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The atypical lipopolysaccharide of *Francisella*

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Abstract

Bacterial lipopolysaccharides (LPSs) are ubiquitous molecules that are prominent components of the outer membranes of most gram-negative bacteria. Genetic and structural characterizations of *Francisella* LPS have revealed substantial differences when compared to more commonly studied LPSs of the Enterobacteriaceae. This review discusses both the general characteristics and the unusual features of *Francisella* LPS.

Keywords

Francisella tularensis; Lipopolysachharide; Lipid A; O-antigen; Kdo hydrolase; Structure; Virulence

1. Introduction

Francisella tularensis is the etiologic agent of tularemia, a potentially fatal human disease. This small, gram-negative, non-motile, facultative intracellular bacterial pathogen has an impressive host range that includes more than two hundred species—vertebrates, invertebrates, and even free-living amoebas.^{1, 2} Fast adaptation and versatility are key features that enable *F. tularensis* to survive in diverse environments, including water, insect vectors, and mammalian hosts. The organism's high degree of infectivity and ease of aerosolization have raised concerns about its potential use as an agent of biological warfare; thus, the Centers for Disease Control and Prevention lists *F. tularensis* as a category A priority pathogen. *F. tularensis* subspecies *tularensis* (type A), *holarctica* (type B), and *mediasiatica* have distinct geographical distributions and levels of virulence. *F. novicida* and *F. philomiragia* are close relatives of *F. tularensis*, sharing many of its phenotypical features. *F. novicida* is considered to be a mouse-adapted member of the genus and *F. philomiragia* is a muskrat pathogen; both of these species are rarely infectious in humans.^{2, 3}

Bacterial lipopolysaccharides (LPSs) are ubiquitous molecules that are prominent components of the outer membranes of most gram-negative bacteria.^{4, 5} Like many LPSs, *F. tularensis* LPS is composed of 1) lipid A, which anchors the LPS to the outer membrane; 2) a core oligosaccharide attached to lipid A by an eight-carbon sugar, 3-deoxy-D-manno-octulosonic acid (Kdo); and 3) an O-polysaccharide (also known as O-antigen), which contains a varying number of tetrasaccharide repeating units. However, several key modifications distinguish *Francisella* LPS from more commonly studied LPSs of the

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Enterobacteriaceae. These alterations lead to very low-level toxicity that *F. tularensis* LPS exhibits. This review discusses both the general characteristics and the unusual features of *Francisella* LPS.

2.1. Structure and biosynthesis of lipid A

In gram-negative bacteria, LPS is embedded in the outer membrane by a hydrophobic lipid A anchor. The composition of lipid A is well characterized in a variety of organisms, including *Escherichia coli* and *Salmonella* species, and substantial differences in *Francisella* lipid A have been noted. Structural analysis of *Francisella* lipid A revealed a β -(1-6)-linked diglucosamine (GlcN) backbone with four long fatty acyl groups that are amide-linked with C18:0 (3-OH) at the 2- and 2'-positions and ester-linked with C18:0 (3-OH) at the 3-position (Figure 1A).⁶⁻⁹ The hydroxy group of the 2'-linked fatty acyl chain is further esterified with C16:0. Unlike *E. coli* lipid A, which has two phosphates at the 1- and 4'-positions (Figure 1E), *Francisella* lipid A has an α -linked galactosamine (GalN) addition at the 1-position and lacks phosphate at the 4'-position (Figure 1A). This major tetraacyl lipid A, typically observed at m/z 1665 by negative ion MALDI mass spectrometry (MS), is present in all *F. tularensis* subspecies, *F. novicida* and *F. philomiragia* when bacteria were grown at 37°C.^{10, 11} The absence of negatively charged phosphate groups affects the overall charge of LPS and has implications in the organism's resistance to antimicrobial peptides. Differences between the presence of phosphate groups in strains of *F. tularensis* and *F. novicida* U112 strain lipid A have been reported. An early report⁸ indicated the lack of a phosphate group at the 1-position of the reducing sugar in the live vaccine strain (LVS) of *F. tularensis* subsp. *holarctica*, but later reports revealed that the majority of lipid A did contain the 1-phosphate group.^{7, 11, 12} A small portion of LVS lipid A was reported to contain galactosamine-1-phosphate,^{12, 13} while in strain 1547-57 of *F. tularensis* subsp. *holarctica* and *F. novicida* U112, it is the major lipid A species.^{7, 11} Reported differences in lipid A structure may arise from differences in the bacterial growth phase when cells are harvested or procedures used for isolation, purification, and analysis of lipid A. Critical proteins involved in modification of the GlcN backbone of *F. novicida* lipid A have been identified: the inner membrane bound phosphatases LpxE and LpxF are responsible for the removal of phosphate groups from the 1- and 4'-positions, respectively;¹⁴⁻¹⁶ FlmK (a homolog of ArnT/PmrK), FlmF2 (ArnC homolog) and NaxD deacetylase are involved in addition of GalN to the phosphate moiety at the 1-position (Figure 2).^{6, 17-19} Modification of lipid A with a mannose at the 4'-position was also reported (Figure 1B).^{17, 20} This modification was detected in *F. novicida* and most isolates of *F. tularensis* subsp. *tularensis*, but not in *F. tularensis* subsp. *holarctica*.¹⁷ FlmF1 and FlmK proteins play roles in mannose addition.^{17, 18}

Another striking difference between *Francisella* lipid A and the lipid A of most other gram-negative bacteria is the length and number of acyl chains in the molecule. *Francisella* lipid A has only four long acyl chains with 16 and 18 carbons, whereas most bacterial lipid A has six acyl chains that are usually shorter (12 to 14 carbons) (Figure 1A & E). Temperature-regulated heterogeneity in *F. novicida* lipid A acyl chains has been reported.^{20, 21} LpxD1 and LpxD2 were identified as N-acyltransferases whose expression and activity increase at 37°C and 18°C, respectively. Thus, when *F. novicida* is grown at 18°C (environmental temperature), lipid A acyl chains at the 2- and 2'-positions are composed primarily of C16:0 (3-OH) (Figure 1B). Conversely, when it is grown at 37°C (mammalian host temperature), lipid A has longer acyl chains C18:0 (3-OH). Minor lipid A species with acyl chain variations include C14:0 or C18:0 fatty acyl branches instead of a C16:0 secondary chain at the 2'-position.^{11, 12, 20} LpxL was identified as a Kdo-dependent acyltransferase responsible for modifying the secondary chain on the 2'-acyl group (Figure 2);²² this function was confirmed by demonstration of the ability of *Francisella* LpxL to complement the acylation of lipid A in an *E. coli* *lpxL* mutant.

In *E. coli*, lipid A with two Kdo sugars (Kdo₂-lipid A) is the minimal LPS molecule required for viability.⁴ While in *Francisella*, >95% of its lipid A is present in “free” form; it is not attached to Kdo, core oligosaccharide, or O-antigen.⁶ The sugar and fatty acid composition of this free lipid A is very similar to lipid A with O-antigen. Both *F. tularensis* and *F. novicida* are reported to have free lipid A.^{6, 8, 12, 23} In *F. novicida*, a small amount of free lipid A contains an α -linked glucose at the 6'-position of distal glucosamine (Figure 1B).⁶ The biosynthesis of these novel lipid As is discussed in detail by Raetz and colleagues.¹⁶

2.2. Structure of core oligosaccharide

Perry and colleagues have made important contributions to the structural elucidation of *Francisella* LPS.^{8, 24, 25} Structural analysis of the *Francisella* core region has revealed an unusual sugar composition. The core is relatively small, and contains two mannose sugars in the inner core instead of the more commonly found heptoses (Figure 1A). *F. novicida* core oligosaccharide differs from *F. tularensis* by the presence of α -glucose attached to the α -glucose branch.^{8, 25} LPS core of LVS was reported to have galactosamine (GalN) instead of N-acetyl galactosamine (GalNAc) as in *F. novicida*; however, a later report suggested GalNAc is present in the core of LVS.²⁶ In gram-negative bacteria, lipid A is linked to the core region through a Kdo moiety (Figure 1A&E). *E. coli* and most other bacteria possess a highly conserved bifunctional glycosyltransferase WaaA (KdtA), which transfers two Kdo sugars onto lipid IV_A, a lipid A precursor. The addition of Kdo sugars to the lipid A precursor is followed by the addition of the rest of the core unit.⁴ *Francisella* LPS has a single non-branched Kdo in its core region (Figure 1A). LPS carrying a single Kdo has also been observed in *Bordetella pertussis* and *Haemophilus influenzae*, but in these bacteria WaaA was shown to be a mono-functional Kdo transferase that could transfer only a single Kdo onto lipid IV_A.^{27, 28} Our laboratory and others have recently discovered a Kdo hydrolase system in *Francisella* composed of *kdhA* and *kdhB* gene products.^{29, 30} It is now known that *Francisella* initially synthesizes its LPS with two Kdo sugars and this novel Kdo hydrolase is involved in removing the second, side-chain Kdo moiety. KdhA and KdhB are inner-membrane proteins and are both required for Kdo hydrolase activity in *Francisella*. KdhA catalyzes the cleavage of the side-chain Kdo and has homology to sialidase-family enzymes (Figure 2). KdhB's function is unknown, but it is predicted to contain a number of alpha-helical transmembrane domains. A similar two-component Kdo hydrolase system has been identified in two other pathogens, *Helicobacter pylori* and *Legionella pneumophila*.^{29, 31}

2.3. Structure of O-antigen

NMR and MS analysis by several laboratories identified *Francisella* O-antigen with its repeating four sugars. LVS, strain 15, OSU10, and SchuS4 strain (members of either subsp. *tularensis* or subsp. *holarctica*) have the O-antigen repeating units α -D-Qui4NFm-(1-4)- α -D-GalNAcAN-(1-4)- α -D-GalNAcAN-(1-3)- α -D-QuiNAc-(1- (Figure 1C)^{26, 32-34} while *F. novicida* strain U112 has an antigenically distinct repeating O-antigen unit of α -D-GalNAcAN-(1-4)- α -D-GalNAcAN-(1-4)- α -D-GalNAcAN-(1-3)- α -D-Qui2NAc4NAc-(1- (Figure 1D).^{24, 35} Reflecting the structural variation, the O-antigen gene clusters of *F. tularensis* and *F. novicida* differ in a number of genes involved in the biosynthesis of individual O-antigen sugars.³³ Present in the gene cluster, *wzx* (FTL_0596) encodes for O-antigen flippase and *wzy* (FTL_0598) encodes for O-antigen polymerase. The Wzx flippase transports a single O-antigen unit linked to the C55-lipid carrier undecaprenyl phosphate (UDP) from the cytoplasmic to the periplasmic face of the inner membrane, whereas the Wzy glycosyltransferase is responsible for the polymerization of O-antigen of various lengths (Figure 2).^{4, 36, 37} Similar to O-antigen polymerase in other bacteria, Wzy of *F. tularensis* LVS is an inner-membrane protein with 11 transmembrane domains.³⁸ The *wzy*

mutant of *F. tularensis* LVS can synthesize lipid A with a single O-antigen. The *wzz* gene, whose product determines O-antigen chain length, appears to be missing in *F. tularensis* and *F. novicida*. In most gram-negative bacteria, a glycosyltransferase, RfaL/WaaL, serves as the O-antigen ligase, transferring O-antigen from its UDP lipid carrier to the lipid A core. A BLAST search for *rfaL* in the *F. tularensis* LVS genome identifies FTL_0706, which is not in the O-antigen gene cluster and displays low homology (41%/25% amino acid sequence similarity/coverage to *E. coli* RfaL). Consistent with the predicted function of FTL_0706, mutants deficient in this gene product lack LPS with O-antigen (Figure 2).^{39, 40} Further studies are needed to identify the enzymatic characteristics of these glycosyltransferases.

The transport of fully mature LPS across the outer membrane of gram-negative bacteria is a process that is not well understood. Several Lpt proteins facilitate the transport in *E. coli*.⁴¹ A BLAST search for similar proteins in the Lpt pathway identifies an LptB homolog with high amino acid similarity and LptD, LptF and LptG homologs with very low similarity in *F. tularensis*. The presence of these proteins suggests a similar pathway may be involved in transporting LPS across the *Francisella* outer membrane.

Apicella and colleagues identified a large-molecular-weight polysaccharide (100–250 kDa) in capsule extracts of type A and type B strains of *F. tularensis*.³⁹ The sugar composition was identical to O-antigen. Analysis determined the capsule to be a polymer of -4)- -D-GalNacAN-(1-4)- -D-GalNacAN-(1-3)- -D-QuiNac-(1-2)- -D-Qui4NFm-(1-. This study did not conclusively reveal the sugar at the reducing end of the molecule, while a tandem MS analysis by Wang et al. suggested the presence of QuiNac at the reducing end when tetrasaccharide repeats are not attached to LPS core.²⁶ These findings indicate that *F. tularensis* synthesizes an O-antigen capsule containing approximately 125 to 300 or more O-antigen repeating units. The presence of O-antigen capsules that are not linked to core oligosaccharide and lipid A is also reported in other bacteria.⁴² Using capsule-specific monoclonal antibodies that do not recognize *F. tularensis* LPS, Apicella's group showed that many of the genes in the O-antigen gene cluster are also required for the synthesis of the O-antigen capsule. Among these genes, *lpxL*, involved in lipid A modification, was required for the synthesis of LPS but not O-antigen capsule.³⁹ Furthermore, the *wzy* mutant lacked both LPS and O-antigen capsule, indicating a common O-antigen polymerase.³⁸

Francisella LPS is an immunodominant molecule with little diversity among *F. tularensis* subspecies. Early on, phase/antigenic variation based on colony morphology, immunogenicity, and virulence was noted.^{43, 44} Analysis of a number of phase variants, which are called "gray" on the basis of colony opacity and morphology, identified alterations in LPS O-antigen (absent or reduced) as well as subtle changes in the core region and lipid A structure.^{13, 44, 45}

3. Biological role of *Francisella* LPS

LPS molecules of gram-negative bacteria play critical roles in the maintenance of outer-membrane integrity, regulation of cell permeability, regulation of the host's immune responses, resistance to antimicrobial peptides, resistance to host antibodies, and prevention of complement activation.⁴ Mutants lacking LPS often exhibit phenotypes that include reduced viability, slow growth, and loss of virulence. *Francisella* LPS is an important virulence factor that contributes to the organism's pathogenesis in numerous ways. Lipid A of many gram-negative bacteria are bioactive molecules that interact with the host immune system.⁴⁶ Unlike others, *Francisella* lipid A is a very poor stimulant of the host's innate immunity.^{10, 47, 48} Recognition of lipid A as foreign is an essential feature of mammalian host defense. Many host cells, such as macrophages and epithelial cells, recognize lipid A by innate immune mechanisms. For example, molecular pattern recognition via the Toll-like

receptor 4 (TLR4)/MD2 receptor complex leads to inflammation by stimulating NF- κ B-mediated gene transcription.^{4, 46} The lack of immune recognition of *Francisella* lipid A has been attributed to several structural differences compared to *E. coli* lipid A, a potent immune activator. These include 1) absence of phosphate at the 4' position as well as the modification of 1-phosphate with GalN and 2) tetraacylation of lipid A with longer acyl chains (16–18 carbons); *E. coli*'s lipid A is hexaacylated with shorter acyl chains (12–14 carbons) (Figure 1 A & E). The lack of phosphate affects the overall charge of lipid A, a frequent target of cationic antimicrobial peptides. Supporting this notion that charge is important to the biology of lipid A, *lpxD1*, *flmK*, and *naxD* mutants of *F. novicida*, which are unable to modify 1- or 4'-phosphate, show increased sensitivity to polymyxin B and attenuation in mice.^{17, 19, 21} Dynamic regulation of lipid A acyl chain length by LpxD1/LpxD2 at varying temperatures appears to be important in maintaining membrane permeability and susceptibility to cationic antimicrobial peptides as well.²¹ Consistent with LpxD1's optimum activity at 37 °C, the *lpxD1* mutant is unable to modify lipid A with longer acyl groups and is severely attenuated in mice, whereas the *lpxD2* mutation has no effect.²¹

An unusual feature of the *Francisella* LPS core region is the presence of a single Kdo unit. This LPS modification was shown to be important for virulence. An *F. tularensis kdhAB* mutant unable to cleave the second Kdo from the remainder of the core oligosaccharide was attenuated in mouse models of infection.⁴⁹ Interestingly, the *kdhAB* mutant activated macrophages through the TLR2 signaling pathway as opposed to the TLR4 pathway that recognizes most gram-negative bacterial LPS. Further investigation suggested that *kdhAB* mutant LPS is not the activator of TLR2 signaling; rather, some other surface components, most likely lipoproteins, are involved in the activation of macrophages. A probable scenario is the presence of an extra Kdo imparts a net negative charge altering the bacterial surface under physiological conditions, resulting in increased access of additional surface molecules to the host's innate immune system.

As in many other bacterial pathogens, O-antigen of *Francisella* plays a role in serum resistance. *F. tularensis wbtA* and *wzy* mutants, which lack O-polysaccharide, are highly sensitive to killing by serum complement.^{49–51} These mutants are also defective in intracellular bacterial growth and are highly attenuated in mice. Interestingly, *F. novicida* core mutants *manB* and *manC*, *F. novicida wbtA*, *F. tularensis FTT1238*, and *F. tularensis wzy* mutants, all lacking O-polysaccharide, were hypercytotoxic in the J774 macrophage-like cell line as well as in primary macrophages.^{40, 52, 53} Cells infected with these mutants secreted high levels of pro-inflammatory cytokines and underwent pyroptosis, a type of programmed cell death. These observations establish a strong connection between *Francisella* LPS and evasion of the host's immune system.

Bacterial O-antigens are known to invoke a strong antibody response due to their repetitive structure and constitutive expression. LPS specific antibodies are often protective and thus useful in vaccine design. A number of *Francisella* O-antigen specific monoclonal antibodies have been shown to be protective in a mouse model of *F. tularensis* infection.⁵⁴ X-ray crystal structure of monoclonal antibody Ab52 in a complex with O-antigen further revealed the basis of O-antigen recognition.⁵⁵ While *Francisella* O-antigen is a dominant antibody target, Boltje et al. showed that mice immunized with *Francisella* LPS produced antibodies that recognized a chemically synthesized core hexasaccharide, but not mice immunized with LVS.⁵⁶ Future studies using these tools will help to better define how the host interacts with *Francisella* LPS and in the design of effective therapeutic antibodies.

4. Concluding remarks

F. tularensis is a highly infectious pathogen and many studies point to its LPS as a key virulence factor. *Francisella* LPS has many unusual characteristics and genetic, biochemical, and structural studies have led the way in the exploration of this unique molecule. The presence of free lipid A is an unexpected finding and its biological role is yet to be investigated. Finally, the mechanism underlying LPS transport to the bacterial surface is not well understood. *Francisella* appears to have several genes in the *lpt* transport pathway, but the relevance of this LPS transport system has not been studied. Further investigations are needed to confirm the findings of these studies and to identify all the genes involved in the biosynthesis, modification and transport of *Francisella* LPS.

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Abbreviations

LPS	Lipopolysachharide
Kdo	3-deoxy-D- <i>manno</i> -octulosonic acid
Qui4NFm	4,6-dideoxy-4-formamido-D-glucose
GalNAcAN	2-acetamido-2-deoxy-D-galacturonamide
QuiNAc	2-acetamido-2,6-dideoxy-D-glucose
Qui2NAc4NAc	2,4-diacetamido-2,4,6-trideoxy-D-glucose

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Highlights

- *Francisella* has an unusual bacterial LPS in many ways.
- *Francisella* lipid A has only four acyl chains, longer than *E. coli* lipid A.
- Majority of *Francisella* lipid A are “free”, which are not linked to core+O-antigen.
- *Francisella* LPS core has only one Kdo, the second Kdo is cleaved off by Kdo hydrolase.
- *Francisella* LPS is an important virulence factor.

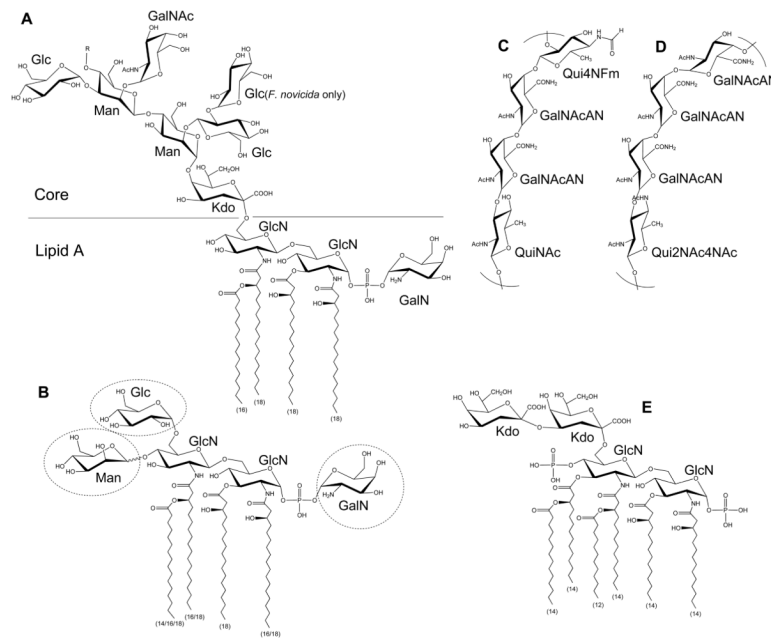


Figure 1. Structure of *Francisella* LPS

Major *Francisella* lipid A molecule with core oligosaccharide (A). The length of each fatty acyl chain is indicated in parenthesis. Chain length variation (16/18) indicates temperature regulation. Less abundant lipid A variants may have various modifications in sugar composition as shown in dashed circles (B). The structures of *F. tularensis* O-antigen (C), and *F. novicida* O-antigen (D) are shown. For comparison, *E. coli* Kdo₂-lipid A is also shown (E). R = O-antigen.

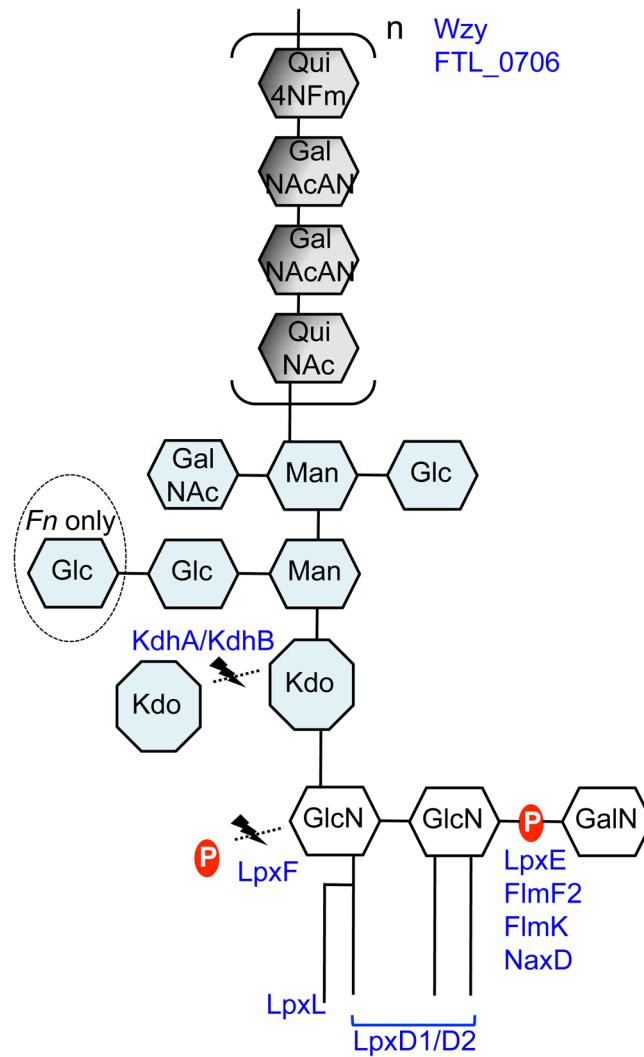


Figure 2. Cartoon representation of *Francisella* LPS and its modifying proteins
 Proteins are located in their areas of action. See text for further details. *Fn*: *F. novicida*.