Supporting Information

Biosynthesis of Undecaprenyl Phosphate-Galactosamine and Undecaprenyl Phosphate-Glucose in *Francisella novicida*

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Supporting Methods

Construction of *F. novicida* *flmF1* and *flmF2* Mutants- The construction of *F. novicida* gene knockout mutants was carried using methods described previously (1, 2). Genomic DNA was isolated from *F. novicida* U112 using the DNA Easy kit. The two *F. novicida* U112 homologues of *E. coli* *arnC* (3), designated *flmF1* and *flmF2*, were amplified by PCR with 2 kb of flanking *F. novicida* genomic DNA. The forward primers *FnflmF1* 5kb+ and *FnflmF2* 5kb+ (Supporting Table 1) were designed with a *SacII* restriction site and a homologous region to the coding strand of chromosomal DNA about 2 kb upstream of *F. novicida flmF1* and *flmF2*. The reverse PCR primers *FnflmF1* 5kb- and *FnflmF2* 5kb- (Supporting Table 1) were designed with a *XhoI* restriction site and a homologous region to the anti-coding strand of chromosomal DNA about 2 kb downstream of the *F. novicida* genes. The PCR was performed using *PfuTurbo* polymerase and *F. novicida* genomic DNA as the template. Amplification was carried out in a 50-µL reaction mixture containing 100 ng of template, 100 ng of primers, and 1 unit of polymerase. The reaction was started at 94 °C for 2 min, followed by 25 cycles of denaturation (1 min at 94 °C), annealing (1 min at 50 °C), and extension (5 min at 72 °C). After the 25th cycle, a 10-min extension time was used. The reaction product was analyzed on a 1% agarose gel, and the desired band was excised and gel-purified. The PCR product was then digested using *XhoI* and *SacII* and ligated into the expression vector pWSK29 (4), which had been similarly processed. The ligation mixture was electroporated into *E. coli* DY330 (5), and colonies were selected at 30 °C for those with the appropriate inserts on LB plates (6) containing 100 µg/mL ampicillin.
The pWSK29 plasmid with the 5-kb insert was isolated using the Qiagen Spin Miniprep kit, and the insert was confirmed by DNA sequencing. Next, each of the *F. novicida* flmF homologues, with their flanking inserts in pWSK29, were replaced with a kanamycin resistance gene. Tn903 from plasmid pWKS130 (4) was amplified by PCR using primers Kan+ and Kan- (Supporting Table 1). It was flanked on its 5’ and 3’ ends by 50 bp of the chromosomal DNA that is immediately upstream or downstream of *F. novicida* flmF1 and flmF2. The PCR was performed using *Pfu Turbo* polymerase. Amplification was carried out in a 50-μL reaction mixture containing 50 ng of template, 100 ng of primers, and 1 unit of *Pfu Turbo* polymerase. The reaction was started at 94 °C for 1 min, followed by 25 cycles of denaturation (45 s at 94 °C), annealing (45 s at 55 °C), and extension (60 s at 72 °C). The PCR product was purified and electroporated into *E. coli* DY330/pWSK29, containing either the flmF1 or flmF2 gene in the 5-kb insert described above, which had been grown at 30 °C until \(A_{600}\) reached 0.5 and then induced at 42 °C for 20 min. After electroporation, recombination occurred between the homologous sequences of the linear PCR product and the flmF1 or flmF2 gene on the pWSK29 hybrid plasmid at 30 °C, resulting in replacement of each flmF homologue with the kanamycin resistance gene. The desired plasmids were then isolated from kanamycin-resistant transformants, selected on LB agar containing 20 μg/mL kanamycin and 100 μg/mL ampicillin, and digested with *XhoI* and *SacII*. About 100 ng of each linear DNA containing the kan insert was gel-purified, and the replacement of *F. novicida* flmF1 and flmF2 with the kanamycin resistance gene on these plasmids was verified by DNA sequencing.
In order to replace each \textit{flmF} homologue present in the genome of \textit{F. novicida} with the kanamycin resistance gene, \textit{F. novicida} U112 cells were grown in 100 mL of Chamberlain's medium (7) to \( A_{600} \) of 0.5. The cells were harvested by centrifugation and resuspended in 5 mL of transformation buffer (8). A volume of 1 mL of the cell suspension was mixed with 100 ng of the linear DNA, prepared as described above, and shaken at 100 rpm at 37 °C for 30 min. Next, 5 mL of Chamberlain's medium was added, and the mixture was shaken at 37 °C for 2 h at 250 rpm. The cells were harvested and resuspended in 1 mL of TSB-C medium (3% trypticase soy broth and 0.1% cysteine). Approximately 100 \( \mu \)L of the cell suspension was spread onto a TSB-C plate containing 10 \( \mu \)g/mL kanamycin. Genomic DNA was isolated from a kanamycin-resistant transformant, and the replacement of each \textit{F. novicida flmF} gene with the kanamycin resistance gene was confirmed by DNA sequencing.

\textit{Cloning of the F. novicida flmF1 and flmF2 Genes.} The \textit{flmF1} and \textit{flmF2} genes of \textit{F. novicida} were amplified by PCR. The forward primers \textit{FnflmF1-clone+} and \textit{FnflmF2-clone+} (Supporting Table 1) were designed with a clamp region, an \textit{NcoI} restriction site, and a homologous region to the coding strand of chromosomal DNA of \textit{F. novicida flmF1} and \textit{flmF2}. The reverse PCR primers \textit{FnflmF1-clone-} and \textit{FnflmF2-clone-} (Supporting Table 1) were designed with a clamp region, an \textit{XhoI} restriction site, and a homologous region to the anti-coding strand of chromosomal DNA of the \textit{F. novicida} genes. The PCR was performed using \textit{Pfu} Turbo polymerase and \textit{F. novicida} genomic DNA as the template. Amplification was carried out in a 50-\( \mu \)L reaction mixture containing 100 ng of template, 100 ng of primers, and 1 unit of polymerase. The reaction was started at 94 °C for 2 min, followed by 25 cycles of denaturation (45 s at 94 °C),
annealing (45 s at 50 °C), and extension (2 min at 72 °C). After the 25th cycle, a 10 min extension time was used. The reaction product was analyzed on a 1% agarose gel, and the desired band was excised and gel-purified. The PCR product was then digested using XhoI and NcoI and ligated into the expression vector pET28b, which had been similarly digested and treated with Antarctic phosphatase (New England Biolabs, Ipswich, MA). The ligation mixture was transformed into XL1-Blue cells, and the kanamycin-resistant colonies were selected on LB plates with 20 µg/mL kanamycin. The plasmids pET28b-flmF1 or pET28b-flmF2 were isolated and transformed into E. coli NovaBlue (DE3) cells after the DNA sequence was confirmed.

**Preparation of Total Lipids and Purification of Free Lipid A from Wild-type and Mutant F. novicida Cells.** One liter of F. novicida U112 wild-type or mutant cells, inoculated from an overnight culture to $A_{600} = 0.02$, was grown at 37 °C in TSB-C medium until the $A_{600}$ reached 1.0. The cells were collected by centrifugation and washed with phosphate-buffered saline (9). The cell pellets were resuspended in 900 mL of a single-phase Bligh-Dyer mixture (10), consisting of chloroform/methanol/water (1:2:0.8, v/v/v), incubated at room temperature for 60 min, and centrifuged to remove insoluble debris. The supernatant (containing most of the glycerophospholipids and free lipid A) was converted to a two-phase Bligh-Dyer system by adding chloroform and water to generate a mixture consisting of chloroform/methanol/water (2:2:1.8, v/v/v). The two phases of Bligh-Dyer system were separated by centrifugation, and the lower phase was recovered and dried by rotary evaporation. Approximately 110 mg lipids were obtained. The dried lipids from the F. novicida, F. novicida flmF1::kan$^{R}$, and F. novicida flmF2::kan$^{R}$ strains were dissolved in chloroform/methanol/water (2:3:1, v/v/v). Each
sample was applied to a 4-mL DEAE-cellulose column in the acetate form equilibrated with the same solvent (11). The column was washed with 10 column volumes of chloroform/methanol/water (2:3:1, v/v/v). The various lipid components were then eluted stepwise with 5 column volumes each of chloroform/methanol/aqueous ammonium acetate (2:3:1, v/v/v), with aqueous ammonium acetate concentrations of 60, 120, 240 and 360 mM (11). Fractions equal to one column volume were collected, and 20 µL of each fraction were spotted onto a TLC plate to monitor the lipid A and phospholipid elution profiles. The plates were developed in the solvent of chloroform/methanol/pyridine/acetic acid/water (25:10:5:4:3, v/v/v/v/v), and the lipids were visualized by spraying the plates with 10% sulfuric acid in ethanol, followed by charring on a hot plate. The *F. novicida flmF1::kan* mutant lipid A species were found to emerge in the DEAE cellulose run-through and the 0-mM ammonium acetate wash fractions, while the *flmF2::kan* mutant lipid A species were found in the 60-mM and 120-mM ammonium acetate fractions, as judged by TLC and ESI/MS. The lipid A-containing fractions were combined and converted to two-phase Bligh-Dyer mixtures. The lower phases were recovered and dried by rotary evaporation. To purify the lipid A species further, preparative TLC was employed. Lipids obtained from the fractions of the DEAE column were dissolved in a small volume of chloroform/methanol (4:1,v/v) and applied along a line at the origin a TLC plate, which was developed with chloroform/methanol/pyridine/acetic acid/water (25:10:5:4:3, v/v/v/v/v). Immediately upon removing the plates from the tank while the plates were drying, the lipid A bands could be seen transiently as white zones. These bands were marked with a pencil and scraped off after the plates were dry. The silica chips were extracted with a single-phase
Bligh-Dyer mixture for 1 h at room temperature. The suspension was centrifuged, and the supernatant was passed through a small column fitted with a small glass wool plug. The flow-through was converted into a two-phase Bligh-Dyer system, the two phases were separated by centrifugation, and the lower phase was recovered and dried. About 3 mg of lipid A component A1 was recovered from the \textit{flmF1::kan} \textsuperscript{R} mutant. About 3 mg of component A3 and ~0.5 mg of component A4 were obtained from the \textit{flmF2::kan} \textsuperscript{R} mutant. The final lipid preparations were analyzed by TLC and ESI/MS. The TLC plate was developed using the solvent chloroform/methanol/pyridine/acetic acid/water (25:10:5:4:3, v/v/v/v/v). Lipids were detected by charring.

Purified A1 from the \textit{flmF1::Kan} \textsuperscript{R} mutant and purified A3 and A4 from the \textit{flmF2::Kan} \textsuperscript{R} mutant were re-analyzed by TLC and negative ion ESI/MS (Supporting Figs. 1A - 1D). For A1 (Supporting Fig. 1B), the peaks at \textit{m/z} 1665.166 and 1637.140 are interpreted as the [M - H]\textsuperscript{-} molecular ions of species differing by two methylene units. The additional ions at \textit{m/z} 1382.922 and 1354.890 are derived by loss of the O-linked 3-hydroxystearoyl group (282.2 amu) from the parent species. The negative ion ESI mass spectra for purified A3 and A4 (Supporting Figs. 1C and 1D) also show the expected pattern, when compared to the spectra of the total lipid extracts. The peaks at \textit{m/z} 1504.135 and 1476.104 for A3, and 1666.194 and 1638.160 for A4, are consistent with the predicted [M - H]\textsuperscript{-} molecular ions and the expected acyl chain length heterogeneity. The peaks in the spectrum of A3 are 162 amu smaller than the corresponding peaks from A4, showing that A4 contains an additional 162 amu substituent, presumably the glucose residue. The peaks in the spectrum of A3 are 161 amu smaller than the corresponding peaks of A1, showing that A3 lacks the galactosamine unit present in A1. Taken together,
the TLC migration (Supporting Fig. 1A), the DEAE cellulose elution behavior (not shown), and the ESI/MS analysis (Supporting Fig. 1B - 1D) strongly support the proposed structures (Fig. 1 of the manuscript).

**Enzymatic synthesis of Undecaprenyl Phosphate-Glucose and Undecaprenyl Phosphate-GalNAc.** The reaction mixture for the synthesis of undecaprenyl phosphate-glucose or undecaprenyl phosphate-GalNAc contained 0.02 mg/mL membranes from *E. coli* NovaBlue(DE3) cells expressing FlmF1 or FlmF2, 100 µM sugar nucleotides (UDP-glucose or UDP-GalNAc), 50 µM undecaprenyl phosphate, 0.2% Triton X-100, and 50 mM HEPES, pH 7.5. After 24 h at 30°C, the mixture was converted to a two-phase Bligh-Dyer system and centrifuged; the lower phase was collected and dried down by rotary evaporation. Next, 5 mL of a chloroform, methanol and water mixture (2:3:1, v/v/v) was added to dissolve the lipids, and the sample was applied to a 3-mL DEAE–cellulose column (acetate form) prepared in chloroform/methanol/water (2:3:1 v/v/v). The column was then washed with 20 mL of the same solvent, and eluted stepwise with 20 mL of the same solvent containing ammonium acetate concentrations of 60, 120, or 240 mM in the aqueous component. Fractions equal to six column volumes were collected, and were converted to two-phase Bligh-Dyer mixtures. The fractions were analyzed by TLC and mass spectrometry. The products of both FlmF1 and FlmF2 were present in the fractions containing 60 mM ammonium acetate as the aqueous component. The crude products obtained from the DEAE columns were further purified using preparative silica Gel 60 TLC plates (10 × 20 cm), which were developed in the solvent chloroform/methanol/water/ammonium hydroxide (65:25:3.6:0.4, v/v/v/v). After locating the desired bands, the plates were scraped and the silica chips were extracted with a
single-phase Bligh-Dyer mixture (10) at room temperature for 1 h with stirring. The suspension was centrifuged and converted to a two-phase Bligh-Dyer mixture (10). The two phases were separated by centrifugation, and the lower phase was recovered and dried using rotary evaporation. The purity of the products was verified using TLC and mass spectrometry.
**Supporting Table 1.** Primers used for deletion and cloning of the *F. novicida* U112 *flmF1* and *flmF2* genes.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequences</th>
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<tr>
<td><em>FnflmF1-5kb+</em></td>
<td>5'-gtattggcaaaaaccgcggtaaaaactctaattttctgttttage-3'</td>
</tr>
<tr>
<td><em>FnflmF1-5kb-</em></td>
<td>5'- gccaatatattttctaatctcgagtaattattatgata atc -3'</td>
</tr>
<tr>
<td><em>FnflmF1-5kb+</em></td>
<td>5'-gtcaatggctcgccgggcgctgatacc-3'</td>
</tr>
<tr>
<td><em>FnflmF2-5kb</em></td>
<td>5'-cttgggttacctacgctataaatgg-3'</td>
</tr>
<tr>
<td><em>FnflmF1-Kan+</em></td>
<td>5'-tattgatagattnaaaataactcaataaatgattataaatcttatagccatattaac-3'</td>
</tr>
<tr>
<td><em>FnflmF1-Kan-</em></td>
<td>5'-atctatgtgttatttacttgagaaagtattagattgagaagcttagagatttagagatttaaaaactcatcg-3'</td>
</tr>
<tr>
<td><em>FnflmF1-clone+</em></td>
<td>5'-cgccgcccatgatatataaagataagctcatgtaataataatccaaacgg-3'</td>
</tr>
<tr>
<td><em>FnflmF1-clone-</em></td>
<td>5'-cgccggtctggtgatgacaagtagaaatgttgataacc-3'</td>
</tr>
<tr>
<td><em>FnflmF2-clone+</em></td>
<td>5'-cgccgctctctcgcggtgcctgacccagaagttattagattagaaac-3'</td>
</tr>
<tr>
<td><em>FnflmF2-clone-</em></td>
<td>5'-cgccgctctctcgcggtgtgacccagaagttattagataacc-3'</td>
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Supporting Figure 1

TLC analysis and negative ion ESI mass spectra of purified species A1, A3 and A4.

Panel A. TLC analysis of lipid A species A1, purified from the \textit{flmF1::Kan}^R mutant (Lane 1), and of A3 (Lane 2) and A4 (Lane 3), purified from the \textit{flmF2::Kan}^R mutant.

Panels B-D. Negative ion ESI mass spectra acquired by LC/MS of purified compounds A1, A3 and A4, respectively.
Supporting Figure 2.

TLC, LC-ESI/MS and LC-ESI/MS/MS in the negative ion mode of undecaprenyl phosphate-glucose and undecaprenyl phosphate-GalNAc. Undecaprenyl phosphate-glucose and undecaprenyl phosphate-GalNAc were synthesized in 1 mL reaction mixtures containing 0.02 mg/mL of recombinant FlmF1 or FlmF2 membranes, with either 100 µM UDP-glucose or 100 µM UDP-GalNAc respectively, 25 µM undecaprenyl phosphate, 0.1% Triton X-100 and 50 mM Hepes (pH 7.5). The mixture was incubated at 30 °C for 24 hours. The purification of the two products is described in the Supporting Materials and Methods section. Panel A. The TLC plate was developed using the solvent chloroform/methanol/water/ammonium hydroxide (65:25:3.6:0.4, v/v/v/v), and the purified compounds were detected by spraying the plate with 10% sulfuric acids in ethanol, followed by charring. Lane 1, UDP-glucose substrate; lanes 2 and 5, undecaprenyl phosphate substrate; lane 3, purified undecaprenyl phosphate-GalNAc; lane 4, purified undecaprenyl phosphate-glucose; and lane 6, UDP-GalNAc substrate. Panels B and C. Negative ion LC-ESI/MS and LC-ESI/MS/MS respectively of purified undecaprenyl phosphate-glucose. Panels D and E. Negative ion LC-ESI/MS and LC-ESI/MS/MS respectively of purified undecaprenyl phosphate-GalNAc. The ion near m/z 845.65 in C and E arises from the undecaprenyl phosphate fragment.
Supporting References


