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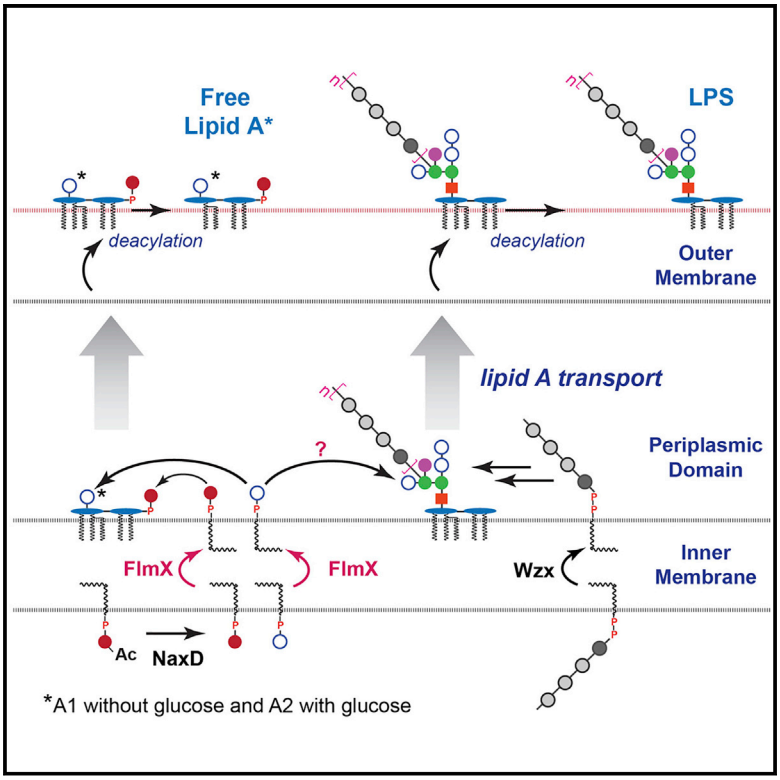
Francisella FlmX broadly affects lipopolysaccharide modification and virulence

Graphical abstract



Authors

Chui-Yoke Chin, Jinshi Zhao, Anna C. Llewellyn, Igor Golovliov, Anders Sjöstedt, Pei Zhou, David S. Weiss

Correspondence

david.weiss@emory.edu

In brief

Lipopolysaccharide (LPS) is a major constituent of the Gram-negative bacterial outer membrane, and specific pathways are involved in its production. Chin et al. show that one protein, FlmX, impacts the modification of each component of LPS, an activity that is critical for the *in vivo* virulence of *Francisella*.

Highlights

- FlmX is a flippase for free lipid A modification in *Francisella*
- FlmX impacts core and O-antigen structure of LPS
- FlmX is essential for pathogenesis of highly virulent *F. tularensis*

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Francisella FlmX broadly affects lipopolysaccharide modification and virulence

Chui-Yoke Chin,[1,2,3,4,8] Jinshi Zhao,[5,8] Anna C. Llewellyn,[2,3,4,9] Igor Golovliov,[6] Anders Sjöstedt,[6] Pei Zhou,[5] and David S. Weiss[1,2,3,4,7,10,*]

[1]Emory Antibiotic Resistance Center, Emory University School of Medicine, Atlanta, GA 30329, USA
[2]Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30329, USA
[3]Yerkes National Primate Research Center, Emory University School of Medicine, Atlanta, GA 30329, USA
[4]Division of Infectious Diseases, Department of Medicine, Emory University School of Medicine, Atlanta, GA 30329, USA
[5]Department of Biochemistry, Duke University School of Medicine, Durham, NC 27710, USA
[6]Clinical Bacteriology, and Laboratory for Molecular Infection Medicine Sweden, Department of Clinical Microbiology, Umeå University, 90185 Umeå, Sweden
[7]Research Service, Atlanta VA Medical Center, Decatur, GA 30033, USA
[8]These authors contributed equally
[9]Present address: Polio Eradication Branch, Global Immunizations Division, Centers for Disease Control and Prevention, Atlanta, GA 30033, USA
[10]Lead contact
*Correspondence: david.weiss@emory.edu
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SUMMARY

The outer membrane protects Gram-negative bacteria from the host environment. Lipopolysaccharide (LPS), a major outer membrane constituent, has distinct components (lipid A, core, O-antigen) generated by specialized pathways. In this study, we describe the surprising convergence of these pathways through FlmX, an uncharacterized protein in the intracellular pathogen *Francisella*. FlmX is in the flippase family, which includes proteins that traffic lipid-linked envelope components across membranes. *flmX* deficiency causes defects in lipid A modification, core remodeling, and O-antigen addition. We find that an *F. tularensis* mutant lacking *flmX* is >1,000,000-fold attenuated. Furthermore, FlmX is required to resist the innate antimicrobial LL-37 and the antibiotic polymyxin. Given FlmX's central role in LPS modification and its conservation in intracellular pathogens *Brucella*, *Coxiella*, and *Legionella*, FlmX may represent a novel drug target whose inhibition could cripple bacterial virulence and sensitize bacteria to innate antimicrobials and antibiotics.

INTRODUCTION

The outer membrane of Gram-negative bacteria is essential for structural integrity and resistance to a panoply of extracellular stresses. Its outer leaflet is comprised of lipopolysaccharide (LPS), which is a glycolipid consisting of lipid A, core, and O-antigen (Wang et al., 2006; Whitfield and Trent, 2014). The lipid A portion of LPS is pivotal for maintaining outer membrane integrity and is essential for bacterial viability (an "essential" LPS component). Modifications to lipid A enhance resistance to antimicrobials such as host cationic antimicrobial peptides (Needham and Trent, 2013; Raetz and Whitfield, 2002), but they are not required for viability ("accessory" LPS components). Similarly, the most external parts of LPS (outer core and O-antigen) are also accessory components that enhance resistance to various environmental stresses but are not required for viability (Raetz and Whitfield, 2002).

Francisella novicida is a model intracellular pathogen, often used as a surrogate for the category A bioterrorism agent *Francisella tularensis* that causes the disease tularemia (Darling et al., 2002). Interestingly, *Francisella* produces LPS species not observed in other bacteria. The lipid A of *Francisella* LPS lacks both the 4′ and 1′ position phosphates and has only four acyl chains that are two to six carbons longer than those of *E. coli* lipid A (Raetz et al., 2009; Raetz and Whitfield, 2002; Trent, 2004). In addition, 70% of total *Francisella* lipid A exists in a "free" form that lacks the core and O-antigen polysaccharides. In contrast to the lipid A in LPS that is attached to core and O-antigen, free lipid A retains a 1′ position phosphate that is modified with a galactosamine (GalN) residue (Wang et al., 2006; Zhao and Raetz, 2010). Modifications of the anionic lipid A phosphate groups with positively charged moieties, such as GalN, lead to decreased negative surface charge and repulsion of cationic antimicrobials, facilitating resistance. Accordingly, *F. novicida* is resistant to cationic antimicrobial peptides (CAMPs), including human LL-37 and the last-resort polymyxin antibiotics (polymyxin B and colistin), which bind to negatively charged lipid A and disrupt membrane integrity (Mohapatra et al., 2007).

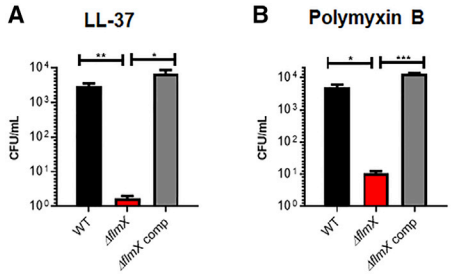


Figure 1. *F. novicida* FlmX is required for resistance to the host antimicrobial peptide LL-37 and polymyxin B

(A and B) Wild-type *F. novicida* U112 (WT), the Δ*flmX* mutant, and the *flmX* complemented strain (Δ*flmX* comp) were treated with 100 μg/mL LL-37 (A) or polymyxin B (B) for 2 h and colony-forming units (CFU) were enumerated after plating. Data are represented as mean ± SD. *p < 0.05, **p < 0.005, ***p < 0.0005.

The lipid A modifications, outer core sugars, and O-antigen are required for *Francisella* pathogenesis (Kanistanon et al., 2008; Llewellyn et al., 2012; Rasmussen et al., 2014). *Francisella* replicates in the cytosol of host cells, including macrophages, which depends on bacterial escape from the phagosome (Jones et al., 2012). While transiently residing in the phagosome, the bacteria must resist innate antimicrobials, including CAMPs, to survive (Jones et al., 2012; Kingry and Petersen, 2014). Thus, the ability of *Francisella* to replicate intracellularly and cause disease requires diverse lipid A/LPS modifications.

Our work presented herein identifies FlmX, a protein with a previously unidentified role in bacterial physiology, as a flippase family member that supports the production of all three components of LPS, that is, lipid A, core, and O-antigen. Consistent with its specific role in generating accessory but not essential LPS components, FlmX was not required for bacterial viability. In contrast, FlmX was indispensable for *Francisella* replication in macrophages and virulence *in vivo*. FlmX was also required for resistance to the cationic antibiotic polymyxin B, highlighting the shared role of this protein in *Francisella* pathogenesis and antibiotic resistance.

RESULTS

Reanalysis of TraSH data identifies *Francisella* virulence genes

We previously used an *in vivo*, genome-wide, negative selection screen (transposon site hybridization [TraSH]) to identify 164 genes required for the growth and/or survival of *F. novicida* in a mouse model of infection (Weiss et al., 2007). The screen used a pool of transposon mutants representing all of the nonessential genes in the genome, and it determined which mutants were recovered in lower numbers after murine infection as compared to the input. When analyzing the results of the screen, we used a very stringent cutoff since, at the time, few of the identified genes had been validated as playing a role in infection. Subsequently, many additional virulence genes have been shown to play a role in infection, and we hypothesized that some may have been excluded by the original TraSH analysis due to the stringent cutoff. We therefore reanalyzed the TraSH data with a less stringent cutoff and identified 100 additional genes predicted to be involved in bacterial replication and/or survival during murine infection. Thirty-eight percent of the identified genes have been shown to be involved in *Francisella* pathogenesis by others (Tables S1 and S2), validating this new analysis. Such genes include the transcriptional regulator MglB (Moule et al., 2010), catalase (Su et al., 2007), thioredoxin (Kraemer et al., 2009), and the transcriptional regulator of numerous oxidative stress resistance genes, OxyR (Moule et al., 2010). Further increasing our confidence in the accuracy of the new data is the fact that some of the newly identified genes are located in an operon with genes known to play a role in virulence, including purine and biotin biosynthetic genes (Napier et al., 2012; Tempel et al., 2006; Weiss et al., 2007). Among the other newly identified genes, more than 30% are annotated as encoding hypothetical proteins of unknown function. Taken together, these data validate the new analysis of the *in vivo* screen and elucidate numerous genes, including *flmX*, identified for the first time as being involved in *Francisella* pathogenesis.

FlmX is required for resistance to an innate immune antimicrobial peptide and polymyxin B

To understand how the newly identified genes might contribute to *in vivo* virulence, we first tested whether any were involved in resistance to the innate immune antimicrobial peptide, LL-37. We screened the LL-37 susceptibility of 168 *F. novicida* transposon mutants representing the 100 newly identified genes. The mutant exhibiting the greatest susceptibility to LL-37 lacked a hypothetical GtrA-like protein of unknown function (FTN_1625), which we have named FlmX (Tables S1 and S2). We generated a deletion mutant lacking *flmX* and observed that it was 1,000-fold more susceptible to LL-37 as compared to wild-type bacteria (Figure 1A). This defect was complemented by restoration of the *flmX* gene in the deletion mutant (Figure 1A). We also investigated whether the *flmX* mutant would exhibit susceptibility to the cationic antibiotic, polymyxin B. Indeed, this strain was highly susceptible to polymyxin B, exhibiting a ~500-fold lower survival than the parental wild-type strain (Figure 1B). These data demonstrate that FlmX plays a critical role in *F. novicida* resistance to cationic antimicrobials.

FlmX regulates LPS remodeling

Modifications to the lipid A portion of LPS can mediate resistance to cationic antimicrobials in Gram-negative bacteria (Raetz et al., 2009; Wang et al., 2006). To determine whether FlmX is involved in lipid A modification, we extracted and analyzed the total crude lipids from the wild-type and *flmX* mutant strains using thin-layer chromatography (TLC) and liquid chromatography-electrospray ionization/mass spectrometry (LC-ESI/MS) (Figure 2; Table S7). TLC analysis revealed the presence of a higher mobility band for free lipid A1 (the tetra-acylated lipid A containing GalN at the 1-phosphate position and lacking the core and O-antigen) in the *flmX* mutant compared to the wild-type and *flmX* complemented strains (Figure 2A; Figure S1). Accordingly, our LC-ESI/MS analysis from the wild-type strain revealed free lipid A1 containing GalN at the 1-phosphate position (*m/z* 1,665.071) (Figure 2B) (Wang et al., 2006). In contrast, free lipid A1 lacking GalN (*m/z* 1,504.011) accumulated in the *flmX* mutant (Figure 2C),

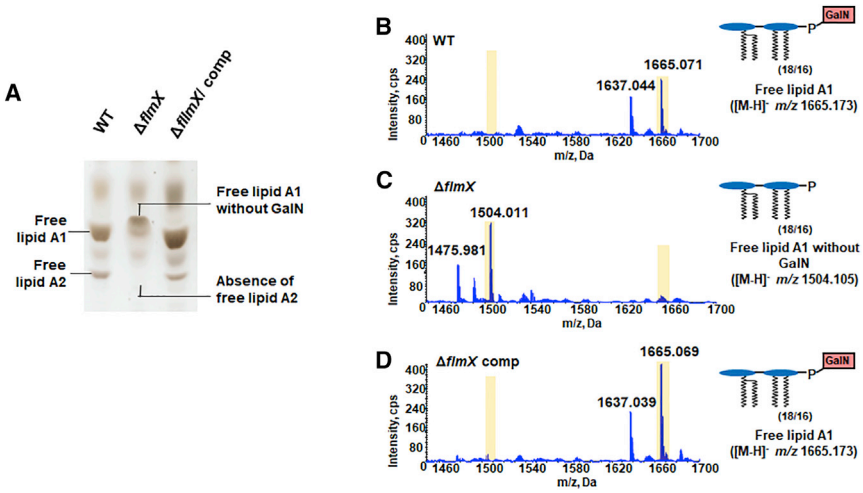


Figure 2. FlmX is required for the galactosamine (GalN) modification of *F. novicida* free lipid A

Lipids were extracted from wild-type *F. novicida* U112 (WT), the Δ*flmX* mutant, and the Δ*flmX* complemented strain (Δ*flmX* comp).

(A) Thin-layer chromatography was performed and revealed lipid A modifications.

(B–D) LC-ESI/MS was performed on total crude lipids from (B) wild-type *F. novicida* U112 (WT), (C) the Δ*flmX* mutant, and (D) the *flmX* complemented strain (Δ*flmX* comp) and the resulting spectra are shown. The peaks represent unmodified free lipid A (*m/z* 1,504.011) and free lipid A modified with GalN (*m/z* 1,665.071). Schematic drawings of free lipid A modified with GalN (*m/z* 1,665) and unmodified free lipid A (*m/z* 1,504) are labeled next to the corresponding mass spectrometry signals.

while the level of free lipid A1 was reduced. The GalN modification was restored when the *flmX* mutant was complemented with *flmX* (Figure 2D). These data show that FlmX plays a critical role in the GalN modification of free lipid A in *F. novicida*.

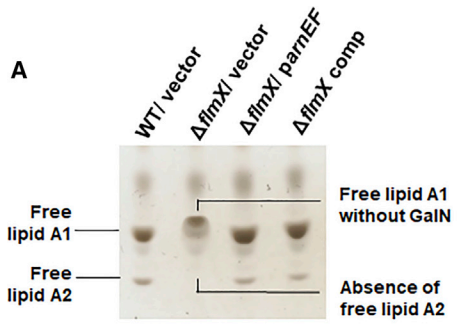
It was unclear how FlmX contributes to the GalN modification of free lipid A1. We previously identified a deacetylase, NaxD, as being required for the GalN modification (Llewellyn et al., 2012). Specifically, NaxD deacetylates *N*-acetyl-galactosamine (GalNAc) to GalN, when GalNAc is linked to the lipid carrier undecaprenyl phosphate (Und-P) (Figure S2). While Und-P-GalN was absent from a *naxD* mutant strain, it was detected in the *flmX* mutant, indicating that FlmX is not involved in generating Und-P-GalN and instead acts downstream of NaxD in the free lipid A modification pathway (Figure S3).

After deacetylation of GalNAc to GalN, Und-P-GalN is translocated across the inner membrane by an unidentified protein, such that GalN can be added to free lipid A1 on the periplasmic side of the inner membrane (Raetz et al., 2009; Song et al., 2009). Bioinformatic analyses predicted FlmX to be a small, hydrophobic, inner membrane protein with four transmembrane domains (Table S3). We hypothesized that FlmX may be a flippase responsible for the translocation of Und-P-GalN across the inner membrane. We first determined whether FlmX is in fact an inner membrane protein using a strain in which FlmX was fused to a FLAG peptide tag at the C-terminus. Fractionation of *F. novicida* expressing FLAG-tagged FlmX indicated that this protein was only present in the inner membrane (Figure S4). As a control, the known inner membrane protein PdpB was also only detected in the inner membrane fraction (de Bruin et al., 2011). Taken together, the data demonstrate that FlmX is an inner membrane protein involved in free lipid A1 modification.

We next investigated whether FlmX is a flippase. Numerous flippases have ATPase domains, and their activities are often ATP-dependent. Bioinformatic analysis, however, revealed that FlmX does not have an ATPase domain. Therefore, this excluded one approach to directly determine whether FlmX is a flippase that relies on using ATP levels to control flippase activity (Raetz and Whitfield, 2002). A second approach that is often used is to determine whether a known flippase protein from another organism can restore activity to a mutant strain lacking the putative flippase of interest (Larrouy-Maumus et al., 2012). To test this, we chose ArnE/F, which is a flippase involved in lipid A modification in *E. coli*, since it translocates a sugar (aminoarabinose) linked to the same Und-P lipid carrier used in *Francisella* free lipid A1 modification (Yan et al., 2007). ArnE and ArnF are also small, cationic, and hydrophobic proteins with four transmembrane domains, similar to FlmX (Table S4). Expression of *E. coli* ArnE/ArnF in the *F. novicida flmX* mutant successfully restored both free lipid A1 modification (Figure 3A; Figure S5) and polymyxin resistance (Figure 3B). Taken together, the following points strongly suggest that FlmX is a flippase: (1) FlmX is essential for free lipid A1 modification with GalN, which requires the translocation of Und-P-GalN across the inner membrane; (2) FlmX is an inner membrane protein; and (3) the *flmX* mutant is complemented by a known flippase from *E. coli*.

FlmX is required for free lipid A2, LPS core, and O-antigen modifications

Further analysis of *Francisella* LPS by TLC and LC-MS revealed that the *flmX* mutant had multiple defects in addition to the lack of the GalN modification on free lipid A1 (Figures 4A–4D; Table S7). There are two main species of free lipid A in *F. novicida*, A1 and A2 (ratio 7:1), which both contain the 1-phosphate that is modified with GalN (Figure S6). In addition, free lipid A2 is further glycosylated at the 6′ position with a hexose group whose identity is unclear and is either a glucose (Wang et al., 2006) or mannose (Kanistanon et al., 2008). Free lipid A2 was absent in the *flmX* mutant, demonstrating that FlmX is required for the hexose addition that generates this lipid species (Figure 4A; Figure S1). The hexose group is not dependent on prior modification of free lipid A with GalN, as the same hexose group is present on the free lipid A2 lacking GalN in the *naxD* mutant, albeit lacking GalN (Figure S3). Taken together, these data show that the role of FlmX in generating free lipid A2 is independent from its role in GalN modification.



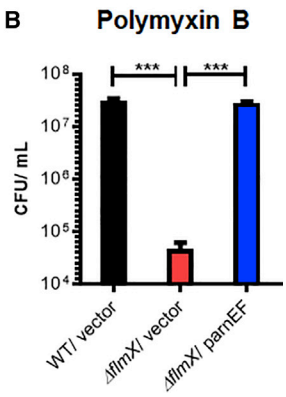


Figure 3. The *E. coli* undecaprenyl phosphate-aminoarabinose flippase, ArnE/ArnF, restores free lipid A modification and polymyxin B resistance to the *F. novicida flmX* mutant

Lipids were extracted from wild-type *F. novicida* U112 with an empty vector (WT/vector), the Δ*flmX* mutant with an empty vector (Δ*flmX*/vector), the Δ*flmX* mutant expressing the *E. coli* undecaprenyl phosphate-aminoarabinose flippase ArnE/ArnF (ArnEF) complemented strain (Δ*flmX*/p*arnEF*), and the Δ*flmX* complemented strain (Δ*flmX* comp).

(A) Thin-layer chromatography was performed on each strain.

(B) The WT/vector, Δ*flmX*/vector, and Δ*flmX*/p*arnEF* strains were treated with 100 μg/mL polymyxin B for 4 h and CFU were enumerated after plating. Data are represented as mean ± SD. ***$p < 0.0005$.

The TLC results also indicated that the core of *Francisella* LPS was altered, and that it lacked a sugar (Figure 4A; Figure S1). To investigate this further, we determined the structure of the core, which first requires the core to be free of O-antigen. Since *wzx* is required for O-antigen (Marolda et al., 2004), we constructed a *flmX* deletion in a *wzx* mutant background. Total crude lipids of the *wzx* mutant and *flmX*/*wzx* double mutant were extracted, purified, and analyzed by LC-ESI/MS for complete core-Kdo-lipid A (Figures 4B–4D) and only core domains (Figure S7; Table S7). A peak at *m/z* 1,330.224, consistent with the $[M-2H]^{2-}$ of the complete core-Kdo-lipid A, was observed only in the *wzx* mutant (Figure 4B). In contrast, a major peak of *m/z* 1,219.190 (complete core-Kdo-lipid A lacking a sugar) was observed in the *flmX*/*wzx* double mutant (Figure 4C), confirming that FlmX is required for a hexose addition and wild-type core structure. Similar results were observed by mass spectrometry of the core domain liberated from lipid A (Figure S7).

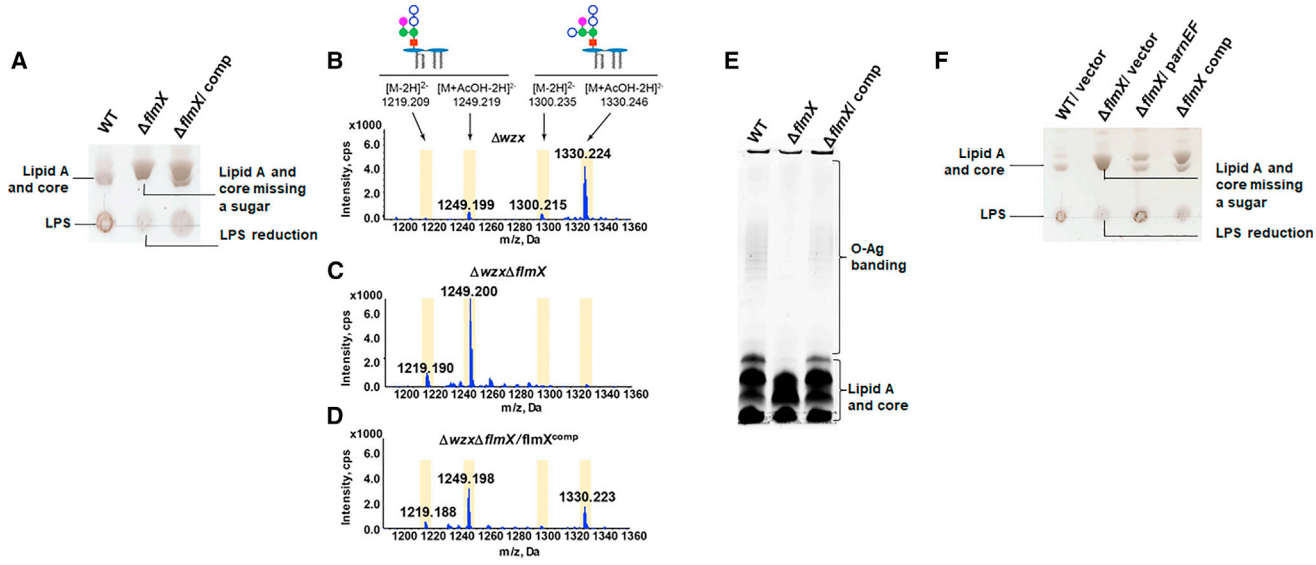


Figure 4. FlmX is required for core and O-antigen modification of *F. novicida* LPS

Lipids were extracted from wild-type *F. novicida* U112 (WT), the Δ*flmX* mutant, and the Δ*flmX* complemented strain (Δ*flmX* comp).

(A) Thin-layer chromatography was performed.

(B–D) Total crude lipids of (B) the Δ*wzx* mutant, (C) the Δ*wzx*Δ*flmX* double mutant, and (D) the Δ*wzx*Δ*flmX* double mutant complemented with *flmX* (Δ*wzx*Δ*flmX*/flmX^{comp}) were purified and mass spectrometry was used to measure the presence of lipid A plus core. The WT and Δ*wzx*Δ*flmX*/flmX^{comp} strains contain the complete lipid A and core including a glucose modification (*m/z* 1,330.224), while the Δ*flmX* mutant contains only lipid A with core lacking the glucose (*m/z* 1,219.190). The schematic species of lipid A + core in the WT and Δ*flmX* mutant are labeled above the corresponding mass spectrometry peaks.

(E) LPS extracted from wild-type *F. novicida* U112 (WT), the Δ*flmX* mutant, and the Δ*flmX* complemented strain (Δ*flmX* comp) was resolved using a Tris-glycine gel stained with Pro-Q Emerald 300. O-antigen banding was observed in the WT and Δ*flmX* complemented strains but not the Δ*flmX* mutant.

(F) Thin-layer chromatography was performed on WT, the Δ*flmX* mutant, the Δ*flmX* mutant expressing the *E. coli* undecaprenyl phosphate-aminoarabinose flippase ArnE/ArnF (ArnEF) complemented strain (Δ*flmX*/p*arnEF*), and Δ*flmX* comp.

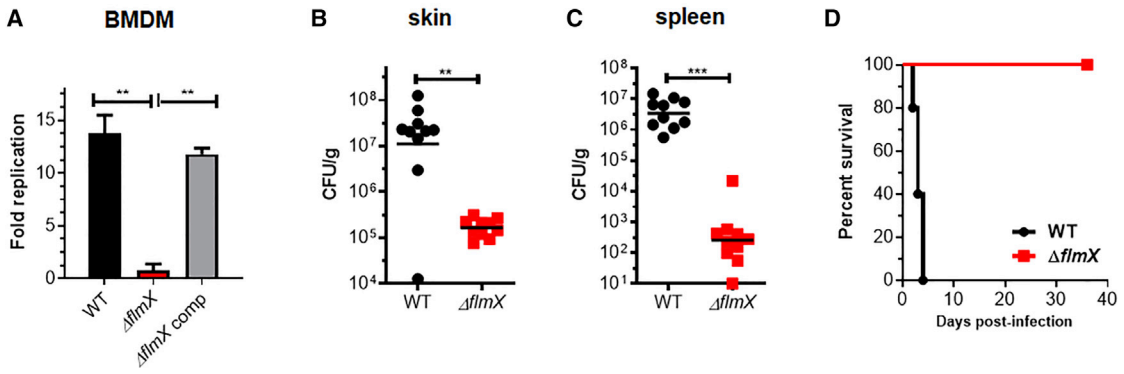


Figure 5. FlmX is required for *F. novicida* pathogenesis

(A) Murine bone marrow-derived macrophages (BMDMs) were infected with a 20:1 multiplicity of infection (MOI) of wild-type *F. novicida* U112 (WT), Δ*flmX*, or the *flmX* complemented strain (Δ*flmX* comp). Data are represented as mean ± SD.

(B and C) Mice were subcutaneously infected with 2×10^5 CFU of either WT or Δ*flmX* mutant. After 48 h, the mice were sacrificed and the (B) skin at the site of infection and (C) spleen were harvested, homogenized, and plated for CFU. Data were pooled from two separate experiments. Bars represent the geometric mean, and significance was determined using a two-tailed Mann-Whitney test.

(D) For survival experiments, mice were subcutaneously infected with 2×10^7 CFU of either WT, Δ*flmX* mutant, or Δ*flmX* comp and sacrificed when they appeared moribund (n = 5 mice). $^{**}p < 0.005$, $^{***}p < 0.0005$.

The *flmX* mutant also exhibited a significant reduction in total LPS as compared to the wild-type and *flmX* complemented strains (Figure 4A; Figure S1). To investigate this reduction of LPS further, we examined the LPS O-antigen content in each strain. The *flmX* mutant had a severe reduction in O-antigen content as evidenced by reduced banding on an LPS staining gel, similar to the extent of O-antigen loss in the *wzx* mutant (Figure 4E; Figure S7). Expression of *flmX* or *E. coli arnE/arnF* complemented O-antigen content in the *flmX* mutant (Figure 4F). However, overexpression of *flmX* failed to complement the *wzx* mutant or *flmX/wzx* double mutant, while overexpression of *wzx* partially restored O-antigen content in the *flmX* mutant and the double mutant (Figure S7C); future studies are required to decipher the interplay between FlmX and Wzx. Taken together, these data indicate that FlmX is essential for the structural modifications to free lipid A species (free lipid A1 and A2) as well as the outer core and O-antigen of LPS, highlighting this protein as having a central role in determining lipid A/LPS structure.

FlmX is required for *Francisella* pathogenesis

We next tested the contribution of FlmX to *Francisella* replication within host cells and in mice. We infected murine bone marrow-derived macrophages (BMDMs) with the wild-type or *flmX* deletion strain and quantified levels of intracellular bacteria 5 h post-infection. In contrast to wild-type bacteria that readily replicated within these macrophages, the *flmX* mutant failed to replicate and was present at roughly 10-fold lower levels (Figure 5A). The *flmX* complemented strain replicated similar to the wild-type, confirming that FlmX is required for *F. novicida* intracellular replication.

Mice were infected subcutaneously with wild-type or *flmX* mutant strains. At 48 h post-infection, mice infected with the *flmX* mutant harbored 100-fold less bacteria in the skin at the site of infection than those infected with the wild-type (Figure 5B). This difference was even greater in the spleen, where *flmX* mutant bacteria were recovered at 10,000-fold lower levels than those in the wild-type (Figure 5C). In survival experiments, all of the mice infected with wild-type bacteria succumbed to infection by day 4, whereas *flmX*-infected mice survived up to 35 days post-infection (Figure 5D). As a control, we observed no significant difference in the ability of the *flmX* mutant strain to grow in rich media (data not shown), indicating that the *in vivo* attenuation phenotype of the mutant was not due to a general fitness defect. Taken together, these data indicate that FlmX plays a critical role in *F. novicida* virulence.

The FlmX ortholog in *F. tularensis* is required for LPS modification and pathogenesis

The FlmX ortholog in *F. tularensis* strain SchuS4 (FTT_0085c) has 100% amino acid identity with *F. novicida* FlmX (Table S5). To determine the contribution of FlmX to the physiology and virulence of highly pathogenic *F. tularensis*, we generated an *flmX* deletion mutant in SchuS4 and tested it for LPS modification and virulence. TLC analysis revealed that similar to the *F. novicida flmX* mutant, the *F. tularensis* mutant lacked modifications to free lipid A1 and had greatly reduced LPS when compared to the wild-type strain (Figure 6A). In contrast to the wild-type (Figure 6B), free lipid A lacking the GalN modification (*m/z* 1,504.055) accumulated in the *flmX* mutant (Figure 6C; Table S7) and a significant reduction of O-antigen was revealed by LPS-specific staining (Figure 6D). The *F. tularensis flmX* mutant was unable to replicate in primary murine BMDMs (Figure 6E), and strikingly, was unable to cause lethal disease in mice even at doses >1,000,000-fold the lethal dose 50 (LD_{50}) of the wild-type strain (Figure 6F). These data demonstrate the conserved role of FlmX in lipid A/LPS modification and virulence in highly virulent, human pathogenic *F. tularensis*.

DISCUSSION

The data presented herein reveal that FlmX is a critical focal point of lipid A/LPS modification (Figure 7). While not required for the

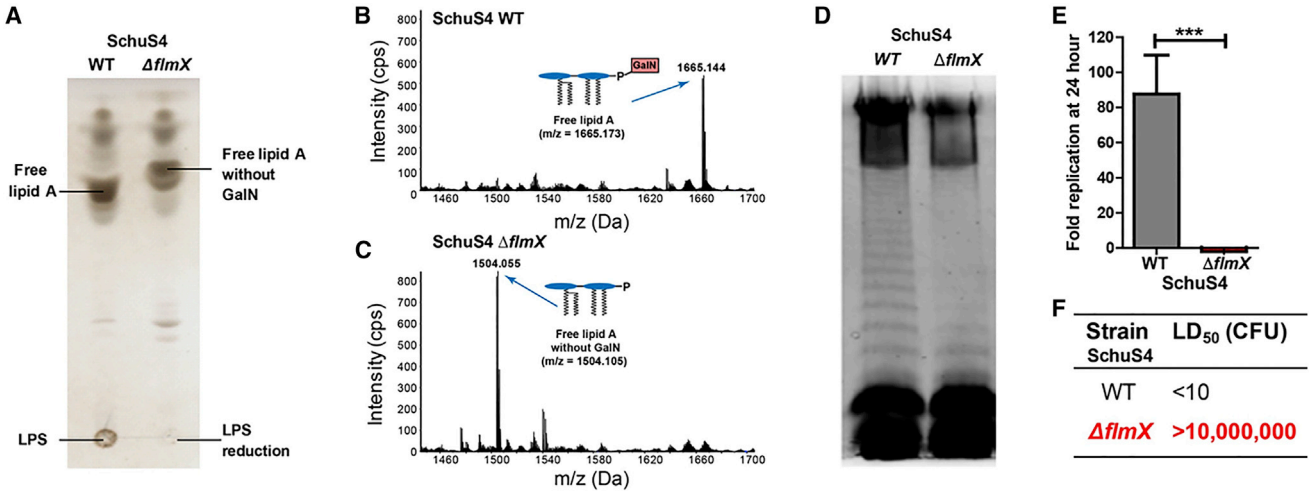


Figure 6. FlmX ortholog in the highly virulent *F. tularensis* is required for lipid A/LPS modification and *in vivo* virulence

(A–C) Lipids were extracted from wild-type *F. tularensis* strain SchuS4 (WT) and an isogenic Δ*flmX* mutant. Total crude lipids were prepared from each strain and (A) thin-layer chromatography and (B and C) LC-ESI/MS were performed as indicated. (B and C) The peaks represent (B) free lipid A modified with GalN (*m/z* 1,665.144) or (C) unmodified free lipid A (*m/z* 1,504.055).

(D) LPS extracted from WT *F. tularensis* and Δ*flmX* mutant strains was resolved using a Tris-glycine gel stained with Pro-Q Emerald 300 to detect the O-antigen banding pattern.

(E) Murine BMDMs were infected with WT *F. tularensis* or the Δ*flmX* mutant. CFU from lysates 30 min post-infection were compared with those from 24 h post-infection to determine fold intracellular replication. Data are represented as mean ± SD. ***$p < 0.0005$.

(F) Susceptibility of mice to intradermal infection with WT *F. tularensis* strain SchuS4 or the Δ*flmX* mutant strain.

biogenesis of the essential components of lipid A/LPS that facilitate bacterial viability, FlmX specifically plays an accessory role in generating diverse, modified lipid A/LPS species that confer stress resistance and virulence. Highlighting the extent of its critical contribution to *in vivo* pathogenesis, a *flmX* mutant of highly virulent, biosafety level 3 (BSL-3) *F. tularensis* was more than 1,000,000-fold attenuated in a murine infection model. The broad control of LPS accessory modifications by FlmX represents an unexpected and novel paradigm in *Francisella* biology and bacterial pathogenesis.

Our data indicate that FlmX is a member of the flippase family, which are integral membrane proteins that translocate lipid-linked sugars across membranes. Protein sequence analysis revealed that FlmX belongs to the GtrA superfamily, whose founding member is a small protein encoded by a *Shigella flexneri* bacteriophage that is involved in the translocation of lipid-linked glucose across the cytoplasmic membrane, and some of whose members are involved in modifications to LPS structure (Guan et al., 1999). The diverse FlmX-dependent lipid A/LPS modifications that are absent in the *flmX* mutant were complemented by exogenous expression of the *E. coli* flippase ArnE/F. While ArnE/F is required for aminoarabinose modification of *E. coli* lipid A, we demonstrated that FlmX is required for the GalN modification of *Francisella* lipid A, in addition to other modifications to LPS. ArnE/F has previously been shown capable of participating in distinct modification pathways when expressed in a non-natural host, such as the restoration of arabinose modification of lipoarabinomannan in a mutant of *Mycobacterium tuberculosis* lacking Rv3789 (Larrouy-Maumus et al., 2012). This indicates that these flippases can have promiscuous substrate specificity.

It was interesting and surprising that FlmX was required for a diverse repertoire of lipid A and LPS modifications. FlmX did not control the generation of the Und-P-hexose substrates required for modification of free lipid A species (such as the GalN modification at the 1-phosphate for lipid A1 and A2, and the glucose modification at the 6′ position for free lipid A2) (Song et al., 2009); it instead acted downstream, consistent with it being the flippase that translocates Und-P-hexose across the inner membrane. It remains uncertain how FlmX influences the addition of glucose to the core or the addition of O-antigen to LPS (Figure 7). Core modifications have previously been described to occur in the cytosol, which suggests either that FlmX is involved in flipping a substrate for a novel periplasmic-based core modification, or that FlmX plays an indirect role. For example, it is possible that in the absence of FlmX, enzymes or the production or translocation of substrates required for the core glucose modification are disrupted. Regarding O-antigen, it is possible that FlmX-dependent outer core modification enhances the efficiency with which O-antigen is added to LPS. Alternatively, deficiency in *flmX* could lead to retention of the Und-P carrier lipid on the inner leaflet of the inner membrane, effectively sequestering this lipid, disrupting its recycling, and preventing other modifications that require Und-P or Und-PP (such as O-antigen) as a carrier lipid. Future research will address numerous remaining questions regarding FlmX biology.

The dependence of multiple lipid A/LPS modifications on FlmX represents a potential Achilles' heel for the bacteria and therefore a potential drug target. A small molecule inhibitor of FlmX would cripple the ability of the bacteria to cause disease and resist stresses such as host cationic antimicrobial peptides, as well as cationic polymyxin antibiotics. Since FlmX is highly

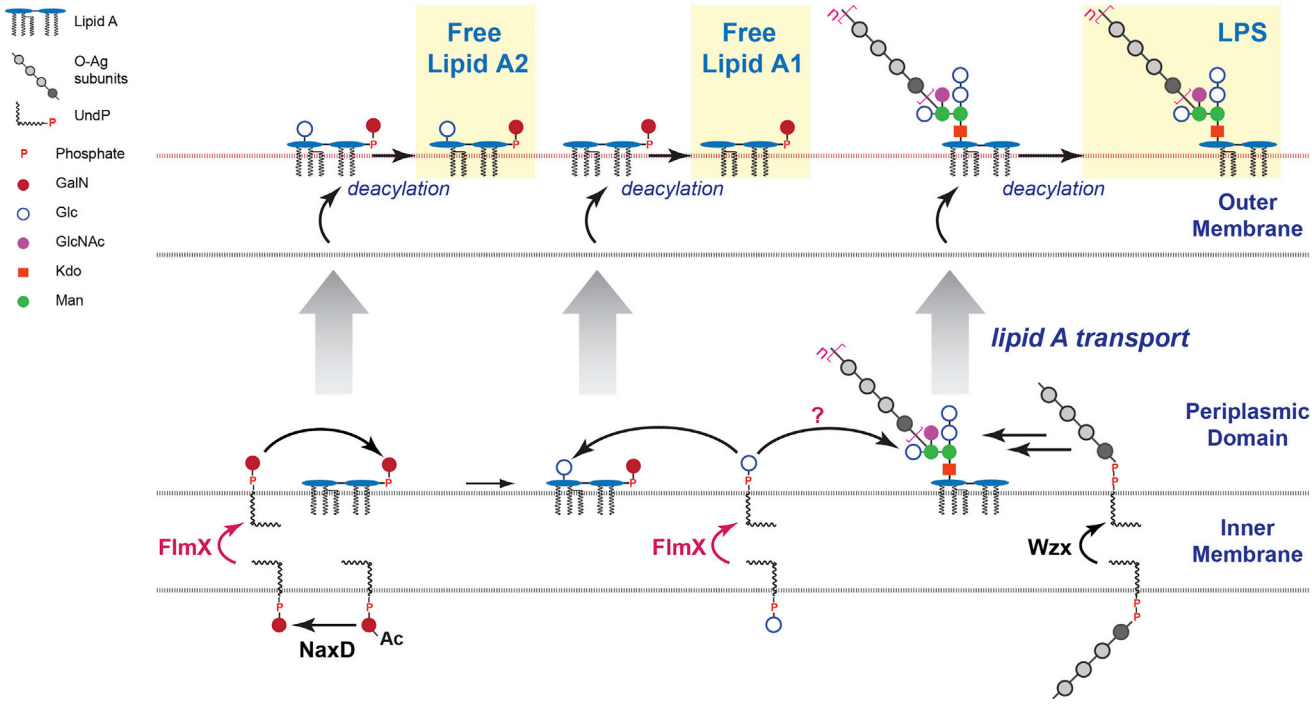


Figure 7. Model of the role of FlmX in controlling the distinct components of *F. novicida* LPS

Our experimental data suggest that FlmX "flips" the undecaprenyl phosphate-GalN (brown circle) and undecaprenyl phosphate-glucosamine (Glc, open blue circle) from the cytosolic to the periplasmic side of the inner membrane, where GalN and Glc are subsequently added to the lipid A to form free lipid A1 and free lipid A2, respectively. FlmX is also required for the addition of a hexose moiety to the core of LPS and for enhanced O-antigen attachment, though the detailed mechanism requires further investigation (indicated by a question mark). The corresponding schematic structures and the color schemes are shown in the key on the top left of the figure. The model was modified from Zhao and Raetz (2010).

conserved in some of the most pathogenic Gram-negative bacteria (Table S5), a FlmX inhibitor would have broad utility and represent a novel component of our antibacterial arsenal.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109247.

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AUTHOR CONTRIBUTIONS

Experiments were conducted by C.-Y.C., J.Z., and I.G. C.-Y.C. performed most of the *F. novicida flmX*-associated *in vitro* and *in vivo* experiments. J.Z. performed all of the TLC- and LC-MS-associated experiments. A.C.L. generated the *F. novicida flmX* deletion mutant and performed preliminary experiments. I.G. and A.S. generated the *F. tularensis flmX* deletion mutant and performed

associated experiments. The manuscript was prepared by C.-Y.C., D.S.W., J.Z., and P.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. *37*, 911–917.

Bryksin, A.V., and Matsumura, I. (2010). Overlap extension PCR cloning: A simple and reliable way to create recombinant plasmids. Biotechniques *48*, 463–465.

Darling, R.G., Catlett, C.L., Huebner, K.D., and Jarrett, D.G. (2002). Threats in bioterrorism. I: CDC category A agents. Emerg. Med. Clin. North Am. *20*, 273–309.

de Bruin, O.M., Duplantis, B.N., Ludu, J.S., Hare, R.F., Nix, E.B., Schmerk, C.L., Robb, C.S., Boraston, A.B., Hueffer, K., and Nano, F.E. (2011). The biochemical properties of the *Francisella* pathogenicity island (FPI)-encoded proteins IglA, IglB, IglC, PdpB and DotU suggest roles in Type VI secretion. Microbiology *157*, 3483–3491.

Gallagher, L.A., McKevitt, M., Ramage, E.R., and Manoil, C. (2008). Genetic dissection of the *Francisella novicida* restriction barrier. J. Bacteriol. *190*, 7830–7837.

Gallagher, L.A., Ramage, E., Jacobs, M.A., Kaul, R., Brittnacher, M., and Manoil, C. (2007). A comprehensive transposon mutant library of *Francisella novicida*, a bioweapon surrogate. Proc. Natl. Acad. Sci. USA *104*, 1009–1014.

Golovliov, I., Sjöstedt, A., Mokrievich, A., and Pavlov, V. (2003). A method for allelic replacement in *Francisella tularensis*. FEMS Microbiol. Lett. *222*, 273–280.

Guan, S., Bastin, D.A., and Verma, N.K. (1999). Functional analysis of the O antigen glucosylation gene cluster of Shigella flexneri bacteriophage SfX. Microbiology (Reading) *145*, 1263–1273.

Jones, C.L., Napier, B.A., Sampson, T.R., Llewellyn, A.C., Schroeder, M.R., and Weiss, D.S. (2012). Subversion of host recognition and defense systems by *Francisella* spp. Microbiol. Mol. Biol. Rev. *76*, 383–404.

Kanistanon, D., Hajjar, A.M., Pelletier, M.R., Gallagher, L.A., Kalhorn, T., Shaffer, S.A., Goodlett, D.R., Rohmer, L., Brittnacher, M.J., Skerrett, S.J., and Ernst, R.K. (2008). A *Francisella* mutant in lipid A carbohydrate modification elicits protective immunity. PLoS Pathog. *4*, e24.

Kingry, L.C., and Petersen, J.M. (2014). Comparative review of *Francisella tularensis* and *Francisella novicida*. Front. Cell. Infect. Microbiol. *4*, 35.

Kraemer, P.S., Mitchell, A., Pelletier, M.R., Gallagher, L.A., Wasnick, M., Rohmer, L., Brittnacher, M.J., Manoil, C., Skerett, S.J., and Salama, N.R. (2009). Genome-wide screen in *Francisella novicida* for genes required for pulmonary and systemic infection in mice. Infect. Immun. *77*, 232–244.

Larrouy-Maumus, G., Škovierová, H., Dhouib, R., Angala, S.K., Zuberogoitia, S., Pham, H., Villela, A.D., Mikušová, K., Noguera, A., Gilleron, M., et al. (2012). A small multidrug resistance-like transporter involved in the arabinosylation of arabinogalactan and lipoarabinomannan in mycobacteria. J. Biol. Chem. *287*, 39933–39941.

Llewellyn, A.C., Zhao, J., Song, F., Parvathareddy, J., Xu, Q., Napier, B.A., Laroui, H., Merlin, D., Bina, J.E., Cotter, P.A., et al. (2012). NaxD is a deacetylase required for lipid A modification and *Francisella* pathogenesis. Mol. Microbiol. *86*, 611–627.

Marolda, C.L., Vicarioli, J., and Valvano, M.A. (2004). Wzx proteins involved in biosynthesis of O antigen function in association with the first sugar of the O-specific lipopolysaccharide subunit. Microbiology (Reading) *150*, 4095–4105.

Mohapatra, N.P., Soni, S., Bell, B.L., Warren, R., Ernst, R.K., Muszynski, A., Carlson, R.W., and Gunn, J.S. (2007). Identification of an orphan response regulator required for the virulence of *Francisella* spp. and transcription of pathogenicity island genes. Infect. Immun. *75*, 3305–3314.

Moule, M.G., Monack, D.M., and Schneider, D.S. (2010). Reciprocal analysis of *Francisella novicida* infections of a *Drosophila melanogaster* model reveal host-pathogen conflicts mediated by reactive oxygen and imd-regulated innate immune response. PLoS Pathog. *6*, e1001065.

Napier, B.A., Meyer, L., Bina, J.E., Miller, M.A., Sjöstedt, A., and Weiss, D.S. (2012). Link between intraphagosomal biotin and rapid phagosomal escape in Francisella. Proc. Natl. Acad. Sci. USA *109*, 18084–18089.

Needham, B.D., and Trent, M.S. (2013). Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. Nat. Rev. Microbiol. *11*, 467–481.

Raetz, C.R., and Whitfield, C. (2002). Lipopolysaccharide endotoxins. Annu. Rev. Biochem. *71*, 635–700.

Raetz, C.R., Guan, Z., Ingram, B.O., Six, D.A., Song, F., Wang, X., and Zhao, J. (2009). Discovery of new biosynthetic pathways: The lipid A story. J. Lipid Res. *50* (*Suppl*), S103–S108.

Rasmussen, J.A., Post, D.M., Gibson, B.W., Lindemann, S.R., Apicella, M.A., Meyerholz, D.K., and Jones, B.D. (2014). *Francisella tularensis* Schu S4 lipopolysaccharide core sugar and O-antigen mutants are attenuated in a mouse model of tularemia. Infect. Immun. *82*, 1523–1539.

Song, F., Guan, Z., and Raetz, C.R. (2009). Biosynthesis of undecaprenyl phosphate-galactosamine and undecaprenyl phosphate-glucose in *Francisella novicida*. Biochemistry *48*, 1173–1182.

Su, J., Yang, J., Zhao, D., Kawula, T.H., Banas, J.A., and Zhang, J.R. (2007). Genome-wide identification of *Francisella tularensis* virulence determinants. Infect. Immun. *75*, 3089–3101.

Tempel, R., Lai, X.H., Crosa, L., Kozlowicz, B., and Heffron, F. (2006). Attenuated *Francisella novicida* transposon mutants protect mice against wild-type challenge. Infect. Immun. *74*, 5095–5105.

Trent, M.S. (2004). Biosynthesis, transport, and modification of lipid A. Biochem. Cell Biol. *82*, 71–86.

Wang, X., Ribeiro, A.A., Guan, Z., McGrath, S.C., Cotter, R.J., and Raetz, C.R. (2006). Structure and biosynthesis of free lipid A molecules that replace lipopolysaccharide in *Francisella tularensis* subsp. *novicida*. Biochemistry *45*, 14427–14440.

Weiss, D.S., Brotcke, A., Henry, T., Margolis, J.J., Chan, K., and Monack, D.M. (2007). In vivo negative selection screen identifies genes required for **Francisella** virulence. Proc. Natl. Acad. Sci. USA *104*, 6037–6042.

Whitfield, C., and Trent, M.S. (2014). Biosynthesis and export of bacterial lipopolysaccharides. Annu. Rev. Biochem. *83*, 99–128.

Yan, A., Guan, Z., and Raetz, C.R. (2007). An undecaprenyl phosphate-aminoarabinose flippase required for polymyxin resistance in *Escherichia coli*. J. Biol. Chem. *282*, 36077–36089.

Zhao, J., and Raetz, C.R. (2010). A two-component Kdo hydrolase in the inner membrane of *Francisella novicida*. Mol. Microbiol. *78*, 820–836.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-Flag M2®	Sigma-Aldrich	Product#F3165
anti-*Francisella* IglC	BEI Resources	RRID: AB_2891308
anti-*Francisella* PdpB	BEI Resources	RRID: AB_2891309
anti-*Francisella* FopA	BEI Resources	Product# (Item discontinued)
anti-mouse IgG	Cell Signaling	Product#7076
Bacterial and virus strains		
NEB 5-alpha competent *E. coli* (High Efficiency)	New England Biolabs (NEB)	Cat#C2987I
Chemicals, peptides, and recombinant proteins		
Phusion® High-Fidelity DNA Polymerase	NEB	Cat#M0530
Amersham Hybond P 0.45 PVDF	GE healthcare	Product#10600029
LL-37	CDC	N/A
Kanamycin sulfate	Thermo Fisher Scientific	Cat#11815024
Colistin	Sigma-Aldrich	Cat#C4461
Polymyxin B	Tokyo Chemical Industries Japan	Product#P1923
Critical commercial assays		
QIAGEN PCR purification kit	QIAGEN	Cat#28104
Gibson Assembly Cloning Kit	NEB	Cat#E2611S
Zyppy Plasmid Miniprep	Zymo Research	Cat#D4036
Zyppy Plasmid Maxiprep kits	Zymo Research	Cat#D4027
QIAquick Gel Extraction Kit	QIAGEN	Cat#28704
Experimental models: Organisms/strains		
Mice: C57BL/6J	Jackson	JAX stock #000664
Mice: BALB/c	Charles River	CRV strain code#028
Oligonucleotides		
Refer to Table S5 all oligonucleotides used in the study	N/A	N/A
Recombinant DNA		
pFFlp	Gallagher et al., 2008	N/A
pBAV1K-T5-GFP	Bryksin and Matsumura, 2010	N/A
Software and algorithms		
ProtParam	ExPASy	https://web.expasy.org/protparam/
TMHMM Server, v 2.0	DTU Bioinformatics	http://www.cbs.dtu.dk/services/TMHMM/
TopPred2	omicX	http://sbcb.bioch.ox.ac.uk/TM_noj/TM_noj.html
PredictProtein	ROSTLAB	https://predictprotein.org/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, David Weiss (david.weiss@emory.edu).

Materials availability

This study did not generate new unique reagents. All recombinant plasmids generated in this study are available on request.

Data and code availability

This study did not generate datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

In vivo animal work

Specific-pathogen free mice were kept in filter-top cages at Yerkes National Primate Center, and provided food and water *ad libitum* (Llewellyn et al., 2012). Emory University Institutional Animal Care and Use Committee (protocol #Y-201700106) approved all procedures (Llewellyn et al., 2012). Female C57BL/6J mice (Jackson) between 7 and 10 weeks were used for all experiments.

Bacterial strains and growth conditions

Francisella novicida U112 was a kind gift from Dr. Denise Monack, Stanford University. Cultures were grown overnight at 37°C with aeration in tryptic soy broth (TSB) supplemented with 0.2% L-cysteine (BD Biosciences, Sparks, MD, USA) or on tryptic soy agar (TSA; BD Biosciences) supplemented with 0.1% L-cysteine. When necessary, media was supplemented with kanamycin (30 μg/mL). For growth curves experiments, the *F. novicida* strains were sub-cultured to an OD_{600} of 0.03 in TSB with 0.2% L-cysteine. Subcultures were read hourly using a SynergyMX BioTek plate reader (Applied Biosystems) for 24hr.

METHOD DETAILS

Mutagenesis and complementation

Mutagenesis and complementation were performed using the primers indicated in Table S6. To generate the *F. novicida flmX* deletion mutants, PCR was used to amplify flanking DNA regions upstream and downstream of the gene of interest using primers in Table S6. A kanamycin resistance cassette was inserted between these flanking regions using overlapping PCR reactions and transformed into chemically competent wild-type strain U112 as previously described (Llewellyn et al., 2012). The primers used to create the kanamycin-resistant deletion mutants contained FRT sites flanking the kanamycin resistance cassette, which allowed a clean deletion of each mutant to be made using the plasmid pFFlp encoding the Flp-recombinase as previously described (Gallagher et al., 2007). The *flmX* strain were complemented *in cis* by overlapping PCR using PCR-amplified fragments of the wild-type gene of interest, upstream and downstream flanking regions, FLAG tag and a kanamycin resistance cassette. The *FTT0453* mutant was generated by introducing the pXBΔ0453 plasmid into *E. coli* S17-1 λ*pir* by electroporation and then transferred to Schu S4 by conjugation as previously described (Golovliov et al., 2003). Clones with plasmid integrated into the chromosome by a single recombination event were selected on plates containing kanamycin and polymyxin B and integration was verified by PCR (Golovliov et al., 2003). Clones with integrations were then subjected to sucrose selection. This procedure selected for a second cross-over event in which the integrated plasmid, encoding *sacB*, was excised from the chromosome. Kanamycin-sensitive clones were examined by PCR confirming the deletion of the gene and confirmed by sequencing.

Plasmid construction

Plasmids were constructed using the primers indicated in Table S6. The broad host range vector pBAV1K-T5-GFP (pBAV) was used as the control plasmid and backbone for the plasmids in all assays (Bryksin and Matsumura, 2010). Plasmids that were used to express *E. coli* undecaprenyl phosphate-aminoarabinose flippase, ArnE/ArnF were made by replacing the promoter and RBS driving expression of *gfp* in the pBav vector with the synthetic constitutive expression in *F. novicida* followed by *E.coli* ArnE/ArnF. Plasmids were constructed using a Gibson Assembly Cloning Kit (NEB) and transformed and isolated from competent *E. coli* (NEB 5-alpha) using Zyppy Mini and Maxi prep kits (Zymo Research).

Preparation of total lipids

Overnight cultures of *F. novicida* U112 wild-type or *flmX* mutant strains were grown at 37°C in TSB supplemented with L-cysteine. The cells were collected by centrifugation (10,000 *g*, 10 min) and washed with PBS. The cell pellets were resuspended in a single-phase Bligh-Dyer mixture (Bligh and Dyer, 1959) consisting of chloroform, methanol and PBS (1:2:0.8, v/v) and centrifuged (4 000 *g*, 30 min) to remove insoluble debris. The supernatant was converted to a two-phase Bligh-Dyer system by adding chloroform and PBS to generate a mixture consisting of chloroform, methanol and aqueous solution (2:2:1.8, v/v). The two phases of Bligh-Dyer system were separated under centrifugation and the lower phase was dried under a stream of nitrogen. The total lipids were analyzed using thin-layer chromatography (TLC) and LC-ESI/MS. The TLC plate was developed in a solvent system containing chloroform, methanol, pyridine, acetic acid and water (25:10:5:4:3, v/v, optimal for separating free lipid A's) or chloroform, methanol, acetic acid and water (25:15:4:4, v/v, optimal for separating lipid A with core). Lipids were detected by spraying 10% of sulphuric acid in ethanol and charring on a hot plate.

Liquid chromatography/mass spectrometry (LC/MS)

Normal phase LC/MS of lipids was performed as previously described (Zhao and Raetz, 2010). Reverse phase LC/MS of lipids was performed using a Shimadzu LC system (comprising a solvent degasser, two LC-10A pumps and an SCL-10A system controller) coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (as above). LC was performed at a flow rate of 200 ml/min with a linear gradient as follows: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 4 min. Mobile phase A consisted of methanol/acetonitrile/aqueous 1 mM ammonium acetate (60:20:20, v/v/v). Mobile phase B consisted of 100% ethanol containing 1 mM ammonium acetate. A Zorbax SB-C8 reversed-phase column (5 m, 2.1 × 50 mm) was obtained from Agilent (Palo Alto, CA). The postcolumn splitter diverted ~10% of the LC flow to the ESI source of the mass spectrometer.

Membrane fractionation and immunoprecipitation

Four liters of *F. novicida* wild-type or FlmX-Flag-tagged overexpression strains were harvested at OD_{600} = 1.0 by centrifugation for 30 min at 5000 *g* at 4°C. Cell pellets were washed with PBS, resuspended in 10 mL of PBS and passed through a sonicator. Unbroken cells were then removed by centrifugation at 10 000 *g* for 20 min at 4°C. Membrane fractions were pelleted from whole-cell lysates by ultracentrifugation at 200 000 *g* for 2 h at 4°C. Immunoprecipitation was performed on each membrane fractions using the FLAG® Immunoprecipitation Kit (Sigma-Aldrich) according to the manufacturer's instructions. *F. novicida* fractionation and protein localization were verified using western blotting.

Western blot

Hundred micrograms of protein from each fraction were run on 15% protein gel (Bio-Rad) along with a broad range protein standard (Bio-Rad). The proteins were next transferred onto PVDF membranes (GE Osmonics). Western blots were performed. Briefly, membranes were blocked 2 hours with 5% skim milk tris-buffered saline with 0.005% Tween-20 (TBST). Next, membranes were incubated with anti-*Francisella* IglC (1:1000; BEI Resources), anti-*Francisella* PdpB (1:4000), anti-*Francisella* FopA (1:2000) for 2 hours at room temperature, respectively. Following washing, membranes were incubated with secondary antibodies against IgG from mouse (1:2000; Cell Signaling) for 2 hours at room temperature, respectively. For FlmX-FLAG tagged detection, membrane was incubated with monoclonal anti-FLAG® M2 (1:1000; Sigma-Aldrich) antibody at 4°C for overnight, followed by anti-mouse IgG (1:2000; Cell Signaling) secondary antibody for 2 hours at room temperature. Membranes were developed using SuperSignal® West Femto Maximum Sensitivity Substrate and imaged using a BioRad Molecular Imager Chemi Doc™ XRS+ Imaging System.

Macrophage infections

Murine bone marrow-derived macrophages (BMDM) were prepared as described previously (Llewellyn et al., 2012; Weiss et al., 2007). Briefly, bone marrow was collected from the femurs of females C57BL/6 mice aged 6 to 10 weeks. Bone marrow cells were plated in sterile Petri dishes and incubated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS)- and 10% macrophage colony-stimulating factor (M-CSF)-conditioned medium. Bone marrow cells were incubated at 37°C with 5% CO2 and harvested after 6 days. All BMDM were incubated before and during infection in 24 well plates at 37°C with 5% CO2. For infection, BMDM were seeded at $5x10^5$ cells per well and incubated overnight at 37°C with 5% CO2. BMDM were infected at a multiplicity of infection (MOI) of 20:1 for *F. novicida* or 50:1 for Schu S4. At indicated time points post infection, macrophages were lysed with 1% saponin. Lysates were serially diluted in phosphate-buffered saline (PBS) and plated onto TSA containing 0.1% cysteine to numerate CFU.

Mouse infections

Female C57BL/6 mice aged 7 to 10 weeks were kept under specific-pathogen free conditions in filter-top cages at Yerkes National Primate Center, and provided food and water *ad libitum*. All experimental procedures were approved by the Emory University Institutional Animal Care and Use Committee. For single infections, mice were infected subcutaneously with $2x\ 10^5$ CFU in 50 μ sterile PBS. After 48 h, the mice were sacrificed, and the spleens, and skin at the site of infection were harvested, homogenized, and plated for CFU on Mueller-Hinton (MH) plates supplemented with 0.1% L-cysteine. For survival experiments, groups of five mice were infected subcutaneously with 1×10^7 cfu bacteria and sacrificed when they appeared moribund.

For testing of Schu S4 and derivatives, BALB/c mice were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). The mice were maintained and used in accordance with the recommendations of the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals in a federally licensed, Select Agent-approved, small animal containment level 3 facility, National Research Council, Ottawa, Canada. *F. tularensis* strains were injected in a volume of 50 μL intradermally in groups of five (n = 5). The mice were examined daily for signs of infection and were euthanized by CO_2 asphyxiation as soon as they displayed signs of irreversible morbidity.

Antimicrobial assays

The indicated strains were grown overnight in TSB with 0.2% L-cysteine (BD Biosciences) and subsequently diluted to an OD_{600} of 0.001 in 30% TSB with 0.2% L-cysteine containing 100 μg/mL of LL-37, polymyxin B (USB Corporation) or colistin (Sigma-Aldrich), respectively. Cultures were incubated at 37°C and plated for colony-forming units at the indicated time points.

Screen for LL-37 resistance determination

Previous *in vivo* genetic screen data that are necessary for virulence (Weiss et al., 2007) with less stringent cut-off (FDR = 19.55%) were reanalysed and additional 100 genes were identified. One hundred and sixty-eight transposon mutants, representing 100 genes were obtained from the *Francisella* two-allele transposon mutant library (Gallagher et al., 2007). Each transposon mutant was grown overnight in TSB with 0.2% L-cysteine (BD Biosciences). Subsequently, each mutant was diluted to an OD_{600} of 0.03 in 30% TSB containing 100 μg/mL of LL-37. Following overnight growth at 37°C with aeration, the OD_{600} was measured and used to calculate the percent growth compared to wild-type bacteria. Strains that grew to an OD_{600} of less than 50% than that of wild-type were deemed to have increased sensitivity.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical methods

Prism 5 Graphpad Software was used for statistical analyses. The significance of the bacterial and culture experiments (macrophage replication, qRT-PCR, killing assays) was determined using a two-tailed, unpaired, Student's t test, for data with normal distribution. Significance was determined using the Mann-Whitney test for the mouse infections, as not all data was normally distributed. Error bars represent standard deviation of the mean in all *in-vitro* data and geometry means in all *in-vivo* data. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$