure.

With regard to vaccine approaches to prevent tularemia, the vaccine that has been used most extensively in humans worldwide and that is in the current FDA pipeline with clinical trials in the US in the LVS.[51,52] As described, variants (commonly gray) of this strain clearly appear that are not protective against virulent Type A or Type B challenge. In addition, it is now clear that a single variant type does not exist, and that there are multiple types of “gray variants.” The growth conditions of the culture can limit the development of gray variants, such as avoiding lengthy batch cultures in which the bacteria are in stationary phase for extended times. Historically, gray variants have been a problem in *Francisella* vaccine lots, as evidenced by the Salk Institute’s vaccine runs in the 1960’s under IND 157 in which a large proportion of the organisms in the vaccine prep gave rise to gray colonies. If there is a genetic basis to the observed colony variation, which is likely for some (e.g., $LVSG^{D}$) but not all colony variants, it may be possible to lock strains in a blue colony type. However, until we gain a more complete understanding of *Francisella* colony variation- at a molecular level understanding both how and why this variation occurs, such phase locked variants are unattainable.

Continued study of *Francisella* LPS will undoubtedly bring about considerable new information and additional therapeutic targets to the field. As technology and genetic analysis of *Francisella* spp. continues to advance, further details will emerge revealing a more comprehensive model of the biosynthesis and variation of *Francisella* LPS and its contribution to human disease.

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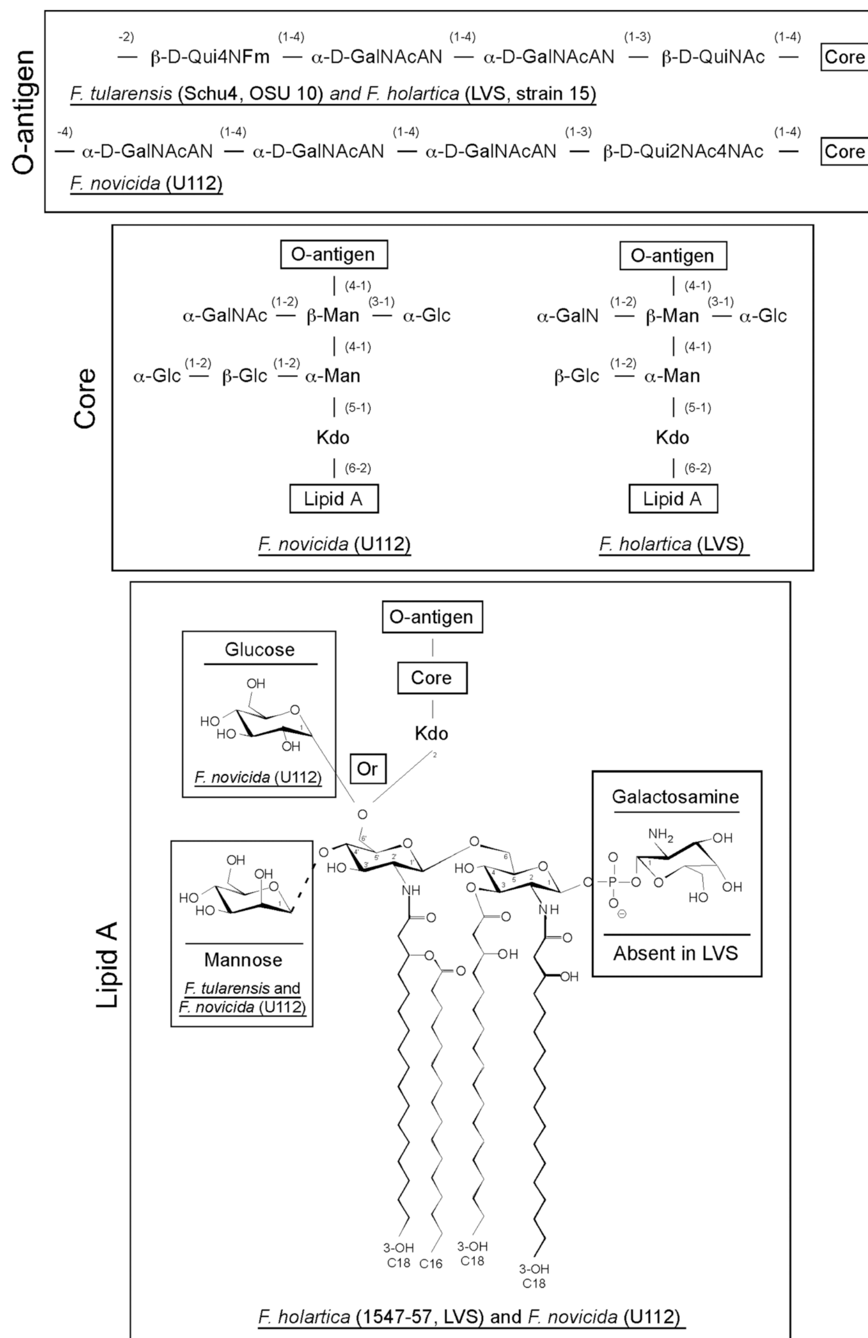

FIGURE 1.

Structure of lipid A, core, and O-antigen molecules synthesized by *Fran-cisella tularensis* subspecies *tularensis, holartica, and novicida*. The lipid A structure consists of a β-(1,6)-linked glucosamine disaccharide with amide-linked fatty acids at the 2- and 2′-positions, and ester-linked fatty acids at the 3-position. Lipid A carbohydrate modifications include the addition of galactosamine through the 1-position phosphate, man-nose at the 4′-position and glucose at the 6′position. Lipid A molecules that have glucose in their structure would not be modified by the addition of Kdo-core-O-antigen. Unless noted on the structure, modifications are present in all *Francisella* subspecies. Linkages of individual carbohydrate residues are shown. Core: Kdo = 3-deoxy-D-manno-octulosonic acid; Man = mannose; Glc = Glucose; GalNAc = N-

acetyl galactosamine. O-antigen = QuiN4Fm = 4,6-dideoxy-4-formamido-D-glucose; GalNAcAN = 2-acetamino-2-deoxy-D-galacturonamide; QuiNAc = 2-acetamino-2,6,dideoxy-D-glucose; Qui2NAc4NAc = 2,4,-diacetamino-2,4,6-trideoxy-D-glucose.

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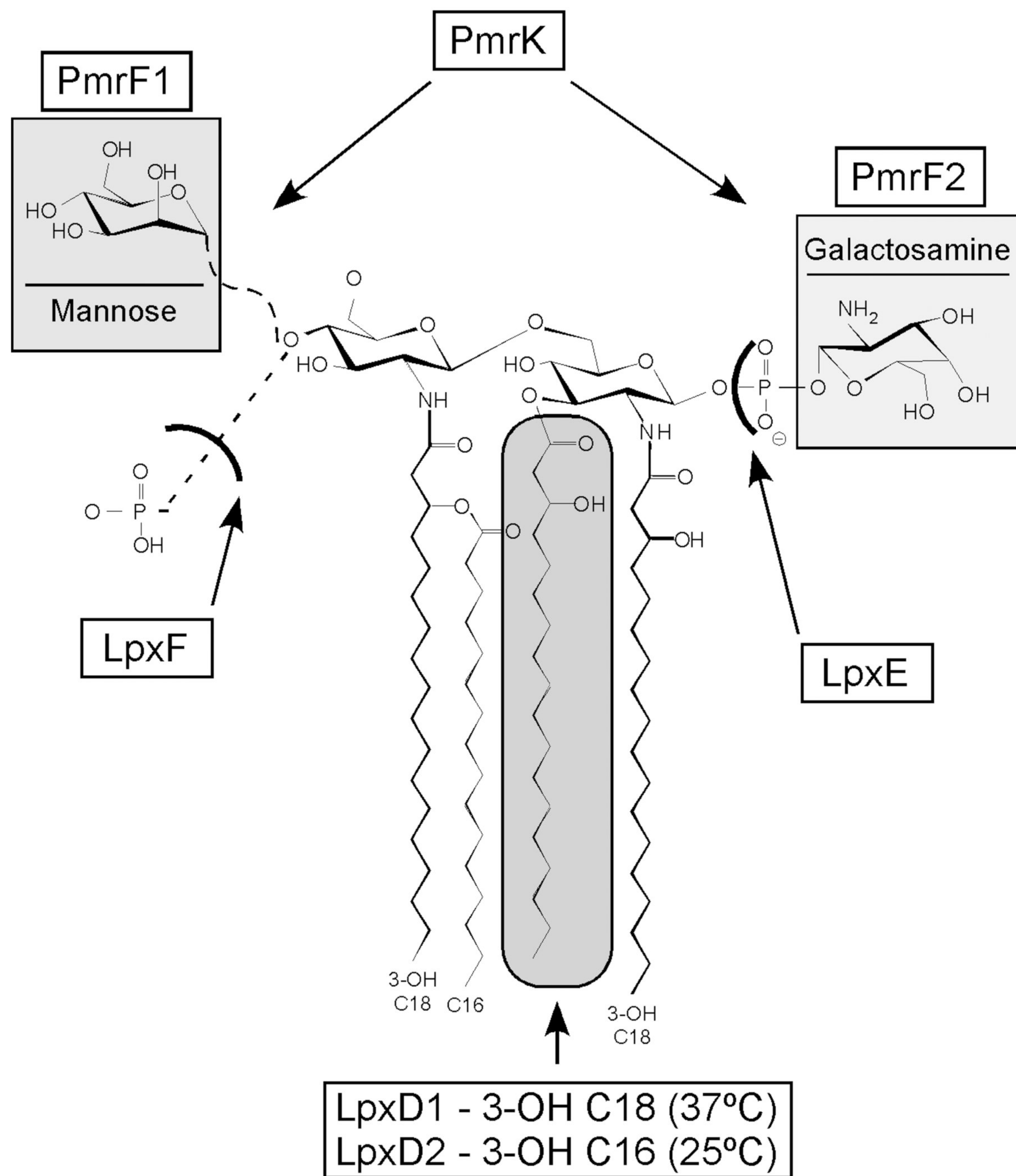

FIGURE 2.

Enzymes required for the synthesis of *F. novicida* lipid A. Genes identified i n *F. novicida* that encode enzymes required for the synthesis of the lipid A. These enzymes include two phosphatases (LpxE and LpxF), two dolichyl phosphate-mannose synthase-like enzymes (PmrF1 and PmrF2), one glycosyltransferase (PmrK), and a second hydroxyacyl acyltransferases (LpxD2).

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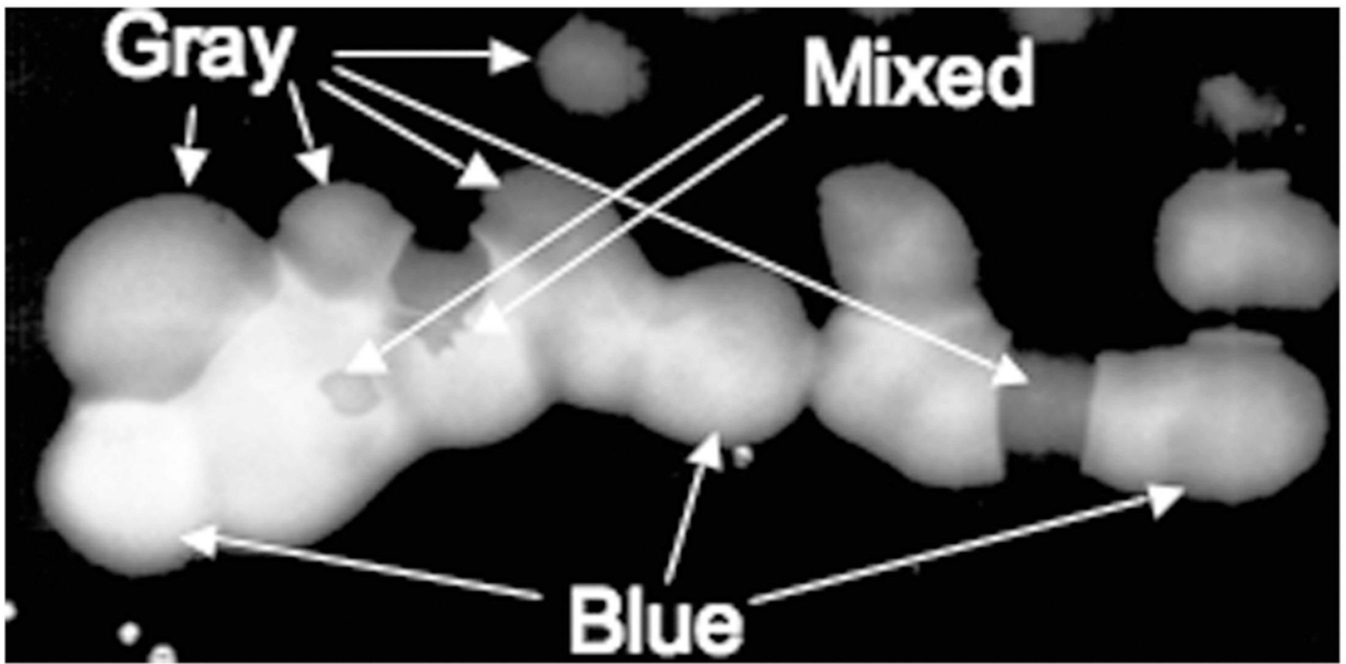

FIGURE 3.
Blue and gray colony variants of *F. tularensis* LVS. Black and white picture taken under an oblique light source illumination of bacterial colonies on the agar surface. Bacteria were grown for ~48 hrs in T-soy broth-cysteine (TSB-C) batch culture followed by plating onto the surface of TSB-C agar. The microscope and light source used were as described by Eigelsbach.[26] Blue, gray and mixed colonies are noted. Courtesy of Brian Bell.

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TABLE 1

Phenotypic comparisons between *F. tularensis* variants

Strain	Colony Morphology	LD_{50}[a]	Intramacrophage Survival[b]	Serum Sensitivity	Protective vs. Schu S4 Challenge[c]	O-Antigen	Reference
LVS	Large, blue, smooth	<10	Survives well	Resistant	Protective	Complete	29,30
LVSG	Small, gray, smooth	$>10^3$	Defective	Sensitive	Not protective	Complete	31, J. Gunn, Unpublished Observation
$LVSG^D$	Small, gray, smooth	$>10^5$	Defective	Sensitive	Not protective	Absent	32
$LVSG^T$	Large, transparent, gray?	ND	Defective	Sensitive	ND	Present but Reduced Length	J. Cannon, Personal Communication
Schu S4	Large, blue, smooth	<10	Survives well	Resistant	NA	Complete	27,28
Schu $S4^{NS}$	Small, non-smooth, gray	10^7	ND	ND	Not protective	ND	27

[a] Routes of delivery of the reported LD_{50}s are as follows: LVS (i.p.), LVSG (i.n.), $LVSG^D$ (s.q.), Schu S4 (i.p.), Schu $S4^{NS}$ (i.p.).

[b] Macrophages (primary or cell lines) used are as follows: LVS (rat, mouse, and human primary or cell lines), LVSG (rat, mouse [reduced proliferation is to a lesser extent than in other macrophage types], and human primary or cell lines), $LVSG^D$ (J774.1), $LVSG^T$ (THP-1).

[c] The route of administration is as described under the LD50 column, and the Schu S4 was via the same route as the variant strain. The dosages of Schu S4 were variable but the results reported in this table are accurate for 10–100 LD50S as the dose.