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

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ABSTRACT: Lipid A of *Francisella tularensis* subsp. *novicida* contains a galactosamine (GalN) residue linked to its 1-phosphate group. As shown in the preceding paper, this GalN unit is transferred to lipid A from the precursor undecaprenyl phosphate-β-D-GalN. A small portion of the free lipid A of *Francisella novicida* is further modified with a glucose residue at position-6′. We now demonstrate that the two *F. novicida* homologues of *Escherichia coli* ArnC, designated FlmF1 and FlmF2, are essential for lipid A modification with glucose and GalN, respectively. Recombinant FlmF1 expressed in *E. coli* selectively condenses undecaprenyl phosphate and UDP-glucose *in vitro* to form undecaprenyl phosphate-glucose. Recombinant FlmF2 selectively catalyzes the condensation of undecaprenyl phosphate and UDP-*N*-acetylgalactosamine to generate undecaprenyl phosphate-*N*-acetylgalactosamine. On the basis of an analysis of the lipid A composition of *flmF1* and *flmF2* mutants of *F. novicida*, we conclude that FlmF1 generates the donor substrate for the modification of *F. novicida* free lipid A with glucose, whereas FlmF2 generates the immediate precursor of the GalN donor substrate, undecaprenyl phosphate-β-D-GalN. A novel deacetylase, present in membranes of *F. novicida*, removes the acetyl group from undecaprenyl phosphate-*N*-acetylgalactosamine to yield undecaprenyl phosphate-β-D-GalN. This deacetylase may have an analogous function to the deformylase that generates undecaprenyl phosphate-4-amino-4-deoxy-α-L-arabinose from undecaprenyl phosphate-4-deoxy-4-formylamino-α-L-arabinose in polymyxin-resistant strains of *E. coli* and *Salmonella typhimurium*.

Fransicella tularensis is a Gram-negative, intracellular pathogen that causes tularemia in a variety of mammalian species, including humans (*1, 2*). Because inhalation of a small dose of *F. tularensis* subsp. *tularensis* can cause fatal tularemia in humans, *F. tularensis* is considered a potential biological weapon (*1, 3*). An environmental isolate, *F. tularensis* subsp. *novicida* U112 (*Fransicella novicida*), is virulent for mice but not humans (*4*), is easy to grow in the laboratory, and provides a practical model system with which to study the biochemistry of *F. tularensis* (*5*−*8*). The genome sequences of several strains of *Francisella* have recently been determined (*9*).

As shown in Figure 1 of the preceding paper (*10*), the lipid A moiety of *Escherichia coli* lipopolysaccharide (LPS)[1] is a hexaacylated disaccharide of glucosamine, which is phosphorylated at the 1- and 4′-positions (*11*−*13*). *F. novicida* lipid A is tetraacylated, and it lacks both the 4′-phosphate residue and the 3′-hydroxyacyl chain (*6, 7, 14, 15*). Over 90% of *F. novicida* lipid A is not covalently bound to core and O-antigen sugars but instead is present in a "free" form that consists of two easily separable species in a ratio of about 7:1 (*7*). The major species is glycosylated at the 1-position with a galactosamine (GalN) residue (Figure 1, compound A1), whereas the minor species is modified with an additional glucose unit at the 6′-position (Figure 1, compound A2) (*7*). Only a small portion of the *F. novicida* lipid A is covalently linked to LPS (*7*); this component is modified with a mannose residue at position 4′ (not shown) (*8*). In addition, *F. novicida* lipid A is characterized by considerable microheterogeneity of its relatively long acyl chains (Figure 1) (*15*). Because of these structural differences, *F. novicida* LPS triggers much less cytokine production than *E. coli* LPS (*16, 17*).

The significance and origin of the free lipid A in *F. novicida* are unclear (*7*). The *F. novicida* genome encodes all of the enzymes needed to make pentaacylated Kdo_2-lipid A, as well as several core glycosyl transferases (*9, 13, 18, 19*). One possible mechanism for free lipid A formation is incomplete Kdo biosynthesis and transfer (*18*). Another possibility is trimming of core sugars from nascent LPS. Known enzymes that modify *F. novicida* lipid A account for some of the structural differences noted above. Two inner membrane phosphatases, LpxE and LpxF, remove the lipid A 1- and 4′-phosphate groups, respectively (*5, 20*). LpxE is Kdo-dependent and therefore does not dephosphorylate free lipid A, whereas LpxF efficiently dephosphorylates both free lipid A and LPS-bound lipid A (*5, 20*). A 3′-*O*-deacylase is

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[1] Abbreviations: L-Ara4N, 4-amino-4-deoxy-L-arabinose; L-Ara4FN, 4-deoxy-4-formylamino-L-arabinose; BCA, bicinchoninic acid; ESI/MS, electrospray ionization/mass spectrometry; *F. novicida*, *Francisella tularensis* subsp. *novicida*; GalN, galactosamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl 1-thio-β-D-galactopyranoside; LPS, lipopolysaccharide; PCR, polymerase chain reaction; TLC, thin-layer chromatography; UDP-GalNAc, UDP-*N*-acetylgalactosamine.

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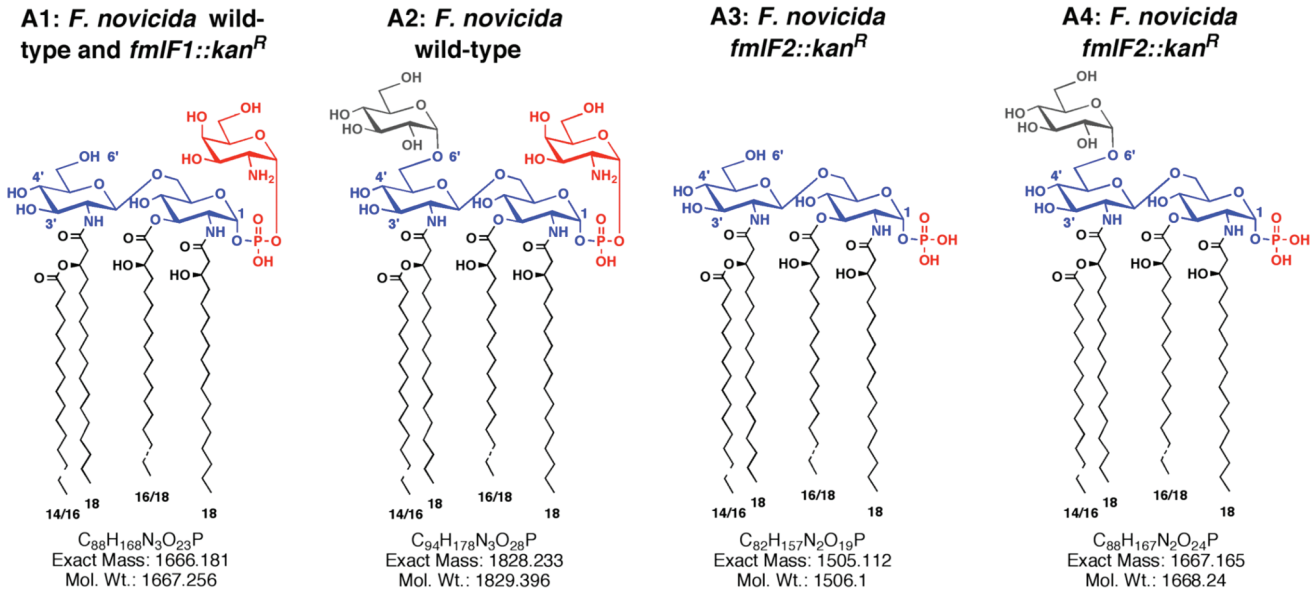
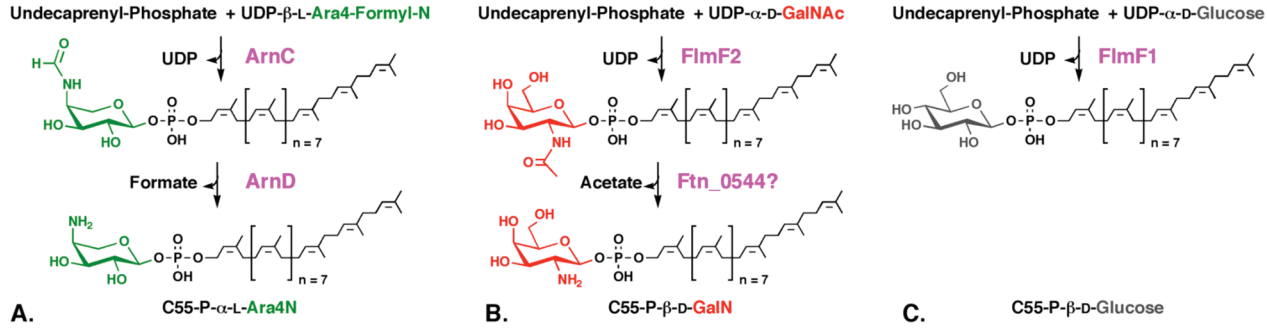


FIGURE 1: Proposed structures of the free lipid A species present in wild-type and *flmF1* and *flmF2* mutant strains of *F. novicida* U112. Species containing the glucose unit are about 10 times less abundant in wild-type (*7*) or *flmF2* mutant cells. They appear to be lacking altogether in mutants defective in FlmK, suggesting that FlmK may have a role in glucose addition to free lipid A (*7*).

Scheme 1: Function of *E. coli* ArnC Compared to *F. novicida* FlmF2 and FlmF1



present that requires prior removal of the 4′-phosphate group for activity (*6*) (C. M. Reynolds and C. R. H. Raetz, unpublished). *F. novicida* also contains a homologue of *E. coli* ArnT (*7, 21, 22*), which is designated FlmK in this system (*8*). ArnT adds the 4-amino-4-deoxy-L-arabinose (L-Ara4N) moiety to lipid A in polymyxin-resistant strains of *E. coli* (*21*−*23*). As shown in the preceding paper, *F. novicida* FlmK uses undecaprenyl phosphate-GalN as the donor substrate for transferring the GalN unit to lipid A (*7, 10*).

The *F. novicida* GalN modification pathway shares several additional features with the L-Ara4N modification system of *E. coli*. In the L-Ara4N pathway, ArnC catalyzes the condensation of undecaprenyl phosphate and UDP-4-deoxy-4-formylamino-L-arabinose (L-Ara4FN) to generate undecaprenyl phosphate-α-L-Ara4FN (Scheme 1A) (*13, 23*). This material is rapidly deformylated by ArnD to produce undecaprenyl phosphate-α-L-Ara4N (Scheme 1A) (*13*), which is transported to the outer surface of the inner membrane by the ArnE/ArnF proteins (*24*), where ArnT transfers its L-Ara4N unit to lipid A (*13*). In *F. novicida*, there are two homologues of *E. coli* ArnC (*13*), designated FlmF1 and FlmF2 (Scheme 1B,C) (*8*). We now demonstrate that recombinant FlmF1 expressed in *E. coli* selectively condenses undecaprenyl phosphate and UDP-glucose *in vitro* to form undecaprenyl phosphate-glucose, whereas FlmF2 condenses undecaprenyl phosphate and UDP-*N*-acetylgalactosamine (UDP-GlaNAc) to generate undecaprenyl phosphate-GalNAc (Scheme 1B,C). We also demonstrate that a specific deacetylase, possibly encoded by the Ftn_0544 gene (*9*), is present in *F. novicida*, which converts undecaprenyl phosphate-GalNAc to undecaprenyl phosphate-GalN in a reaction that is analogous to the deformylation of undecaprenyl phosphate-α-L-Ara4FN in *E. coli* (Scheme 1B) (*13*).

EXPERIMENTAL PROCEDURES

Materials and Reagents. F. novicida strain U112 (*9*) was obtained from Dr. F. Nano, University of Victoria, British Columbia, Canada. Trypticase soy broth, yeast extract, and tryptone were purchased from Difco (Detroit, MI). The Easy DNA kit and isopropyl 1-thio-β-D-galactopyranoside (IPTG) were purchased from Invitrogen (Carlsbad, CA). The spin miniprep and gel extraction kits were from Qiagen (Valencia, CA). Shrimp alkaline phosphatase was purchased from the USB (Cleveland, OH). Bicinchoninic (BCA) protein assay reagents (*25*) were from Thermo Fisher Scientific (Waltham, MA). All restriction enzymes, Antarctic phosphatase, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). *Pfu Turbo* DNA polymerase was purchased from Stratagene (La Jolla, CA). Oligonucleotide primers were custom-synthesized by MWG Biotech (Huntsville, AL). DNA sequencing was performed by the DNA Sequencing Facility of Duke University.

UDP-glucose and UDP-GalNAc were purchased from Sigma/Aldrich (St. Louis, MO). UDP-[U-^{14}C]glucose and UDP-GalN[^{14}C]Ac were purchased from American Radiolabeled Chemicals (St. Louis, MO). GDP-[U-^{14}C]mannose was from PerkinElmer (Waltham, MA). UDP-GalN was synthesized according to a published procedure (*26*). Undecaprenyl phosphate was purchased from the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland.

Strain Construction and Isolation of Free Lipid A. The construction of the *F. novicida flmF1* and *flmF2* gene knockout mutants, the expression cloning of the *flmF* genes in *E. coli*, and the purification of the free lipid A from each of the *F. novicida* mutants are described fully in the Supporting Information section.

Membrane Preparation. Wild-type and mutant *F. novicida* U112 cultures (100 mL each) were grown at 37 °C in 3% trypticase soy broth supplemented with 0.1% cysteine and 20 μg/mL kanamycin and were harvested by centrifugation at 4 °C when A_{600} = 1.0. *E. coli* NovaBlue(DE3) strains (100 mL cultures), harboring either pET28b (Novagen/EMD Chemicals Inc., Gibbstown, NJ), pET28b-*flmF1*, or pET28b-*flmF2*, were grown at 37 °C in LB broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) with 20 μg/mL kanamycin and were induced with 1 mM IPTG when A_{600} = 0.8. The cells were harvested by centrifugation at 4 °C when A_{600} = 2.0. All subsequent steps were carried out at 4 °C or on ice. The cell pellets were washed once with 50 mM HEPES (pH 7.5) and resuspended in a final volume of 10 mL of the same buffer. The cells were disrupted by one passage through a French pressure cell at 18000 psi. Unbroken cells and large debris were removed by centrifugation at 10000*g* for 20 min. The membranes were collected by centrifugation at 100000*g* for 1 h, washed once by suspension in 10 mL of 50 mM HEPES, pH 7.5, centrifuged a second time, and then resuspended in the same buffer at a protein concentration of about 5 mg/mL, as determined by the BCA assay with bovine serum albumin as the standard (*25*).

In Vitro FlmF Assays with Radioactive Substrates. To maximize radioactive product formation and to facilitate the detection and further analysis of lipid products, the following incubation conditions were employed in the initial experiments. The 10 μL reaction mixtures contained 0.5 mg/mL membrane protein from *E. coli* NovaBlue(DE3) cells, harboring either pET28b, pET28b-*flmF1*, or pET28b-*flmF2*; additional assay components included 10 μM UDP-[U-^{14}C]glucose (66000 cpm) or 10 μM UDP-GalN[^{14}C]Ac (11000 cpm), 50 μM undecaprenyl phosphate, 0.1% Triton X-100, and 50 mM HEPES (pH 7.5). The reactions were incubated for 60 min at 30 °C. A 1 μL sample of the reaction mixture was then spotted onto a silica gel 60 TLC plate, which was developed in the solvent chloroform/methanol/water/ammonium hydroxide (65:25:3.6:0.4 v/v/v/v). The plate was dried, exposed to a phosphorimager screen (Molecular Dynamics Inc., Sunnyvale, CA), and analyzed with a Storm 840 phosphorimager (GE Healthcare, Chalfont St. Giles, Buckinghamshire, U.K.) to determine the percent of substrate that was converted to product.

To quantify the specific activities of membranes from *E. coli* NovaBlue(DE3) cells, harboring either pET28b, pET28b-*flmF1*, or pET28b-*flmF2*, 0.02 mg/mL membrane protein was used in a 10 μL reaction mixture. For FlmF1, the other components of the assay system included 200 μM UDP-[U-^{14}C]glucose (3300 cpm/μL), 200 μM undecaprenyl phosphate, 0.1% Triton X-100, and 50 mM HEPES (pH 7.5). For the FlmF2 assay system, 200 μM UDP-GalN[^{14}C]Ac (1100 cpm/μL) was used in place of UDP-glucose along with the other components listed above. For quantitative assays of membranes from wild-type *F. novicida*, the *flmF1* null mutant, or the *flmF2* null mutant, 2 mM $MnCl_2$ was also included in the assay system, along with 0.02 mg/mL membrane protein. The 10 μL reaction mixtures were incubated at 30 °C. A 1 μL sample of the reaction mixture was spotted onto a silica gel TLC plate at different times. The plate was then developed in the solvent chloroform/methanol/water/ammonium hydroxide (65:25:3.6:0.4 v/v/v/v), dried, and subjected to phosphorimager analysis.

Preparation of the FlmF1 and FlmF2 Products Generated in Vitro for LC-ESI/MS Analysis. Typically, the 50 μL reaction mixtures contained 0.5 mg/mL membrane protein from *E. coli* NovaBlue(DE3) cells, harboring either pET28b, pET28b-*flmF1*, or pET28b-*flmF2*. Other components of the assay system included either 200 μM UDP-glucose or 200 μM UDP-GalNAc, 200 μM undecaprenyl phosphate, 0.1% Triton X-100, and 50 mM HEPES, pH 7.5. The reaction mixtures were incubated for 60 min at 30 °C. The reaction was stopped by the addition of appropriate amounts of chloroform and methanol to make a two-phase Bligh−Dyer system (*27*), which was centrifuged to separate the phases. The lower phase was dried under a stream of nitrogen and analyzed using LC-ESI/MS.

In Vitro Detection of an Undecaprenyl Phosphate-GalNAc Deacetylase by LC-ESI/MS. The 200 μL reaction mixture included 0.5 mg/mL wild-type *F. novicida* U112 membranes, 4 μM undecaprenyl phosphate-GalNAc, prepared as described in the Supporting Information, 2 mM $MnCl_2$, 150 mM KCl, 0.5 mg/mL *E. coli* phospholipids, 1.0 mg/mL BSA, 0.1% Triton X-100, and 50 mM HEPES, pH 7.5. The reaction mixture was incubated at 30 °C. A 50 μL sample was removed at 0 and 5 h and converted to a two-phase Bligh−Dyer system by the addition of appropriate amounts of chloroform and methanol (*27*). After centrifugation, the lower phase was recovered and dried under nitrogen and analyzed using LC-ESI/MS.

Electrospray Ionization/Mass Spectrometry (ESI/MS) in the Negative Mode. All ESI/MS and ESI/MS/MS spectra were acquired on a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems, Foster City, CA) equipped with an ESI source. Lipid samples were dissolved in ~200 μL chloroform/methanol (2:1 v/v) for direct infusion ESI/MS or in DMSO/methanol (1:1 v/v) for LC-ESI/MS analysis. The samples were subjected to ESI/MS in the negative ion mode (*28*). Nitrogen was used as the collision gas for MS/MS experiments. Data acquisition and analysis were performed using the instrument's Analyst QS software.

Liquid Chromatography/Mass Spectrometry. LC-ESI/MS of various 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 (see above). Reverse-phase LC (*28*) was performed at a flow rate of 200 μL/min with a linear gradient as follows: 100% mobile phase A was held isocratically for 2 min and then

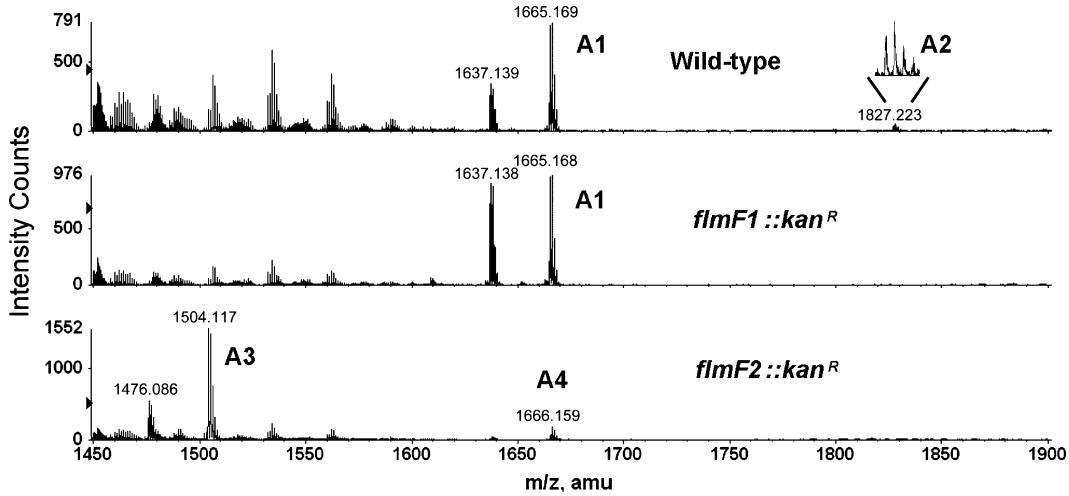


FIGURE 2: Negative ion ESI/MS of the free lipid A species in total lipids of wild-type *F. novicida* and the *flmF* insertion mutants. The proposed structures of the free lipid A species A1, A2, A3, and A4 are shown in Figure 1. The observed peaks are within experimental error of the expected $[M - H]^-$ ions predicted from these structures. The microheterogeneity of the acyl chains (*15*) accounts for the additional peaks that are 28 amu smaller than the major ones.

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 μm, 2.1 $\times$ 50 mm) was obtained from Agilent (Palo Alto, CA). The postcolumn splitter diverted ~10% of the LC flow into the ESI source of the mass spectrometer.

RESULTS

Free Lipid A Species in F. novicida FlmF1 and FlmF2 Null Mutants. In order to verify the proposed functions of the *F. novicida flmF1* and *flmF2* genes (Scheme 1), each one was replaced with a kanamycin resistance cassette. The *F. novicida* U112 wild type and the *flmF1::Kan*^{R} and *flmF2::Kan*^{R} mutants were grown to late log phase. The total lipids were extracted and analyzed directly by ESI/MS. Similar to previous results (*7*), we found that the *F. novicida* U112 phospholipids consist mainly of phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine (not shown), along with two free lipid A species, designated A1 and A2, in a ratio of about 7:1 (Figures 1 and 2). Free lipid A comprises ~15% of the total *F. novicida* phospholipid fraction (*7*). The 1-phosphate groups of both A1 and A2 are modified with an α-linked D-GalN residue, whereas an additional α-linked glucose moiety is attached to the 6′-position of A2 (Figure 1) (*7*).

In the lipids of wild-type *F. novicida*, peaks corresponding to lipid A1 and A2 are readily apparent in the ESI/MS spectrum (Figure 2, upper panel). The calculated exact masses of lipid A1 and A2 are 1666.181 and 1828.233, respectively (Figure 1). Figure 2 (upper panel) shows two peaks at *m/z* 1665.169 and 1827.223, which are interpreted as the $[M - H]^-$ molecular ions of the major monoisotopic peaks of A1 and A2 (Figure 1). The peak at *m/z* 1637.139 is interpreted as an acyl chain variant of A1 that is two methylene units smaller. For the *flmF1::Kan*^{R} and *flmF2::Kan*^{R} mutants, the phospholipid compositions match that of the wild-type strain (not shown), whereas the lipid A species differ (Figure 2, middle and lower panels). In the *flmF1::Kan*^{R} mutant, only the peaks corresponding to A1 (*m/z* 1665.168 and 1637.138), but not of A2 (*m/z* 1827.223), are detected by ESI/MS. This result indicates that the free lipid A is not modified with a glucose residue at the 6′-position when the *flmF1* gene is inactivated. In the *flmF2::Kan*^{R} mutant, neither A1 nor A2 is present; instead, two new species at *m/z* 1504.117 (A3) and 1666.159 (A4) are observed in the ESI/MS spectrum (Figure 2, lower panel). The 162 amu difference between these two species is consistent with the presence of a hexose unit in the larger molecule, most likely a glucose residue, given the composition of wild-type free lipid A (Figure 1) (*7*). Moreover, there is a 161 amu difference between A3 (*m/z* 1504.117) and wild-type A1 (*m/z* 1665.169) and between A4 (*m/z* 1666.159) and wild-type A2 (*m/z* 1827.223), indicative of the loss of the GalN unit in the free lipid A of the *flmF2* mutant. The proposed structures of A3 and A4 are shown in Figure 1. Our findings are consistent with those of Ernst and co-workers (*8*), who demonstrated the loss of the GalN unit in the LPS-bound lipid A of an *flmF2* mutant; however, the composition of free lipid A was not investigated (*8*).

Purification of Free Lipid A Species from F. novicida FlmF1 and FlmF2 Null Mutants. The altered free lipid A species present in the *flmF1::Kan*^{R} and the *flmF2::Kan*^{R} mutants (Figure 2) were purified by DEAE-cellulose column chromatography and preparative TLC (see Supporting Information) (*29, 30*). The free lipid A of the *flmF1::Kan*^{R} mutant (like the wild type) eluted together with phosphatidylethanolamine and phosphatidylcholine in the run-through of the DEAE-cellulose column, indicating that it has no net negative charge (data not shown), consistent with its proposed structure (Figure 1). The *flmF2::Kan*^{R} species A3 and A4 (Figure 1) bound to the DEAE-cellulose column and eluted with chloroform/methanol/60 mM aqueous ammonium acetate (2:3:1 v/v/v), confirming that the free lipid A species from the FlmF2 mutant have a net negative charge (Figure 1). After preparative TLC, about 3 mg of A1, 3 mg of A3, and ~0.5 mg of A4 were recovered from 1 L cultures. TLC analyses and mass spectra of these compounds are shown in Supporting Information Figure 1.

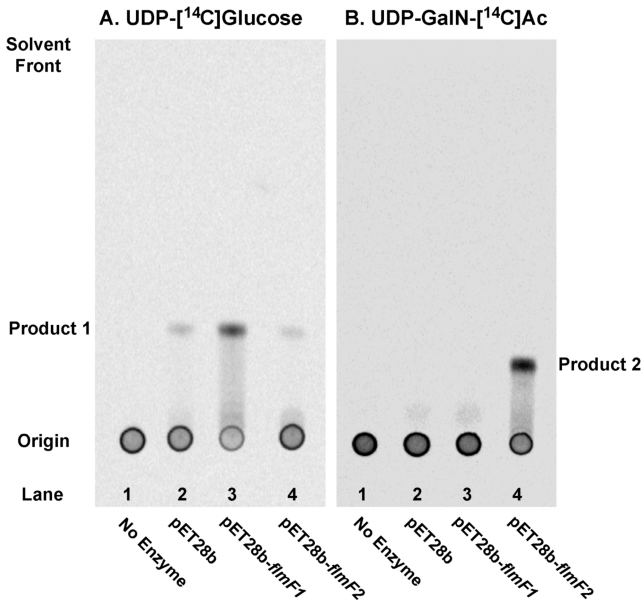


FIGURE 3: A radiochemical *in vitro* assay for recombinant FlmF1 and FlmF2. This thin-layer chromatography shows the lipid products generated in 60 min from undecaprenyl phosphate and UDP-[U-^{14}C]glucose (panel A) or UDP-GalN[^{14}C]Ac (panel B) by 0.5 mg/mL membranes from the three indicated *E. coli* NovaBlue(DE3) strains. Lane 1, no enzyme control; lane 2, pET28b vector control; lane 3, pET28b-*flmF1*; lane 4, pET28b-*flmF2*.

Heterologous Expression and in Vitro Activity of F. novicida FlmF1 and FlmF2. In order to test for the predicted glycosyl transferase activities of *F. novicida* FlmF1 and FlmF2 (Scheme 1), the two genes were cloned from *F. novicida* U112 DNA, ligated into pET28b, and expressed in *E. coli* NovaBlue(DE3). Both FlmF1 and FlmF2 were expressed at high levels without formation of inclusion bodies, as judged by SDS−PAGE analysis of cell-free extracts and membrane preparations (data not shown).

The ability of recombinant *F. novicida* FlmF1 and FlmF2 to catalyze the condensation of undecaprenyl phosphate and UDP-glucose or UDP-GalNAc was initially tested with a qualitative radiochemical assay, in conjunction with TLC and phosphorimager analysis. UDP-[U-^{14}C]glucose (Figure 3A) or UDP-GalN[^{14}C]Ac (Figure 3B) was used at 10 μM as the donor substrate in the presence of 50 μM undecaprenyl phosphate. The reaction mixtures were incubated for 60 min at 30 °C in the presence of 0.5 mg/mL membrane protein, as described in Experimental Procedures. Membranes of *E. coli* NovaBlue(DE3)/pET28b were used as the vector control. The spot seen at the origin in Figure 3A is the unreacted substrate, UDP-[U-^{14}C]-glucose. For the control without enzyme (Figure 3A, lane 1), there is no formation of a rapidly migrating lipid band. For the reactions containing added membrane proteins, a new spot (Figure 3A, product 1) is observed, which migrates with an R_f suggestive of undecaprenyl phosphate-glucose. The yields of this material with the vector control and the FlmF2 membranes (Figure 3A, lanes 2 and 4, respectively) are ~5%, whereas the yield with the FlmF1 membranes is ~50% under these conditions (Figure 3A, lane 3), consistent with Scheme 1C. The background seen with the vector control and the FlmF2 membranes likely represents the activity of a chromosomally encoded undecaprenyl phosphate-glucose synthase of *E. coli*.

The corresponding TLC assay with radiolabeled UDP-GalN[^{14}C]Ac as the donor substrate (Figure 3B) gives results consistent with Scheme 1B. The spot at the origin represents unreacted UDP-GalN[^{14}C]Ac. No lipid product is observed for the reaction mixtures lacking enzyme (Figure 3B, lane 1), the vector control membranes (Figure 3B, lane 2), or the FlmF1 membranes (Figure 3B, lane 3). However, a new radioactive lipid (Figure 3, product 2) is efficiently formed in the reaction mixture containing the FlmF2 membranes (Figure 3B, lane 4). This material has an R_f consistent with undecaprenyl phosphate-GalNAc and related molecules (*22*). The absence of activity in the vector control and the FlmF1 membranes suggests that *E. coli* does not contain an endogenous enzyme of this kind and that FlmF2 and FmlF1 are highly selective for their respective sugar nucleotides (Scheme 1).

Specific Activity of Recombinant FlmF1 and FlmF2. To quantify the specific activities of recombinant FlmF1 and FlmF2 in *E. coli* membranes, each preparation at 0.02 mg/mL was assayed in a modified system containing 200 μM undecaprenyl phosphate and 200 μM UDP-glucose or UDP-GlaNAc, under conditions where product formation was linear with time and protein concentration (Figure 4). The specific activity of the FlmF1 membranes with UDP-glucose as the donor substrate (8.0 nmol min^{-1} (mg of protein)$^{-1}$) was ~100-fold higher than that of the vector control or the FlmF2 membranes (Figure 4A and Table 1). Conversely, the specific activity of the FlmF2 membranes with UDP-GalNAc as the donor substrate (5.5 nmol min^{-1} mg^{-1}) was at least ~200-fold greater than that of the FlmF1 or the vector control membranes (Figure 4B and Table 1), approaching the limits of detection.

FlmF1 and FlmF2 Activity in Wild-Type and Mutant F. novicida Membranes. To confirm the results obtained with recombinant FlmF1 and FlmF2 expressed in *E. coli*, membranes of *F. novicida* and of the *F. novicida flmF1* and *flmF2* knockout mutants were assayed (Figure 4 and Table 1). Wild-type *F. novicida* membranes and *flmF2* mutant membranes catalyzed the condensation of undecaprenyl phosphate and UDP-[U-^{14}C]glucose with similar specific activities (3.1 and 2.9 nmol min^{-1} mg^{-1}, respectively), whereas the *flmF1* mutant membranes were inactive (Figure 4C and Table 1). Conversely, the wild-type *F. novicida* and *flmF1* mutant membranes catalyzed the condensation of undecaprenyl phosphate and UDP-GalN[^{14}C]Ac with similar specific activities (1.6 and 1.4 nmol min^{-1} mg^{-1}, respectively), whereas the *flmF2* mutant membranes were inactive (Figure 4D and Table 1). The data further support the idea that *F. novicida* FlmF2 produces undecaprenyl phosphate-GalNAc, which may be the precursor of undecaprenyl phosphate-GalN (Scheme 1), the sugar donor that leads to GalN modification of *F. novicida* lipid A. By analogy to *E. coli* (Scheme 1) (*13*), we would therefore expect that *F. novicida* cells contain a novel deacetylase that removes the acetyl group of undecaprenyl phosphate-GalNAc to form undecaprenyl phosphate-GalN (Scheme 1).

Based on their sequences (*8, 9*), both FlmF1 and FlmF2, which show significant homology to *E. coli* ArnC, are predicted to be integral membrane proteins with at least two transmembrane helices near their C-termini (*31*). Assays of *E. coli* cytosols from cells expressing recombinant FlmF1 or FlmF2 confirmed this prediction. The specific activity of the FlmF1 membranes with UDP-[U-^{14}C]glucose as the donor (Table 1) was ~100 fold higher than that of its

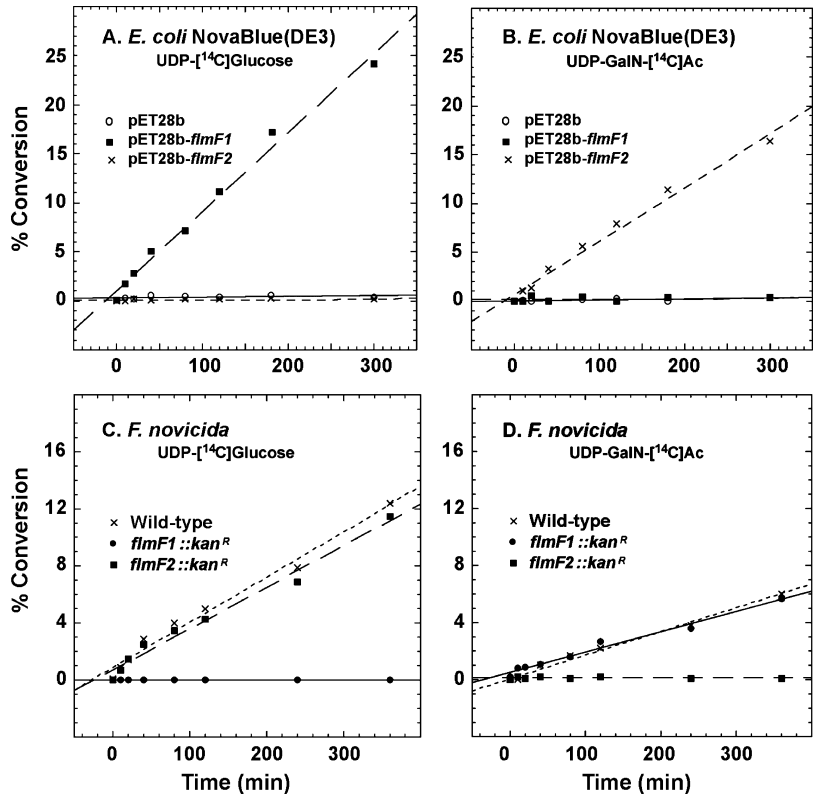


FIGURE 4: Quantification of FlmF1 and FlmF2 specific activities in selected strains. The upper panels show the time courses of the condensation reactions of undecaprenyl phosphate and UDP-[U-^{14}C]glucose (panel A) or undecaprenyl phosphate and UDP-GalN[^{14}C]Ac (panel B), catalyzed by membranes of *E. coli* NovaBlue(DE3), harboring either the pET28b vector, pET28b-*flmF1*, or pET28b-*flmF2*. The lower panels (C and D) show the corresponding assays for membranes of wild-type *F. novicida* U112, *F. novicida flmF1::Kan*^{R}, or *F. novicida flmF2::Kan*^{R}. The conditions for these quantitative assays are described in Experimental Procedures.

Table 1: Specific Activities of Recombinant FlmF1 and FlmF2 Expressed in *E. coli* or in the Corresponding *F. novicida* Mutants[a]

source of membranes	specific activity (nmol min^{-1} mg^{-1})	
	UDP-[U-^{14}C]glucose	UDP-GalN[^{14}C]Ac
E. coli NovaBlue/pET28b	0.08	<0.025
E. coli NovaBlue/pET28b-*flmF1*	8.0	<0.02
E. coli NovaBlue/pET28b-*flmF2*	0.06	5.5
F. novicida U112 wild type	3.1	1.6
F. novicida flmF1:kan^R	<0.01	1.4
F. novicida flmF2:kan^R	2.9	<0.01

[a] The specific activities of the condensation reactions of undecaprenyl phosphate and UDP-[U-^{14}C]glucose or UDP-GalN[^{14}C]Ac, catalyzed by the indicated membranes, were determined as described in Experimental Procedures.

corresponding cytosol (0.06 nmol min^{-1} mg^{-1}), whereas the specific activity of the FlmF2 cytosol with UDP-GalN[^{14}C]Ac as the donor (0.65 nmol min^{-1} mg^{-1}) was ~9-fold lower than that of the corresponding membranes (Table 1).

ESI/MS Analysis of the Products Generated by Recombinant FlmF1 and FlmF2. For the LC-ESI/MS analysis of the lipid products, 0.5 mg/mL membrane protein, 200 μM sugar nucleotide (nonradioactive UDP-glucose or UDP-GalNAc), and 200 μM undecaprenyl phosphate were incubated at 30 °C for 1 h in 50 mM HEPES, pH 7.5, and 0.1% Triton X-100, as described in Experimental Procedures. The results are shown in Figure 5. The peak near *m/z* 845.65, which is seen in both of the no-enzyme controls (Figure 5A,C), is the $[M - H]^-$ molecular ion of the undecaprenyl phosphate substrate. The peak at *m/z* 1007.708 (Figure 5B), which is generated by recombinant FlmF1, is consistent with the predicted exact mass for the $[M - H]^-$ molecular ion of undecaprenyl phosphate-glucose, confirming the identity of the radiochemical experiment (Figure 3A). The results with UDP-GalNAc (Figure 5C,D) show that *E. coli* membranes expressing FlmF2 generate a product at *m/z* 1048.746, which is within experimental error for the predicted $[M - H]^-$ of undecaprenyl phosphate-GalNAc. FlmF1 and vector control membranes (not shown) do not generate any undecaprenyl phosphate-GalNAc under these conditions. However, small amounts of undecaprenyl phosphate-glucose are seen with vector and FlmF2 membranes (not shown), consistent with the radiochemical results (Figure 3A).

Based on LC-ESI/MS analysis with nonradioactive UDP-GalN as the donor substrate, membranes expressing either FlmF1 or FlmF2 do appear to generate small amounts of a product with $[M - H]^-$ near *m/z* 1006.7, consistent with the formation of undecaprenyl phosphate-GalN. The vector control membranes are inactive. However, we estimate that UDP-GalN is utilized at no more than 1% of the rate of either UDP-glucose or UDP-GalNAc for FlmF1 and FlmF2. Therefore, UDP-GalN is unlikely to be the physiological substrate for either enzyme.

As described in the Supporting Information section, the products of the FlmF1- and FlmF2-catalyzed *in vitro* reactions were purified using DEAE-cellulose and preparative TLC. Analysis by TLC and ESI/MS/MS (Supporting Information Figure 2) verified the FlmF1 product as undecaprenyl phosphate-glucose and the FlmF2 product as undecaprenyl phosphate-GalNAc.

Undecaprenyl Phosphate-GalNAc Deacetylase Activity in Extracts of F. novicida U112. To detect *F. novicida*

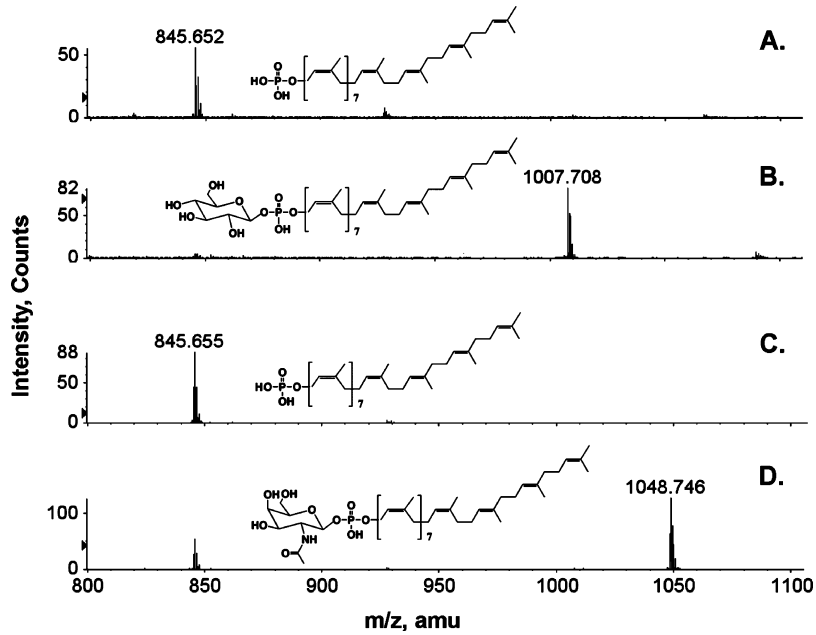


FIGURE 5: LC-ESI/MS analysis in the negative ion mode of products generated *in vitro* by recombinant FlmF1 and FlmF2. The LC-ESI mass spectra of the reaction products, generated in 60 min from undecaprenyl phosphate and UDP-glucose (panels A and B) or undecaprenyl phosphate and UDP-GalNAc (panels C and D), are shown. Panels A and C are the no enzyme controls, whereas panels B and D contained 0.5 mg/mL membranes from *E. coli* NovaBlue(DE3)/pET28b-*flmF1* or *E. coli* NovaBlue(DE3)/pET28b-*flmF2*, respectively. The structures of undecaprenyl phosphate, undecaprenyl phosphate-glucose, and undecaprenyl phosphate-GalNAc are inserted.

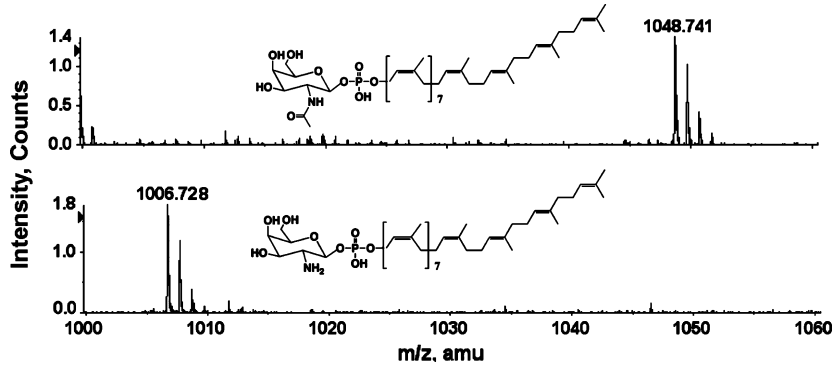


FIGURE 6: Evidence for an undecaprenyl phosphate-GalNAc deacetylase activity in membranes of *F. novicida* U112. Negative ion LC-ESI mass spectra indicate the presence of an undecaprenyl phosphate-GalNAc deacetylase activity in *F. novicida* U112 membranes. Upper panel: The 0 time control showing only substrate. Lower panel: The product generated after a 5 h incubation in the presence of 0.5 mg/mL membranes.

undecaprenyl phosphate-GalNAc deacetylase activity, undecaprenyl phosphate-GalNAc was incubated with *F. novicida* U112 membranes (0.5 mg/mL), and the reaction was monitored using LC-ESI/MS. The results are summarized in Figure 6. The molecular ion peak $[M - H]^-$ of the starting material, near *m/z* 1048.7, is present without detectable product peak at time 0 (Figure 6, upper panel). However, after a 5 h incubation, the substrate disappears, and a new peak near *m/z* 1006.7 is observed (Figure 6, lower panel), which is the same as that seen with purified undecaprenyl phosphate-GalN, as described in the preceding paper (*10*). This putative deacetylase activity is completely absent in extracts of *F. novicida* deleted in Ftn_0544 (Figure 7), a gene adjacent to *flmF2* and *flmK* on the *F. novicida* chromosome (F. Song and C. R. H. Raetz, in preparation).

DISCUSSION

F. novicida encodes all of the enzymes of the constitutive pathway for Kdo_2-lipid A biosynthesis (*9, 13*), but the structure of *F. novicida* lipid A is strikingly different from that of *E. coli* (Figure 1 of the preceding paper). Although a small amount of LPS can be detected, over 90% of the *F. novicida* lipid A is present in a "free" state, lacking the usual Kdo, core, and O-antigen sugars (*7*). The major free lipid A species (designated A1) is glycosylated at the 1-position with a GalN residue (Figure 1). The additional minor species (A2) is modified with GalN at the 1-position and with glucose at the 6′-position (Figure 1). Both free lipid A species lack the usual 4′-phosphate moiety and the 3′-hydroxyacyl chain (Figure 1) (*7*). *F. novicida* mutants lacking Kdo transferase are viable, but, as expected, they do not synthesize LPS (J. Zhao and C. Raetz, in preparation). However, these mutants continue to make free lipid A, which is required for growth, given that the early genes of the constitutive pathway (i.e., *lpxA* and others) are essential (*9*).

The modification pathway for the addition of the GalN unit to *F. novicida* lipid A shares some common features with the L-Ara4N modification pathway present in polymyxin-resistant

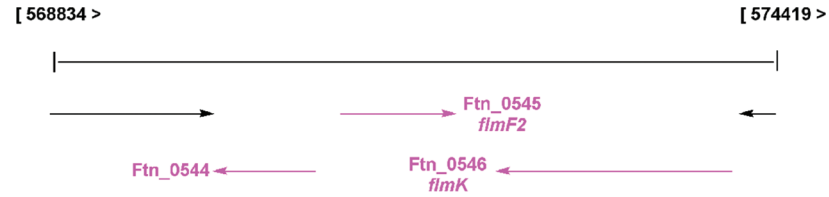


FIGURE 7: Genes clustered with *flm 2* (Ftn_0545) on the *F. novicida* chromosome. Mutants lacking the Ftn_0544 gene are unable to deacetylate undecaprenyl phosphate-GalNAc *in vitro* under the conditions shown in Figure 6. These mutants also accumulate undecaprenyl phosphate-GalNAc in living cells and do not make undecaprenyl phosphate-GalN (F. Song and C. R. H. Raetz, in preparation). The three genes involved in lipid A modification with GalN are highlighted in magenta, and their direction of transcription is indicated. The black flanking genes are involved in unrelated processes.

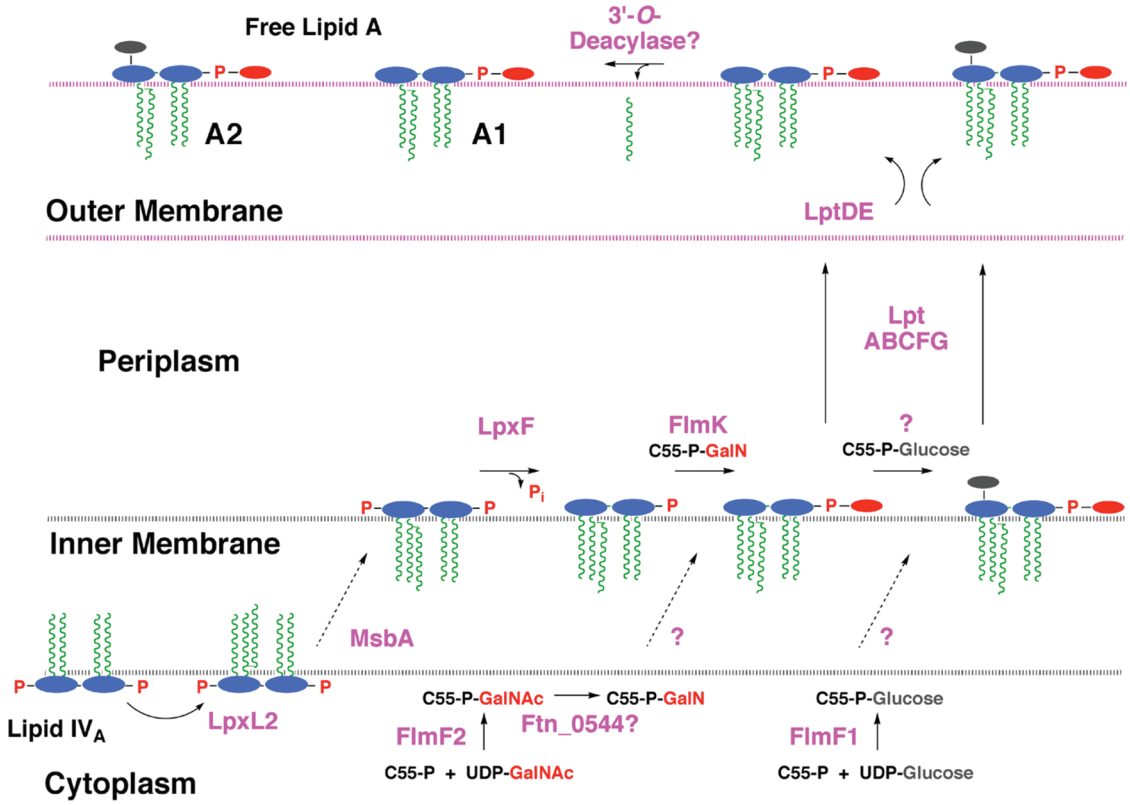


FIGURE 8: Proposed topography of the enzymes that modify free lipid A in *F. novicida*. A similar pathway is also proposed for the modification and export of intact LPS (not shown), except that LpxE (*20*), which is Kdo-dependent in *F. novicida*, may remove a portion of the 1-phosphate moieties of the lipid A unit (*40*). Blue ovals indicate the lipid A glucosamine residues, red ovals designate GalN units, and black ovals represent glucose moieties. Fatty acids are shown in green. Proteins involved in lipid A biosynthesis, modification, and transport are highlighted in magenta. The gene encoding the 3′-*O*-deacylase has not yet been identified in *F. novicida*.

mutants of *E. coli* and *Salmonella* (Scheme 1) (*13*). Specifically, the GalN donor is not a sugar nucleotide, but rather it is a lipid, undecaprenyl phosphate-GalN (Scheme 1), strongly suggesting that GalN addition to lipid A occurs on the periplasmic surface of the inner membrane (Figure 8). Furthermore, as shown in the preceding paper (*10*), the single *F. novicida* homologue of the *E. coli* L-Ara4N transferase ArnT (*7*), which is designated FlmK in *F. novicida* (*8*), is capable of transferring the GalN unit from purified undecaprenyl phosphate-GalN to lipid IV_A *in vitro*. L-Ara4N addition to lipid A also requires the MsbA flippase (*32*) in living cells of *E. coli*, but conditional MsbA mutants of *F. novicida* (Figure 8) are not yet available to test this idea.

There are two *arnC*(*pmrF*) (*9, 13*) homologues in the *F. novicida* genome, which are designated *flmF1* and *flmF2* (Scheme 1). The amino acid sequence identity between *F. novicida* FlmF1 (FTN_1403, 314 amino acid residues) and FlmF2 (FTN_0545, 318 amino acid residues) is 37% over the full lengths of these proteins. *F. novicida* FlmF1 and *E. coli* ArnC are 31% identical, whereas *F. novicida* FlmF2 and *E. coli* ArnC are 28% identical. All three proteins are members of the dolichyl phosphate-mannose synthase family, which provided the first clue that they might function in the biosynthesis of undecaprenyl phosphate sugars (*29*). *F. novicida* FlmF1 and FlmF2 are annotated as glycosyl transferases in the NCBI database without specifying their substrate selectivity. The sequence homology and the genomic context of *flmF2* (Ftn_0545), which is located next to *flmK* on the *F. novicida* genome (Figure 7), are consistent with our finding that FlmF2 catalyzes the formation of undecaprenyl phosphate-GalNAc, the proposed precursor of undecaprenyl phosphate-GalN (Scheme 1), whereas FlmF1 (Ftn_1403) synthesizes undecaprenyl phosphate-glucose (Figures 2−5 and Table 1). Neither FlmF2 nor FlmF1 appears to utilize UDP-GalN efficiently *in vitro*.

Several independent criteria were used to validate the functions of *flmF1* and *flmF2*. In the *F. novicida flmF1::Kan*^{R} total lipids, only component A1 (Figure 1) was detected

(Figure 2, middle panel), whereas neither A1 nor A2 (Figure 1) was present in the *flmF2::Kan*^{R} knockout (Figure 2, lower panel); instead, two novel species lacking the GalN unit accumulated, which are designated A3 and A4 (Figure 1). These results are consistent with the proposed roles of FlmF1 and FlmF2 (Scheme 1). In *in vitro* assays with *E. coli* membranes, recombinant *F. novicida* FlmF1 was 100 times more active than FlmF2 or the vector control in generating undecaprenyl phosphate-glucose, whereas FlmF2 was over 200 times more active than FlmF1 and the vector control in generating undecaprenyl phosphate-GalNAc (Figure 4 and Table 1). In addition, *F. novicida flmF1::Kan*^{R} membranes do not synthesize undecaprenyl phosphate-glucose *in vitro*, while *flmF2::Kan*^{R} and wild-type membranes do so with similar specific activities (Figure 4 and Table 1). Conversely, *F. novicida flmF2::Kan*^{R} membranes cannot synthesize undecaprenyl phosphate-GalNAc, whereas *flmF1::Kan*^{R} and wild-type membranes do so with comparable specific activities (Figure 4 and Table 1).

E. coli membranes from cells expressing FlmF2 or the vector control showed similar, albeit very low, activities with undecaprenyl phosphate and UDP-glucose (Figure 3A and Table 1). *E. coli* membranes from cells expressing FlmF1 or the vector control showed little or no activity with undecaprenyl phosphate and UDP-GalNAc (Figure 3B and Table 1). The low, but significant, residual activity seen with UDP-glucose in these controls (Figure 3A and Table 1) may reflect the presence of a chromosomally encoded undecaprenyl phosphate glucose synthase in *E. coli*. There are two *flmF* homologues in the *E. coli* genome: NCBI AP_002950, annotated as a bactoprenol glucosyl transferase, and AP_002851, the *E. coli arnC* gene encoding the undecaprenyl phosphate-L-Ara4FN synthase (Scheme 1).

As noted above, *F. novicida* FlmF2 has a much higher specific activity with UDP-GalNAc than with UDP-galactosamine *in vitro* (data not shown), strongly suggesting that undecaprenyl phosphate-GalNAc is its physiological product. Yet, in wild-type *F. novicida* lipids (*10*), only undecaprenyl phosphate-GalN is detected by mass spectrometry, as shown in the preceding paper. The explanation for this anomaly is the presence of an undecaprenyl phosphate-GalNAc deacetylase in *F. novicida* membranes (Figure 6 and Scheme 1). This activity is missing in mutants lacking Ftn_0544 (Figure 7), a gene that is distantly related to the YdjC class of peptidoglycan deacetylases (*33*). Mutants lacking Ftn_0544 do not synthesize GalN-modified lipid A and accumulate the expected undecaprenyl phosphate-GalNAc in their total lipid fraction (F. Song and C. R. H. Raetz, in preparation). The function of this proposed deacetylase (Scheme 1) may be analogous to that of the deformylase of the L-Ara4N pathway (*23*), possibly preventing the reversal of undecaprenyl phosphate-GalNAc formation by FlmF2. Furthermore, UDP-GalNAc and UDP-GlcNAc themselves are synthesized from precursors containing the *N*-acetyl group (*34*).

A recent study by Kanistanon et al. (*8*) reported that deletion of *flmK* not only eliminated the GalN residue that is present on LPS-bound lipid A but also eliminated a mannose unit (not shown) attached to the 4′-position of LPS-bound lipid A, which is not present in free lipid A (Figure 1) (*7*). One possibility is that FlmK has a dual catalytic function, thereby incorporating both the GalN and the 4′-mannose units. In principle, the GalN modification of lipid A might be required before the action of a separate enzyme that incorporates the mannose residue. This scenario is excluded, however, because strains lacking FlmF2 still incorporate the 4′-mannose unit into their LPS-bound lipid A (*8*). The mannose residue of LPS-bound lipid A cannot be derived from a sugar nucleotide because the 4′-phosphate group must be removed by LpxF prior to mannosylation, and the LpxF active site faces the periplasm (Figure 8) (*5*), where sugar nucleotides are not available. A logical donor for the mannose unit would therefore be undecaprenyl phosphate-mannose, but neither FlmF1 nor FlmF2 is active with GDP-[U-^{14}C]mannose as the donor substrate (data not shown). Kanistanon et al. did not examine the composition of free lipid A in their mutants (*8*). However, deletions of *flmF1* failed to incorporate mannose into LPS-bound lipid A (*8*), suggesting that undecaprenyl phosphate-mannose is derived from undecaprenyl phosphate-glucose by the action of a novel epimerase. This scenario might also explain our observation that deletion of FlmK(ArnT) results in the loss of both the GalN and glucose substituents present on free lipid A in *F. novicida* (*7*).

Our current hypothesis for the topography of free lipid A modification in *F. novicida* U112 is shown in Figure 8. The gene encoding the 3′-*O*-deacylase has not yet been identified; this enzyme is placed in the outer membrane by analogy to *Salmonella* LpxR with which it shares no obvious sequence similarity (*35*). Furthermore, it is unclear how undecaprenyl phosphate-glucose and undecaprenyl phosphate-GalN gain access to the outer surface of the inner membrane (Figure 8). There are no significant *F. novicida* homologues of the *E. coli* proteins ArnE and ArnF, which are the proposed inner membrane transporters for undecaprenyl phosphate-L-Ara4N (*24*). The *F. novicida* genome (*9*) does encode strong homologues of MsbA and at least of some of the Lpt proteins, which presumably transport LPS and free lipid A to the outer surface of the outer membrane, as proposed in *E. coli* and *Neisseria meningitidis* (*36*−*39*).

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SUPPORTING INFORMATION AVAILABLE

Detailed methods for the preparation of substrates, lipids, and other reagents, including the construction of *F. novicida flmF1* and *flmF2* mutants and the cloning of the *F. novicida flmF1* and *flmF2* genes. This material is available free of charge via the Internet at http://pubs.acs.org.

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