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Trent, M., Ribeiro, A., Doerrler, W., Lin, S., Cotter, R., & Raetz, C. (2001). Accumulation of a polyisoprene-linked amino sugar in polymyxin-resistant Salmonella typhimurium and Escherichia coli: Structural characterization and transfer to lipid A in the periplasm. *Journal of Biological Chemistry, 276* (46), 43132-43144. https://doi.org/10.1074/jbc.M106962200
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Accumulation of a Polyisoprene-linked Amino Sugar in Polymyxin-resistant Salmonella typhimurium and Escherichia coli

STRUCTURAL CHARACTERIZATION AND TRANSFER TO LIPID A IN THE PERIPLASM

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Polymyxin-resistant mutants of Escherichia coli and Salmonella typhimurium accumulate a novel minor lipid that can donate 4-amino-4-deoxy-L-arabinose units (L-Ara4N) to lipid A. We now report the purification of this lipid from a psa pmrA mutant of E. coli and assign its structure as undecaprenyl phosphate-α-L-Ara4N. Approximately 0.2 mg of homogeneous material was isolated from an 8-liter culture by solvent extraction, followed by chromatography on DEAE-cellulose, C18 reverse phase resin, and silicic acid. Matrix-assisted laser desorption ionization/time of flight mass spectrometry in the negative mode yielded a single species [M – H]− at m/z 977.5, consistent with undecaprenyl phosphate-α-L-Ara4N (M+, 987.4). 31P NMR spectroscopy showed a single phosphorus atom at −0.44 ppm characteristic of a phosphodiester linkage. Selective inverse decoupling difference spectroscopy demonstrated that the undecaprenyl phosphate group is attached to the anomic carbon of the L-Ara4N unit. One- and two-dimensional 1H NMR studies confirmed the presence of a polyisoprene chain and a sugar moiety with chemical shifts and coupling constants expected for an equatorially substituted arabinopyranoside. Heteronuclear multiple-quantum coherence spectroscopy analysis demonstrated that a nitrogen atom is attached to C-4 of the sugar residue. The purified donor supports in vitro conversion of lipid IVβ to lipid IIβ, which is substituted with a single L-Ara4N moiety. The identification of undecaprenyl phosphate-α-L-Ara4N implies that L-Ara4N transfer to lipid A occurs in the periplasm of polymyxin-resistant strains, and establishes a new enzymatic pathway by which Gram-negative bacteria acquire antibiotic resistance.

Polysaccharide-linked sugars function as donor substrates for many types of glycosyltransferases (1). In eubacteria, undecaprenyl moieties (1, 2) serve as lipid carriers for sugar residues that are transferred to acceptors located outside of the cytoplasm, where sugar nucleotides are not available. Undecaprenyl diphosphate-sugars (generally oligosaccharides) are precursors for polymerization of O-antigen (3–6), enterobacterial common antigen (7, 8), and peptidoglycan (1, 9–11). Undecaprenyl phosphate-sugars (typically monosaccharides) are thought to be donors for processes that include bacteriophage-mediated O-antigen conversion (12, 13), glycosylation of teichoic acids (14–17), and biosynthesis of mycobacterial lipoglycans (18–21). In eucaryotic cells, the structurally related dolichyl phosphate-sugars and dolichyl diphosphate-sugars (1, 22–24) play important roles in various stages of protein glycosylation and in the assembly of phosphatidylinositol-linked glycans.

As demonstrated in the preceding article (25), a membrane-bound donor, proposed to be undecaprenyl phosphate-α-L-Ara4N1 (Fig. 1)2 based upon bioinformatic considerations (26, 27), is required for the modification of lipid A with 4-amino-4-deoxy-L-arabinose (L-Ara4N) units in polymyxin-resistant mutants of Escherichia coli and Salmonella typhimurium. A novel L-Ara4N transferase, encoded by arnT (previously designated orf5 or pmrK) (28, 29), catalyzes L-Ara4N transfer to lipid A-like molecules in vitro when membranes of polymyxin-resistant mutants are employed as the source of the L-Ara4N donor (25). The formation of L-Ara4N and its transfer to lipid A are induced by activation of the transcription factor PmrA, which may occur by mutation (30–32), by activation of PhoP (33, 34), or by exposure of cells to mildly acidic pH, ferric ions, or metavanadate (26, 35, 36). Attachment of the positively charged L-Ara4N moiety to lipid A is critical for resistance to the antibiotic polymyxin and to certain cationic antimicrobial peptides present inside phagocytic cells (37, 38).

We now report the purification and structural characterization of a novel, minor lipid that accumulates in polymyxin-resistant mutants of E. coli and S. typhimurium. The purified lipid functions as a donor of L-Ara4N residues in the ArnT-catalyzed modification of lipid A in vitro. MALDI/TOF mass spectrometry and high resolution NMR spectroscopy strongly
support the proposal (25, 26) that the donor lipid has the structure undecaprenyl phosphate-α-L-Ara4N (Fig. 1). The ambiguous demonstration of an undecaprenyl-linked intermediate indicates that lipid A modification with the 1-Ara4N moiety occurs on the periplasmic surface of the inner membrane. Modification of lipid A with L-Ara4N units may provide a new biochemical marker for lipid A flip-flop (39) across the inner membrane.

EXPERIMENTAL PROCEDURES

Materials—32P, and [γ-32P]ATP were obtained from PerkinElmer Life Sciences. Silica Gel 60 (0.25-mm) thin layer plates were purchased from EM Separation Technologies. Tryptone and yeast extract were from Difco. Triton X-100 and biocinchoninic acid were from Pierce. CDCl3, CD3OD, and D2O were purchased from Aldrich. All other chemicals were reagent-grade and were purchased from either Sigma or Mallinkrodt.

Bacterial Strains—The strains used in the present study are described in Table I. Bacteria were usually grown at 37°C in LB medium (40), which contains 10 g of NaCl, 10 g of tryptone, and 5 g of yeast extract per liter. When needed, cultures were supplemented with 100 μg/ml kanamycin, 12 μg/ml tetracycline, 30 μg/ml chloramphenicol, or 30 μg/ml kanamycin.

Preparation of Radiolabeled Substrates—[4,32P]Lipid IVα was prepared using 100 μCi of [γ-32P]ATP, tetraacyldisaccharide 1-phosphate, and membranes of E. coli cells overexpressing the 4'-kinase (LpxK), as described previously (41-43). To prepare Kdo-[4,32P]Lipid IVα, the purified E. coli Kdo transferase (KdtA) was used in tandem with LpxK (42, 43).

Preparation of Cell-free Extracts and Membranes—Typically, 100-ml cultures of bacteria were grown to an optical density of 1.0 at 37°C and harvested by centrifugation at 7,000 × g for 15 min. All steps were carried out at 4°C. Cell pellets were resuspended in 50 mM Hepes, pH 7.5, at a protein concentration of 5–10 mg/ml, and broken by passage through a French press at 18,000 pounds/square inch. The crude extract was cleared by centrifugation at 7,000 × g for 15 min. Membranes were prepared by ultracentrifugation at 149,000 × g for 60 min, followed by resuspension and a second ultracentrifugation step to remove all cytosolic components. The final membrane pellet was resuspended in 50 mM Hepes, pH 7.5, at a protein concentration of 5–10 mg/ml. The supernatant from the first ultracentrifugation step was subjected to a second ultra-centrifugation to remove residual membrane particles. All samples were stored as aliquots at −80°C. Protein concentrations were determined with biocinchoninic acid (44) using bovine serum albumin as the standard.

L-Ara4N Transferase (ArnT) Assay Conditions—The 1-Ara4N transferase (ArnT) was assayed under optimized conditions in a 10-μl reaction mixture containing 50 mM Mes, pH 6.5, 0.2% Triton X-100, and 10 μM of [4,32P]Lipid IVα (20,000 cpm/nmol) (25). Reactions were incubated at 30°C at the indicated protein concentration and times and were stopped by spotting 5-μl portions onto a Silica Gel 60 TLC plate. The substrate and product(s) were separated by developing the plate with chloroform, pyridine, and water (50:50:16.5, v/v), and quantified using a Molecular Dynamics PhosphorImager equipped with ImageQuant software. ArnT-specific activity was calculated from the percentage of substrate converted to product and expressed as pmol/min/mg (25).

Construction of a Polymyxin-resistant Mutant of E. coli K-12—Wild-type E. coli K-12 cells (W3110) were treated with 50 μg/ml of the mutagen N-methyl-N-nitro-N-nitosoguanidine (40). Approximately 107 cells were plated on LB agar containing 2 μg/ml polymyxin B sulfate (45). At this plating density, no growth of untreated cells was observed, but with mutagenesis, polymyxin-resistant colonies were recovered at a frequency of about 10−7. Several such colonies were purified twice on LB agar containing 10 μg/ml polymyxin B sulfate. The polymyxin resistance phenotype of a representative isolate was moved into a wild-type background (W3110) by P1 transduction (40) by selecting directly for polymyxin resistance at 10 μg/ml on LB agar. To validate the chromosomal location of the resistance gene in the transductant (designated WD101), pmrA+ (basR) of E. coli was re-introduced into WD101 by co-transduction with a linked Tn10 transposon at 37°C, using a P1vir lysate prepared on strain AKK211 (zjd-2211::Tn10). Several of the tetracycline-resistant transductants of WD101 (2 of 14) generated in this manner had lost their polymyxin resistance, when tested for growth on LB agar containing 10 μg/ml polymyxin B sulfate. This finding supports the idea that a mutation in pmrA is responsible for the polymyxin resistance of WD101. To validate this hypothesis, however, the pmrA and pmrB genes were amplified together by subjecting WD101 chromosomal DNA to polymerase chain reaction, using Pfu DNA polymerase (Stratagene) according to the manufacturer’s instructions. Both genes were sub-cloned into the vector pT7Blue-3 (Novagen) using blunt end ligation at the EcoRV site. The sequences of the forward and reverse primers were 5′-CAGGCCTGCG-GATGATATTCTGC-3′ and 5′-GTTTTAATCACCCTTACGCCGTG-3′ respectively. The sequences of both genes were then determined to locate mutation(s). Two amino acid residues were altered in PmrA of WD101, demonstrating that this mutant is a likely pmrA constitutive (pmrA50). No changes were found in the pmrB sequence.

Construction of a pmrA50 Mutant of E. coli K12 Lacking Phosphatidylethanolamine—The presence of phosphatidylethanolamine interferes with the purification of undecaprenyl phosphate-α-L-Ara4N, since both compounds are zwitterionic phospholipids. The E. coli K-12 strain AD90 contains a kan insertion in the phospholipid biosynthetic gene pps, resulting in the complete absence of phosphatidylethanolamine in its membranes (46). AD90 cells harboring the temperature-sensitive

| Strains and plasmids | Description | Source or Ref. |
|----------------------|-------------|---------------|
| S. typhimurium       |             |               |
| ATCC 14028s          | Wild type   | E. coli Genetic Stock Center, Yale University |
| CS092                | phe-24 (PhoP-constitutive) | This work |
| CS095                | pheP102–Tn10d-cam | This work |
| CS330                | pheP102–Tn10d-cam | This work |
| JSG435               | pmrA50 zjd–Tn10d-cam (PmrA-constitutive) | E. coli Genetic Stock Center, Yale University |
| JSG421               | pmrA–Tn10d  | This work |
| E. coli              |             |               |
| W3110                | Wild type, F−, λ− | Novagen |
| W3101                | W3110, pmrA2 | Novagen |
| WD102                | zjd-2211::Tn10 derivative of WD101 (AKK211 donor) | This work |
| AKK211               | λ−, relA1, spot71, ilv-299, metB1, zjd−, 2211::Tn10, ampcP1, mcrB1, creC510 | This work |
| AD90                 | pss93–kan nadB | E. coli Genetic Stock Center, Yale University |
| WD901                | pmrA2–zjd-2211::Tn10 derivative of AD90 (WD20 donor) | This work |
| BLR(DE3)             | Δ(lsr-recA506–Tn10DE3), Tet′ | Novagen |
| NovaBlue(DE3)        | Δ(lsr-recA506–Tn10DE3), Tet′ | Novagen |
| Plasmids             |             |               |
| pET21a               | Vector containing a TIlac promoter, Amp′ | Novagen |
| pARN1E               | pET21a containing E. coli arnT | Novagen |
| pARN6E               | pET21a containing S. typhimurium arnT | 25 |
| pDD72                | pss′ covering plasmid with temperature-sensitive ori, Cam′ | 46 |
| pT7Blue-3           | Cloning vector, Kan′, Amp′ | Novagen |
| pWTD5                | pT7Blue-3 containing pmrA1/pmra operon from wild-type W3110 | This work |
| pWTD6                | pT7Blue-3 containing pmrA1/pmra operon from WD101 | This work |
| pLysS                | pACYC184 encoding T7 lysozyme, Cam′ | Novagen |

| Strains and plasmids | Description | Source or Ref. |
|----------------------|-------------|---------------|
| E. coli              |             |               |
| CS022                | phe-24 (PhoP-constitutive) | This work |
| CS025                | pheP102–Tn10d-cam | This work |
| JSG435               | pmrA50 zjd–Tn10d-cam (PmrA-constitutive) | This work |
| JSG421               | pmrA–Tn10d  | This work |

TABLE I

Bacterial strains and plasmids
**Undecaprenyl Phosphate-α-L-Ara4N in Polymyxin-resistant Bacteria**

Fig. 1. Structure of undecaprenyl phosphate-α-L-Ara4N and proposed transfer of the L-Ara4N moiety to core-lipid A by ArnT. The numbering scheme for the undecaprenyl phosphate-α-L-Ara4N molecule is used for the NMR analysis presented. The 4'-' and 1-positions of lipid A are also indicated. The active site of ArnT may be located on the periplasmic surface of the inner membrane, given the involvement of an undecaprenyl phosphate-linked donor substrate (25). ArnT can also transfer the L-Ara4N moiety to the 1-position of lipid A (25) (not shown). In acceptor substrates lacking the Kdo domain of the core, such as lipid IV$_\alpha$ (not shown), ArnT incorporates the L-Ara4N unit exclusively at the 1-position (25).

L-Ara4N Moiety

Isoprene Units

\[ \begin{align*}
\text{L-Ara4N} & : \alpha \rightarrow \text{Core-Lipid A} \\
\text{Undecaprenyl Phosphate} & \\
\text{(L-Ara4N) Modifed Lipid A} &
\end{align*} \]

pss$^+$ covering plasmid pDD72 grew normally at 30°C on LB broth, because normal amounts of phosphatidylethanolamine are synthesized under these conditions (46). Growth of AD90 cured of its covering plasmid after a shift to 42°C is slow and requires supplementation of the medium with 50 mM MgCl$_2$ (46).

A tetracycline-resistant derivative of WD101 was selected by transducing zjd-2211:Tn10 from AKK211 into WD101, as described above, and retrieving one of the polymyxin-resistant recombinants. The de- 

Fig. 1. Structure of undecaprenyl phosphate-α-L-Ara4N and proposed transfer of the L-Ara4N moiety to core-lipid A by ArnT. The numbering scheme for the undecaprenyl phosphate-α-L-Ara4N molecule is used for the NMR analysis presented. The 4'- and 1-positions of lipid A are also indicated. The active site of ArnT may be located on the periplasmic surface of the inner membrane, given the involvement of an undecaprenyl phosphate-linked donor substrate (25). ArnT can also transfer the L-Ara4N moiety to the 1-position of lipid A (25) (not shown). In acceptor substrates lacking the Kdo domain of the core, such as lipid IV$_\alpha$ (not shown), ArnT incorporates the L-Ara4N unit exclusively at the 1-position (25).

glass tube. The supernatant was transferred to a two-phase Bligh/Dyer system consisting of chloroform/methanol/water (2:2:1.8 v/v) by adding 1.2 ml of chloroform, 0.42 ml of methanol, and 1.17 ml of water. The phases were separated by low speed centrifugation, and the lower phase was removed. The sample was dried under a stream of N$_2$ and re-dissolved in a small volume of chloroform/methanol (4:1, v/v). The $^{32}$P-labeled phospholipids (100,000 cpm/lane) were separated on Silica Gel 60 TLC plates in the solvent chloroform/methanol/water/NH$_3$OH (65:25.3:6.0:0.4, v/v). The plate was dried and exposed to a PhosphorImager screen overnight to visualize the $^{32}$P-containing bands.

Extraction of Lipid A from $^{32}$P-Labeled Cells—$^{32}$P-Labeled cells were grown, harvested, and extracted as described above with 3 ml of a single phase Bligh/Dyer mixture. The insoluble residue, which contains the $^{32}$P-labeled lipid A still covalently bound to lipopolysaccharide, was recovered by centrifugation and subjected to hydrolysis at 100°C in 12.5 mM sodium acetate buffer, pH 4.5, in the presence of 1% SDS to cleave the Kdo-lipid A linkage (26, 48). The released $^{32}$P-labeled lipid A species were extracted by the Bligh/Dyer method, and a portion was spotted onto a Silica Gel 60 TLC plate (~10,000 cpm/lane) that was developed in the solvent chloroform, pyridine, 88% formic acid, water (50:50:16.5:0, v/v). The plate was dried and exposed to a PhosphorImager screen overnight to visualize the $^{32}$P-labeled lipid A species.

Large Scale Purification of the Putative Donor Lipid Undecaprenyl Phosphate-α-L-Ara4N—A 200-ml culture of WD901 (pss93::kan pmrA C zjd-2211::Tn10) was grown for ~36 h in LB broth containing 50 mM MgCl$_2$ to an$A_{600}$ of ~2.0. This culture was used to inoculate four 2-liter cultures, each in a 6-liter Erlenmeyer flask, at a starting $A_{600}$ of 0.05. The cells were grown in a rotary shaker at 37°C until $A_{600}$ reached ~2.0, harvested by low speed centrifugation at 4°C, and resuspended in a total of 120 ml of phosphate-buffered saline, pH 7.4. The cell suspen-
Undecaprenyl Phosphate-α-L-Ara4N in Polymyxin-resistant Bacteria

Fig. 2. Accumulation of a novel minor phospholipid in the polymyxin-resistant E. coli mutant WD101. 32P-Labeled lipid A species and phospholipids were separated by TLC and visualized with a PhosphorImager. The lipid A species were resolved using the solvent chloroform, pyridine, 88% formic acid, water (50:50:16.5, v/v/v), and the phospholipids were separated in chloroform/methanol/water/NH4OH (65:25:3.6:0.4, v/v/v/v). The position of the putative L-Ara4N donor lipid, seen only in the polymyxin-resistant mutant, is indicated.

Mass Spectrometry—Spectra of the purified donor lipid were acquired in the negative and positive linear modes using a time of flight matrix-assisted laser desorption ionization (MALDI/TOF) mass spectrometer (Compact MALDI 4, Kratos Analytical Manchester, UK), equipped with a nitrogen laser (337 nm), 20-kV extraction voltage, and time-delayed extraction. Each spectrum was the average of 50 laser shots. The instrument was operated at a resolution of about ±1 atomic mass units for compounds with M<sub>r</sub> ~ 2000. Saturated 6-aza-2-thiophenylglycerol and Cardiolipin served as the matrix in both the positive ion and the negative ion modes. The lipid samples were dissolved in chloroform/methanol (4:1), mixed 1:1 with the specified matrix, and dried at room temperature prior to analysis.

NMR Spectroscopy—Approximately 0.2 mg of the purified donor lipid was dissolved in 0.6 ml of CDCl<sub>3</sub>/CD<sub>3</sub>OD/D<sub>2</sub>O (2:3:1, v/v) in a 5-mm NMR tube. NMR spectra were obtained at 25 °C using Varian Inova 800, Inova 600, or Unity 500 spectrometers, as indicated, equipped with 200-MHz proton-detected broadband Carr-Purcell-Meiboom-Gill (CPMG) pulse sequences. The spectra were assigned by 1-D HMQC, HMQC-UV, and HSQC experiments.
Sun Ultra 10 computers and 5-mm Varian probes. The 2H signal of CD3OD was used for a field frequency lock. Use of the CDCl3/CD3OD/D2O solvent system introduces four solvent resonances. The signals from CH3OD (3.3 ppm) and CHCl3 (7.6 ppm) do not overlap with the donor lipid resonances. The HOD (4.6 ppm) and CD3OH (4.8 ppm) signals are removed with a presaturation sequence.

1H NMR spectra at 800 MHz were obtained with 8.2-kHz spectral width, 73° pulse flip angle (7.5 s), 5.0 s acquisition time, and 1.2-s relaxation delay. The spectra were digitized using 82,000 points yielding a digital resolution of 0.2 Hz/point. 1H NMR spectra at 600 MHz were obtained with 5.7-kHz spectral width, 63° pulse flip angle (6.0 s), 5.6-s acquisition time, and 1.2-s relaxation delay and were digitized using 64,000 points to give a digital resolution of 0.18 Hz/point. 1H NMR spectra at 500 MHz were obtained with 4.5-kHz spectral width, 74° pulse flip angle (7.5 μs), 5.0-s acquisition time, and 1.2-s relaxation delay and were digitized using 45,000 points to give a digital resolution of 0.20 Hz/point. 31P NMR spectra at 202 MHz (500 MHz field) and selective inverse (31P) decoupling difference spectra were obtained as described previously (51–53). Two-dimensional-COSY, HMQC, and HMBC analyses were performed at the 800- and 600-MHz fields, based on the experiments described previously (51) at 500 MHz.

RESULTS

Isolation and Characterization of a Polymyxin-resistant Mutant of E. coli K-12—To facilitate the purification of the proposed undecaprenyl phosphate-α-L-Ara4N donor lipid, a new polymyxin-resistant mutant of E. coli K-12, designated WD101, was isolated by chemical mutagenesis of W3110. WD101 formed single colonies on agar containing 10 μg/ml polymyxin. The resistance phenotype was located near the pmrA/pmrB locus at minute 93 on the E. coli chromosome (30), as judged by P1vir transduction. Polymerase chain reaction-based cloning and DNA sequencing of the pmrA/B cluster of WD101 showed that two amino acids were altered in the transcription factor PmrA (A42T and G53E). No mutations were present in pmrB.

TLC analysis of 32P-labeled lipid A species isolated from WD101 showed extensive modification with polar moieties, when compared with wild-type W3110 (Fig. 2, left panel). The modified lipid A components of WD101 were generally more hydrophilic, indicating the presence of additional L-Ara4N and/or pEtN substituents (26, 53), as judged by TLC analysis (Fig. 2, left panel) and mass spectrometry (not shown). Lipid A species containing an extra palmitoyl group were not detected in large amounts in WD101, consistent with the activation of pmrA but not phoP. These findings are in accord with earlier studies (32) of polymyxin-resistant mutants of E. coli, which were not characterized by DNA sequencing.

Accumulation of a Novel Minor 32P-Labeled Lipid in WD101 and in Certain Strains of S. typhimurium—The major phospholipids of WD101 consist of phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin, as in the wild-type W3110 (Fig. 2, right panel). However, in contrast to E. coli W3110 (Fig. 2), the lipid is also detectable in wild-type S. typhimurium (Fig. 3, lane 1).
This finding is consistent with the fact that lipid A of wild-type S. typhimurium grown on LB broth contains some L-Ara4N-modified species, albeit at lower levels than in pmrAC mutants (26, 53).

The putative donor lipid was absent in a S. typhimurium pmrA/H11002 mutant (Fig. 3, lane 5) and was also missing in pmrA mutants containing deletions in either the pmrE or pmrF genes (data not shown). The latter are required for the modification of lipid A with the L-Ara4N moiety and for the maintenance of polymyxin resistance (28). Furthermore, phoP/phoQ regulation was evident in an otherwise wild-type background, given the increase in the minor lipid in a PhoPC setting (Fig. 3, lane 2) and its complete absence in a phoP− mutant (Fig. 3, lane 3). Because its presence or absence was regulated in the same manner as reported previously for L-Ara4N addition to lipid A in both E. coli (30, 32) and S. typhimurium (28, 29, 55), we decided to purify and characterize the compound.

Construction a pmrA E. coli Mutant (WD901) Lacking Phosphatidylethanolamine—Purification of the putative donor lipid from WD101 or from a pmrA mutant of S. typhimurium was not feasible because it could not be separated from phosphatidylethanolamine, the major phospholipid of these bacteria (56), in various large scale chromatography steps (data not shown). To solve this problem, the pmrAC allele of WD101 was transferred into E. coli AD90/pDD72 (46) by P1 vir transduction, generating WD901/pDD72. After being cured of the covering plasmid pDD72, these strains lack phosphatidylethanolamine because of an insertion mutation in their chromosomal copy of the pss gene. As shown in Fig. 4, lane 2, the presence of the pmrAC mutation in WD901/pDD72 resulted in the accumulation of the putative L-Ara4N donor lipid. Once cured of pDD72, the donor lipid was still produced by WD901, despite the complete absence of phosphatidylethanolamine (Fig. 4, lane 4). Neither AD90/pDD72 nor AD90 contained this minor lipid (Fig. 4, lanes 1 and 3).

To make certain that the pss mutation had no effect on the transfer of the i-Ara4N moiety to lipid A, 32P-labeled lipid A species were prepared from AD90 (pss−) and WD901 (pss− pmrAC) in the presence or absence of the pss− covering plasmid pDD72. As judged by TLC analysis, the pattern of lipid A modifications of WD901/pDD72 (Fig. 5, lane 2) was identical to that of WD101 (pss− pmrAC) (Fig. 2, left panel). Upon removal of the pss− covering plasmid pDD72, however, the three lipid A species containing the pEtN moiety disappeared (Fig. 5, lane 4), whereas incorporation of the i-Ara4N moiety was unaffected. The lipid A species of AD90 grown with or without the covering plasmid (Fig. 5, lanes 1 and 3) were essentially the same as wild type (Fig. 2). These findings demonstrate that...
phosphatidylethanolamine is the source of the pEtN moieties attached to lipid A in pmrA C mutants. About 70% of the lipid A of WD901 consisted of a species with a single L-Ara4N substituent (Fig. 5, lane 4), presumably attached to the 4'-phosphate position (52).

The relative resistance of AD90 and WD901 to polymyxin could not be evaluated, because 50 mM Mg^{2+} (required for growth of both strains) interfered with the disc diffusion assays (data not shown).

**Purification of the Putative L-Ara4N Donor Lipid from WD901**—A 100-mg sample of WD901 phospholipids (Fig. 6, lane 1) was applied to a 20-ml DEAE-cellulose column equilibrated in chloroform/methanol/water (2:3:1, v/v). As expected, phosphatidylglycerol and cardiolipin bound to the column, whereas the putative donor lipid emerged in the run through (Fig. 6, lane 2). This finding is consistent with the proposed zwiterionic character of the donor lipid (Fig. 1). If pss C cells had been employed, phosphatidylethanolamine would also have been present in the run through.

The DEAE-cellulose run-through fraction was applied to a 4.0-ml C18 reverse phase column, prepared in an acetonitrile/isopropanol system. Three major bands were present in the run through. The apparent Rf of the donor lipid increased slightly after the reverse phase and silicic acid steps (Fig. 6, lanes 3 and 4), possibly arising from interaction with contaminating tetra-butyl ammonium dihydrogen phosphate. When most of this material was removed by two-phase Bligh/Dyer partitioning, the purified lipid (0.2 mg) migrated exactly like the desired component of the original sample (Fig. 6, lane 5).

**MALDI/TOF Mass Spectrometry of the Purified Lipid**—When analyzed in the negative mode, the purified material yielded a major ion at m/z 977.5 (Fig. 7A), consistent with [M–H] C for the proposed structure (Fig. 1), undecaprenyl phosphate–L-Ara4N (M C = 978.41). No peak was observed at m/z 1057.4 atomic mass units, the [M–H] C expected for a hypothetical undecaprenyl diphasphate–L-Ara4N. Analysis of the purified sample in the positive ion mode produced a major peak at m/z 767.9 atomic mass units, which could be interpreted as arising from the fragment [undecaprenol + H] C (Fig. 7B). The molecular weight of undecaprenol is 767.3.

**Characterization of the Purified Lipid by One-dimensional 1H NMR Spectroscopy**—The NMR spectra of the purified lipid show all the features expected for a monoglycosylated polyisoprenyl phosphate (18, 19). The one-dimensional 800-MHz 1H NMR spectra (Figs. 8A to 10A, and Fig. 1 in the Supplemental Material) reveal the following: 1) six resolved 1H resonances between 5 and 3.2 ppm that can be assigned to the L-Ara4N unit (Fig. 8A) (26, 57); 2) the CH (dd, 5.41 ppm) and the proximal CH2OP (dd, 4.45 ppm) signals of the adjacent isoprene unit (Fig. 1 and Table III). After correcting for the overlap of the C-2 methylene proton signal arising from residual tetra-butyl ammonium dihydrogen phosphate (designated × at the top of Figs. 9B and 10B), the 1H NMR signals between 1.6 and 1.75 ppm integrate to 12 methyl groups. The methyl signal at 1.74 ppm (d, long range  J = 1.0 Hz) (see Fig. 1 in the Supplemental Material) corresponds to the methyl protons that are in cis configuration relative to the monohydroxy proton of the α isoprene residue (Figs. 1, 9B, and 10B). The methyl signals at 1.70 and 1.69 ppm integrate to 6 and 2 methyl groups, respectively, corresponding to the 7 cis-methyls of the interior isoprene units and the cis-methyl of the α isoprene unit (Figs. 1, 9B, and 10B and Table III). The methyl signals at 1.63 and 1.61 ppm (Figs. 9B and 10B) integrate to 1 and 2 methyl groups, respectively, and are ascribed to the three trans-methyls (as defined in relation to their respective methine protons) of the α, β, and γ isoprene units (Fig. 1 and Table III).

**Characterization of the Purified Lipid by Two-dimensional 1H NMR Spectroscopy**—To our knowledge, intact samples of undecaprenyl phosphate derivatives isolated from natural sources have not been studied previously by two-dimensional NMR methods because of their instability in the available solvents (18, 19). However, as noted in our previous work (51) with E. coli lipid A, many natural lipids, including undecaprenyl phosphate–L-Ara4N, are stable in CDCl 3/CD3OD/D2O (2:3:1, v/v) for weeks and display sharp, well resolved resonances. The positions of the individual protons of the arabino sugar were derived from a two-dimensional COSY analysis (Fig. 8C, Table II, and Fig. 1 in the Supplemental Material). The doublet-doublet at 4.90 ppm (J2,3 = 6.4, J3,4 = 7.8 Hz) is assigned to the anomic H-1 proton of the α-L-Ara4N moiety (57). The COSY cross-peak from H-1 (Fig. 8C) locates the H-2 signal at 3.61 ppm (dd, J2,3 = 6.4, J3,4 = 8.0 Hz). A second COSY cross-peak from H-2 connects to H-3 at 3.81 ppm (dd, J2,3 = 8.0, J3,4 = 4.3 Hz) which, in turn, is coupled to H-4 at 3.29 ppm (broad...
Further tracing of the COSY connectivities locate the remaining L-Ara4N protons, i.e. H-5 (3.71 ppm; dd, $J_{4,5}/H_{11005}$ 2.4, $J_{5}/H_{11032}$ 12.8 Hz) and H-5' (3.96 ppm; dd, $J_{4,5}/H_{11032}/H_{11005}$ 4.1, $J_{5}/H_{11005}$ 12.8 Hz).

The two-dimensional COSY also revealed a strong cross-peak between the CH and proximal CH$_2$ groups of the α isoprene unit (Fig. 8C). The CH and proximal CH$_2$ of the α isoprene unit each showed a weak cross-peak to the resolved methyl signal at 1.74 ppm (see Fig. 1 in Supplemental Material). These weak cross-peaks arise from four- and five-bond long range couplings and strengthen the assignment of the 1.74 ppm methyl signal to the methyl group of the α isoprene unit. The major unresolved polyisoprene CH signals (~5.1 ppm) similarly show strong cross-peaks to the upfield CH$_2$ resonances and weaker (long range) cross-peaks to the upfield CH$_3$ signals (see Fig. 1 in the Supplemental Material).

The low field shift of H-1 (4.90 ppm) and the large $J_{1,2}$ coupling constant (6.4 Hz) indicate that H-1 of the L-Ara4N residue is in the axial position (57), so that the undecaprenyl phosphate chain must be situated equatorially. The large $J_{2,3}$ coupling (8 Hz) indicates that H-2 and H-3 are axially disposed relative to each other. The intermediate $J_{3,4}$ coupling (4.3 Hz) indicates an axial-equatorial interaction, implying that H-4 is equatorial and that the amino group is axial (as shown in Fig. 1). The measured values of 2.4 and 4.1 Hz for $J_{4,5}$ and $J_{4,5}'$ do not allow distinction between the axial and equatorial H-5 protons. However, two-dimensional NOESY data (see Fig. 2 in the Supplemental Material) revealed strong NOE cross-peaks.
between H-1, H-3, and H-5, supporting the idea that these are all axial protons on the same face (below plane in Fig. 1) of the sugar. The noise streak at 4.56 ppm arises from the HOD signal. Resonances designated × are due to residual solvents or other impurities. B, this expansion shows the partially resolved isoprenyl CH₂ and CH₃ groups and their directly bonded carbon atoms. The cis, trans, and adjacent configurations are defined in Table III. The butyl chains of tetra-butyl ammonium dihydrogen phosphate, carried over from the reverse phase chromatography step of the purification, give rise to multiplet ¹H resonances at 3.20, 1.66, 1.44, and 1.04 ppm (see Supplemental Material Fig. 1). The tetra-butyl ammonium dihydrogen phosphate resonance (x) at 1.66 ppm partially overlaps with two of the undecaprenyl CH₂ signals of the donor lipid. The donor lipid sample used for the HMQC and HMBC experiments also displayed CH₂ (1.19 ppm) (not shown) and CH₃ (3.83 ppm) signals arising from small amounts of an ethanol impurity in the sample, which resulted in masking of the ¹-Ara₄N H-2 by the ethanol CH₃ signal in A. This impurity is not present in the experiment shown in Fig. 8, in which an additional two-phase Bligh-Dyer partitioning was carried out to remove the contaminating ethanol.

## Table II

| Ara₄N   | ¹H chemical shifts (ppm from internal TMS) | ¹³C chemical shifts (ppm from internal TMS) |
|---------|-------------------------------------------|-------------------------------------------|
| δH      | H-1 (δ₁,₂) (δ₁,₅) H-2 (δ₂,₃) H-3 (δ₃,₄) H-4 (δ₃,₄) H-5 (δ₄,₅) (δ₄,₅) (δ₅,₅) | C-1 79.5 C-2 72.4 C-3 70.8 C-4 61.0 C-5 63.0 |
|         | (4.90) (3.61) (3.81) (3.29) (3.71) (3.96) (7.8) (12.8) (12.8) | |

### Selective ³¹P-Decoupled ¹H Difference Spectroscopy

The linkage between the undecaprenyl chain and the arabinose sugar unit was investigated. One-dimensional ³¹P NMR spectroscopy revealed a single phosphorus resonance near −0.44 ppm, consistent with a phosphodiester linkage (see Fig. 3 in the Supplemental Material).

FIG. 9. Partial HMOC spectra of the purified donor lipid. A, single bond ¹H–¹³C correlations of the ¹-Ara₄N moiety, part of the proximal isoprene unit, and the unresolved olefinic protons of the undecaprenyl chain are shown. The noise streak at 4.56 ppm arises from the HOD signal. Resonances designated × are due to residual solvents or other impurities. B, this expansion shows the partially resolved isoprenyl CH₂ and CH₃ groups and their directly bonded carbon atoms. The cis, trans, and adjacent configurations are defined in Table III. The butyl chains of tetra-butyl ammonium dihydrogen phosphate, carried over from the reverse phase chromatography step of the purification, give rise to multiplet ¹H resonances at 3.20, 1.66, 1.44, and 1.04 ppm (see Supplemental Material Fig. 1). The tetra-butyl ammonium dihydrogen phosphate resonance (x) at 1.66 ppm partially overlaps with two of the undecaprenyl CH₂ signals of the donor lipid. The donor lipid sample used for the HMQC and HMBC experiments also displayed CH₂ (1.19 ppm) (not shown) and CH₃ (3.83 ppm) signals arising from small amounts of an ethanol impurity in the sample, which resulted in masking of the ¹-Ara₄N H-2 by the ethanol CH₃ signal in A. This impurity is not present in the experiment shown in Fig. 8, in which an additional two-phase Bligh-Dyer partitioning was carried out to remove the contaminating ethanol.
Supplemental Material (58). Next, subtraction of two 1H NMR spectra obtained with on and off resonance selective decoupling of the –0.44 ppm phosphate signal (51, 52) revealed simultaneous decoupling changes at the anomeric H-1 signal of the L-Ara4N moiety and at the proximal CH2 signal of the α isoprene residue (Fig. 8B), thereby establishing the presence of a single phosphate group linking the arabinose C-1 carbon and the proximal CH2 carbon of the α isoprene unit of the undecaprenyl chain (Fig. 1).

**Evaluation of the Carbon Structure of the Donor Lipid by HMBC Spectroscopy**—To confirm the assignments derived from the 1H NMR analysis, 13C data for the donor lipid (–0.2 mg) were obtained indirectly through 1H-detected HMQC and HMBC two-dimensional NMR experiments. The partial two-dimensional HMQC 1H-13C correlation map (Fig. 9A) reveals six direct 1H-13C single-bond correlations in the sugar region (Table II). The 1H-13C correlations discussed below, the anomeric carbon resonance near 99.5 ppm (C-1), the H-5 and H-5’ multiplets connect to carbon resonances at 72.4 (C-2) and 70.8 (C-3) ppm, respectively, as predicted for oxygen-substituted carbon atoms of sugars. However, nitrogen-substituted carbons of amino sugars resonate near 50–55 ppm (51, 59). The H-4 multiplet shows a prominent cross-peak near 51 ppm, confirming C-4 as the site of the amino group substitution. Fig. 9A also shows the direct bond correlations from the major unresolved methine proton signals of the undecaprenyl chain to unresolved olefinic carbon signals near 126 ppm, and from the methine and the proximal methylene protons of the α isoprene unit to carbon resonances at 122.8 and 63.5 ppm, respectively.

The bulk CH2 protons of the undecaprenyl chain yield four distinct 1H-13C HMQC cross-peaks (Fig. 9B and Table III). Based upon the multibond correlations discussed below, the major carbon peak at 27.2 ppm is assigned to the proximal CH2 groups of the bulk isoprene units (i.e., the ones adjacent to a methine proton as shown in Table III), whereas the 33.0 ppm peak of about equal intensity is assigned to those CH2 groups of the bulk isoprene units that are trans relative to a methine proton (Fig. 1 and Table III). The smaller carbon cross-peak at 40.5 ppm arises from the cis-CH2 groups of the ω-1 and ω-2 isoprene units (Fig. 1). The small cross-peak at 32.8 ppm arises from the trans (distal) CH2 group of the α isoprene unit (Fig. 1 and Table III).

The CH3 protons yield six distinct HMQC peaks (Fig. 9B). The cis-CH3 protons of the α isoprene unit (1.74 ppm), six of the seven cis-CH3 protons of the interior β to ω–3 isoprene units (1.70 ppm), and the remaining interior cis-CH3 group that overlaps with the cis-CH2 of the ω unit (1.69 ppm) yield distinct carbon cross-peaks at 23.8, 24.1, and 26.3 ppm, respectively (also see Table III). The three trans methyl groups of the ω-2, ω-1, and ω-3 isoprene units yield three distinct carbon cross-peaks at 16.8, 16.8, and 18.2 ppm, respectively (Fig. 9B and Table III).

**Evaluation of the Carbon Structure of the Donor Lipid by HMBC Spectroscopy**—The HMBC multibond correlations in the sugar region (Fig. 10A) verify the L-Ara4N assignments derived from the COSY and HMQC experiments. For example, H-5 at 3.96 ppm shows distinct multibond correlations to C-4 (51.0 ppm), C-3 (70.8 ppm), and C-1 (99.5 ppm). The HMBC correlations also yield a complete analysis of the α isoprene unit. The CH2OP proton signal at 4.44 ppm (also see Fig. 8A and Table III) shows distinct multibond correlations to carbon resonances at 122.8 ppm (the methine carbon of the α isoprene unit) and 142.0 ppm (the quaternary carbon of the α isoprene unit). The methine proton signal at 5.41 ppm yields multibond carbon peaks at 23.8 ppm (the cis-CH3 group of the α isoprene unit) and at 32.8 ppm (the distal trans CH2 of the α isoprene unit). Scrutiny of the cross-peaks from 1.74 ppm methyl proton signal (Fig. 10B) shows the corresponding multibond correlations to 32.8, 122.8, and 142.0 ppm, thus verifying the assign-

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**Table III**

| Isoprene Unit | Methyl Groups | δC  | δH [multiplicity, J(Hz)] |
|--------------|--------------|-----|-------------------------|
| α            | H_3C(α)      | 23.8| 1.743 [d, 1.0 Hz]       |
| β to α-3     | H_3C(β)      | 24.1| 1.698, 6 methyl groups  |
| α            | H_3C(α)      | 26.3| 1.686, 1 methyl group   |
| α-1, α-2     | H_3C(α)      | 16.8| 1.629, 1 methyl group   |
| α            | H_3C(α)      | 18.2| 1.610, 1 methyl group   |

| Isoprene Unit | Methylene Groups | δC  | δH [multiplicity, J(Hz)] |
|--------------|-----------------|-----|-------------------------|
| α            | (αβ)POH_2C      | 63.5| 4.446 [dd, 6.8, 6.8**]  |
| β to α       | (αβ)H_2         | 32.8| 2.11                    |
| β to α-3     | (αβ)H_2         | 33.0| 2.05                    |
| α-1 to α-2   | (αβ)H_2         | 40.5| 1.99                    |

| Isoprene Unit | Methine Groups | δC  | δH [multiplicity, J(Hz)] |
|--------------|---------------|-----|-------------------------|
| α            | POH_2CCH_3     | 122.8| 5.41 [t, 6.8]          |
| β to α-3     | CH_3           | 126.8| ~5.15                 |
| α-1 to α-2   | CH_3 or CH_3   | 126.0| ~5.11                |

| Isoprene Unit | Quaternary Carbons | δC  |
|--------------|---------------------|-----|
| α            | POH_2CCH_3         | 142.0|
| β to α-3     | CH_3 or CH_3       | 137.0|
| α-1 to α-2   | CH_3 or CH_3       | 136.5|

The abbreviations used are: adj, adjacent in relation to a methine proton of an isoprene unit; likewise, cis and trans are used throughout to designate the configurations of various groups in relation to a methine proton of an isoprene unit, as shown above.

**Proton-phosphorus coupling constant.**
ment of the 1.74 ppm signal as the cis-methyl group of the \( \alpha \) isoprene unit.

Similarly, the major unresolved CH proton signals (~5.1 ppm) of the undecaprenyl chain yield multibond correlations to various CH\(_3\) and CH\(_2\) carbon resonances between 16 and 41 ppm (Fig. 10A). The major CH\(_3\) and CH\(_2\) proton signals show multibond correlations to methine carbon peaks near 126 ppm and to quaternary carbon signals near 136 ppm (Fig. 10). The trans-CH\(_3\) signals of the \( \omega-1 \) and \( \omega-2 \) units show a distinct connectivity to the cis-CH\(_2\) carbons at 40 ppm, thus verifying assignment of the upfield shifted CH\(_2\) proton signals (at 2.0 ppm) as cis-CH\(_2\) groups of the \( \omega-1 \) and \( \omega-2 \) units of the undecaprenyl chain. The interior \( \beta \) to \( \omega-3 \) cis-CH\(_3\) signals at 1.70 and 1.69 ppm show multibond correlations exclusively to the CH\(_2\) carbons at 33.0 ppm, and not to the CH\(_2\) carbons at 27.0 ppm, thus yielding the assignment of the 33.0 ppm CH\(_2\) carbon signals to the CH\(_2\) groups trans from the CH\(_3\) proton (Table III). Finally, multibond correlations are seen in Fig. 10B from the trans CH\(_2\) proton signals at 2.06 ppm to the adjacent CH\(_2\) carbon signals at 27.0 ppm, and from the adjacent CH\(_2\) proton signals at 2.08 ppm to the trans CH\(_2\) carbon signals at 33.0 ppm. This cross-peak pattern is the reverse of that observed in the direct correlation experiment (Fig. 9B).

The \(^1\)H and \(^{13}\)C NMR assignments derived for the sugar and the undecaprenyl moieties of the donor lipid are summarized in Tables II and III. The \(^{13}\)C NMR assignments derived for the \( \omega \) to \( \omega-2 \) units of the undecaprenyl chain are in excellent agreement with the \(^{13}\)C NMR assignments of farnesol, \( \beta \)-farnesene, \( \beta \)-springene, elegantediol, and other linear terpenes (60, 61). Taken together with the coupling constants and the mass spectrometry, the results provide unequivocal proof for the novel glycolipid structure proposed in Fig. 1, i.e. undecaprenyl phosphate-\( \alpha \)-L-Ara4N.

Reconstitution of L-Ara4N Transferase Activity in Vitro with Purified Undecaprenyl Phosphate-\( \alpha \)-L-Ara4N—Transfer of the L-Ara4N moiety to lipid IV\(_A\) to form lipid II\(_A\) proceeds rapidly when ArnT is overexpressed in the polymyxin-resistant \( E. coli \) host BLR(DE3) (Fig. 11) (25). However, when ArnT is overexpressed in the \( E. coli \) K-12 strain NovaBlue(DE3), transferase activity is not observed (Fig. 11). Like all other \( E. coli \) K-12 strains, NovaBlue(DE3) does not synthesize the L-Ara4N donor lipid (data not shown) and is sensitive to polymyxin (25). However, addition of purified undecaprenyl phosphate-\( \alpha \)-L-Ara4N to membranes of \( E. coli \) K-12 NovaBlue(DE3) cells overexpressing arnT reconstitutes robust lipid II\(_A\) formation in vitro in a concentration-dependent manner, whereas donor lipid addition to membranes from the vector control cells NovaBlue(DE3)/pET21 does not (Fig. 11). Efficient reconstitution was also seen.
when using membranes of E. coli K-12 NovaBlue(DE3) overexpressing S. typhimurium arnT (data not shown). ArnT activity, which was absent in a pmrE− or pmrF− derivative of a pmrAC mutant of S. typhimurium, was likewise recovered by the addition of purified donor lipid to membranes (data not shown). These findings, in conjunction with product analysis presented in the accompanying article (25), demonstrate conclusively that the addition of the L-Ara4N moiety to lipid A requires undecaprenyl phosphate-α-L-Ara4N as the donor substrate.

**DISCUSSION**

The lipid A moiety of lipopolysaccharide of certain Gram-negative bacteria can be modified with the positively charged sugar L-Ara4N (58, 62–66) in a regulated manner (26, 28, 55). By masking of the negative phosphate groups of lipid A, the L-Ara4N residue prevents binding of cationic antimicrobial peptides and polymyxin to the outer membrane, rendering the bacteria resistant to killing by these substances (30–32, 37). In the intracellular pathogen S. typhimurium, modification with the L-Ara4N unit may occur on both the 1- and the 4-phosphogroups of lipid A (53, 58). Modification of the 4-position is predominant under most conditions (52, 53) but appears to be Kdo-dependent.

As was suggested by our bioinformatic analysis of the pmrF operon (26), a polysoprenolinked intermediate is required for the transfer of the L-Ara4N moiety to lipid A. Here we have presented the identification, purification, and biophysical characterization of this novel substance, which is now properly described as undecaprenyl phosphate-α-L-Ara4N or undecaprenyl 4-amino-4-deoxy-α-L-arabinopyranosyl phosphate. This material was first detected as a minor phospholipid that accumulates in the polymyxin-resistant mutant of E. coli (Fig. 2). In S. typhimurium, the lipid is also present in wild-type cells, but its level is increased in pmrAC and PhoP−/−mutants. It is absent in pmrC− strains (Fig. 3).

The purification to near-homogeneity of the donor lipid required as a first step the construction of a special pmrAC mutant of E. coli lacking phosphatidyethanolamine to simplify all subsequent procedures (Figs. 4–6). MALDI/TOF mass spectrometry and high resolution NMR spectroscopy were then used for the actual structure determination. As noted in previous NMR studies of lipid A, the mixture CDC18/CDDOD/D2O (2:3:1, v/v) is ideal for NMR analyses of small lipid samples (51, 52, 67), because there is no measurable decomposition for weeks under these conditions. The data (shown in Figs. 7–10, Tables II and III, and see Figs. 1–3 in the Supplemental Material) confirm unequivocally that the L-Ara4N donor substrate has the structure undecaprenyl phosphate-α-L-Ara4N. The proposed structure and conformation of the sugar is supported by its coupling constants and chemical shifts, summarized in Table II, which are in accord with a synthetic standard of L-Ara4N (57). The attachment of the nitrogen atom to position 4 of the sugar is established by the HMBC experiment (Fig. 9A). The size and configuration of the polysoprene chain are confirmed by mass spectrometry (Fig. 7) and by the HMBC/HMBC analyses (Figs. 9 and 10).

Reconstitution of the purified lipid donor with the cloned E. coli or S. typhimurium L-Ara4N transferase (ArnT) resulted in robust *in vitro* conversion of the precursor lipid-IVₐ to lipid-IIₐ (Fig. 11), a compound that contains a single L-Ara4N moiety on its 1-phosphate residue (50, 52, 58). ArnT is a complex enzyme with 12 predicted trans-membrane helices (25) and is a member of an ancient family of glycosyltransferases that includes the eucaryotic protein mannoseyltransferases (23, 68). All the enzymes of this family appear to use polysoprene-linked sugars as their donor substrates (23, 68). However, prior to the present work, identification of undecaprenyl phosphate-α-L-Ara4N and *in vitro* systems for detecting the transfer of L-Ara4N to lipid A or lipid A precursors had not been reported.

Many interesting questions regarding the possible translocation of the donor lipid across the inner membrane for utilization by ArnT in the periplasm remain unanswered. The relevant transporter has not been identified. It is unlikely that Wzx (RfhX), the putative O-antigen flippase (5, 69), is responsible for the translocation of undecaprenyl phosphate-α-L-Ara4N into the periplasm, because Wzx proteins are thought to transport undecaprenyl diphostate oligosaccharides (70). Nevertheless, wzx-deficient mutants should be tested for their sensitivity to polymyxin. Most importantly, perhaps, in *in vitro* systems for measuring flip-flop of undecaprenyl phosphate-α-L-Ara4N must be devised. A new enzymatic system for the conversion of UDP-glucuronic
acid to UDP-\(\iota\)-Ara4N has recently been identified in extracts of polymyxin-resistant mutants of \(E.\ coli\), and should facilitate the preparation of undecaprenyl phosphate-\(\iota\)-Ara4N.

Acknowledgments—We thank Professor Lauren Anderson of the University of Wisconsin, Madison, for the long term interest in our work and helpful advice. The Duke University NMR Center was supported in part by NCI Grant P30-CA14236 from the National Institutes of Health (to A. A. Ribeiro). Instrumentation in the Duke NMR Center was funded by grants from the National Science Foundation, the National Institutes of Health, the North Carolina Biotechnology Center, and Duke University.

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