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M. Stephen Trent
Duke University Medical Center

Anthony A. Ribeiro
Duke University Medical Center

William T. Doerrler
Duke University Medical Center

Shanhua Lin
Johns Hopkins School of Medicine

Robert J. Cotter
Johns Hopkins School of Medicine

See next page for additional authors

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Authors

M. Stephen Trent, Anthony A. Ribeiro, William T. Doerrler, Shanhua Lin, Robert J. Cotter, and Christian R.H. Raetz

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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M. Stephen Trent^{‡§}, Anthony A. Ribeiro^{‡¶}, William T. Doerrler[‡], Shanhua Lin^{||}, Robert J. Cotter^{||},
and Christian R. H. Raetz^{‡**}

From the [‡]Department of Biochemistry and the [¶]Duke NMR Spectroscopy Center and Department of Radiology, Duke University Medical Center, Durham, North Carolina 27710 and the ^{||}Middle Atlantic Mass Spectrometry Laboratory, Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2185

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 *pss⁻pmrA^C* 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 ($M_r = 978.41$). 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 anomeric 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 IV_A to lipid II_A, 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.

Polyisoprene-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

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains Figs. 1–3.

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** To whom correspondence should be addressed. Tel.: 919-684-5326; Fax: 919-684-8885; raetz@biochem.duke.edu.

¹ The abbreviations used are: L-Ara4N, 4-amino-4-deoxy-L-arabinose; pEtN, phosphoethanolamine; Mes, 2-[N-morpholino]ethanesulfonic acid; Kdo, 3-deoxy-D-manno-octulosonic acid; MALDI/TOF mass spectrometry, matrix-assisted laser desorption ionization/time of flight mass spectrometry; HMQC, heteronuclear multiple-quantum coherence spectroscopy; HMBC, heteronuclear multiple-bond correlation spectroscopy.

² A systematic chemical name for undecaprenyl phosphate- α -L-Ara4N would be undecaprenyl 4-amino-4-deoxy- α -L-arabinopyranosyl phosphate.

TABLE I
Bacterial strains and plasmids

Strains and plasmids	Description	Source or Ref.
<i>S. typhimurium</i>		
ATCC 14028s	Wild type	ATCC
CS022	<i>pho-24</i> (PhoP-constitutive)	71
CS015	CSO22, <i>phoP102::Tn10d-cam</i>	54
CS330	CSO22, <i>pagP::TnphoA</i>	72
JSG435	<i>pmrA505 zjd::Tn10d-cam</i> (PmrA-constitutive)	33
JSG421	<i>pmrA::Tn10d</i>	33
<i>E. coli</i>		
W3110	Wild type, F ⁻ , λ^-	<i>E. coli</i> Genetic Stock Center, Yale University
WD101	W3110, <i>pmrA^C</i>	This work
WD102	<i>zjd-2211::Tn10</i> derivative of WD101 (AKK211 donor)	This work
AKK211	λ^- , <i>relA1</i> , <i>spoT1</i> , <i>ilv-299</i> , <i>metB1</i> , <i>zjd-</i> , <i>2211::Tn10</i> , <i>ampCp-1</i> , <i>mcrB⁻</i> , <i>creC510</i>	<i>E. coli</i> Genetic Stock Center, Yale University
AD90	<i>pss93::kan nadB⁺</i>	46
WD901	<i>pmrA^Czjd-2211::Tn10</i> derivative of AD90 (WD102 donor)	This work
BLR(DE3)	Δ (<i>srl-recA</i>)306::Tn10(DE3), Tet ^r	Novagen
NovaBlue(DE3)	Δ (<i>srl-recA</i>)306::Tn10(DE3), Tet ^r	Novagen
Plasmids		
pET21a	Vector containing a T7lac promoter, Amp ^r	Novagen
pArnTEc	pET21a containing <i>E. coli arnT</i>	25
pArnTSt	pET21a containing <i>S. typhimurium arnT</i>	25
pDD72	<i>pss⁺</i> covering plasmid with temperature-sensitive <i>ori</i> , Cam ^r	46
pT7Blue-3	Cloning vector, Kan ^r , Amp ^r	Novagen
pWTD5	pT7Blue-3 containing <i>pmrA/pmrB</i> operon from wild-type W3110	This work
pWTD6	pT7Blue-3 containing <i>pmrA/pmrB</i> operon from WD101	This work
pLysS	pACYC184 encoding T7 lysozyme, Cam ^r	Novagen

support the proposal (25, 26) that the donor lipid has the structure undecaprenyl phosphate- α -L-Ara4N (Fig. 1). The unambiguous demonstration of an undecaprenyl-linked intermediate indicates that lipid A modification with the L-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_i 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 bicinchoninic 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 Mallinckrodt.

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 μ g/ml ampicillin, 12 μ g/ml tetracycline, 30 μ g/ml chloramphenicol, or 30 μ g/ml kanamycin.

Preparation of Radiolabeled Substrates—[4'-³²P]Lipid IV_A was prepared using 100 μ Ci 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 Kdo₂[4'-³²P]lipid IV_A, 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 A₆₀₀ = 1.0 at 37 °C and harvested by centrifugation at 7,000 \times 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 \times g for 15 min. Membranes were prepared by ultracentrifugation at 149,000 \times 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 149,000 \times g centrifugation step was subjected to a second ultracentrifugation to remove residual membrane particles. All samples were stored as aliquots at -80 °C. Protein concentrations were determined with bicinchoninic acid (44) using bovine serum albumin as the standard.

L-Ara4N Transferase (ArnT) Assay Conditions—The L-Ara4N transferase (ArnT) was assayed under optimized conditions in a 10- μ l reac-

tion mixture containing 50 mM Mes, pH 6.5, 0.2% Triton X-100, and 10 μ M of [4'-³²P]lipid IV_A (20,000 cpm/nmol) (25). Reactions were incubated at 30 °C at the indicated protein concentration and times and were stopped by spotting 5- μ l portions onto a Silica Gel 60 TLC plate. The substrate and product(s) were separated by developing the plate with chloroform, pyridine, 88% formic acid, 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 nmol/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 μ g/ml of the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (40). Approximately 10⁷ cells were plated on LB agar containing 2 μ g/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 μ g/ml polymyxin B sulfate. The polymyxin resistance phenotype of a representative isolate was moved into a wild-type background (W3110) by P1vir transduction (40) by selecting directly for polymyxin resistance at 10 μ g/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 of single colonies on LB agar containing 10 μ g/ml polymyxin. 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'-CAGGCTGCG-GATGATATTCTGC-3' and 5'-GTTTAACCTACCGTGTTCAGCGTG-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 (*pmrA^C*). No changes were found in the *pmrB* sequence.

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

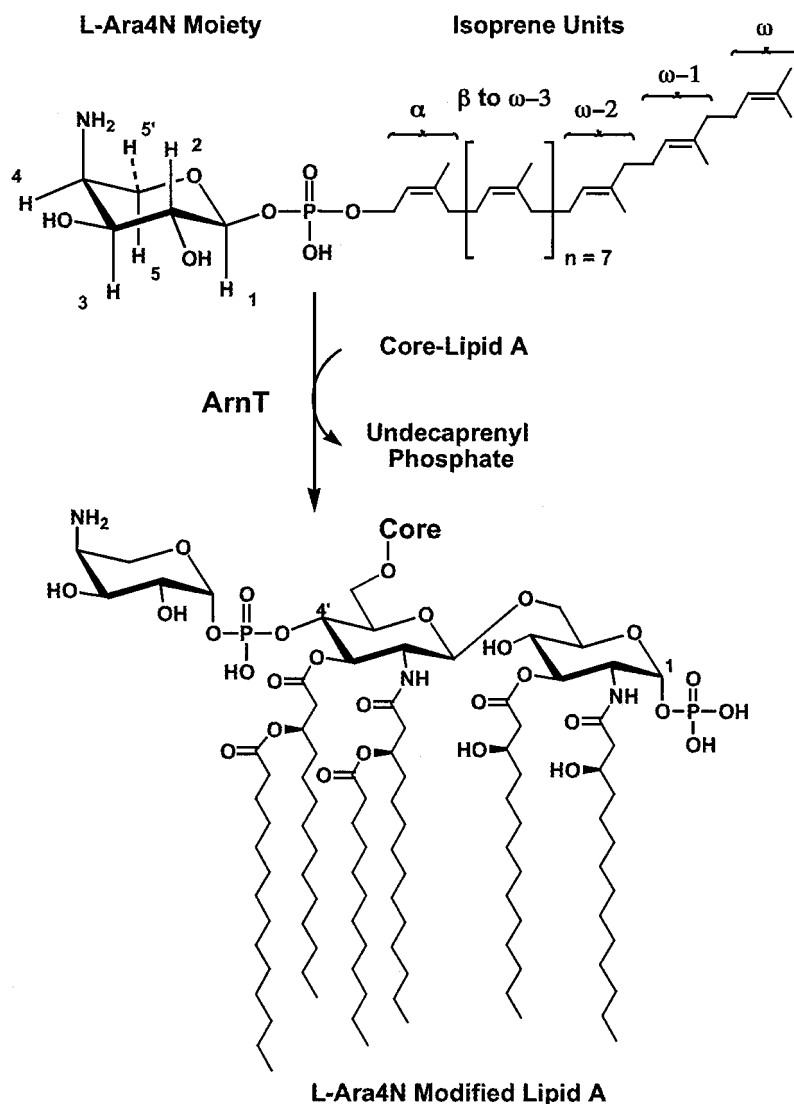


FIG. 1. Structure of undecaprenyl phosphate- α -L-Ara4N and proposed transfer of the L-Ara4N moiety to core-lipid A by ArnT. The numbering scheme for the undecaprenyl phosphate- α -L-Ara4N molecule is used for the NMR analysis presented. The 4'- and 1-positions of lipid A are also indicated. The active 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_A (not shown), ArnT incorporates the L-Ara4N unit exclusively at the 1-position (25).

pss⁺ covering plasmid pDD72 grow 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 MgCl₂ (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 desired strain, WD102 (*pmrA*^C *zjd-2211::Tn10*), was used to generate another P1vir lysate with which the *pmrA*^C allele could be transferred into AD90/pDD72 by selecting for tetracycline resistance (12 µg/ml) at 30 °C on LB agar, followed by screening of individual colonies for polymyxin resistance at 10 µg/ml as the unselected marker. A *pmrA*^C *zjd-2211::Tn10* transducant of AD90/pDD72 was then cured of its covering plasmid at 42 °C, as described previously (46), generating the phosphatidylethanolamine-deficient strain WD901 (*pss93::kan pmrA*^C *zjd-2211::Tn10*). Like AD90, WD901 grew slowly on LB broth in the presence of 50 mM MgCl₂.

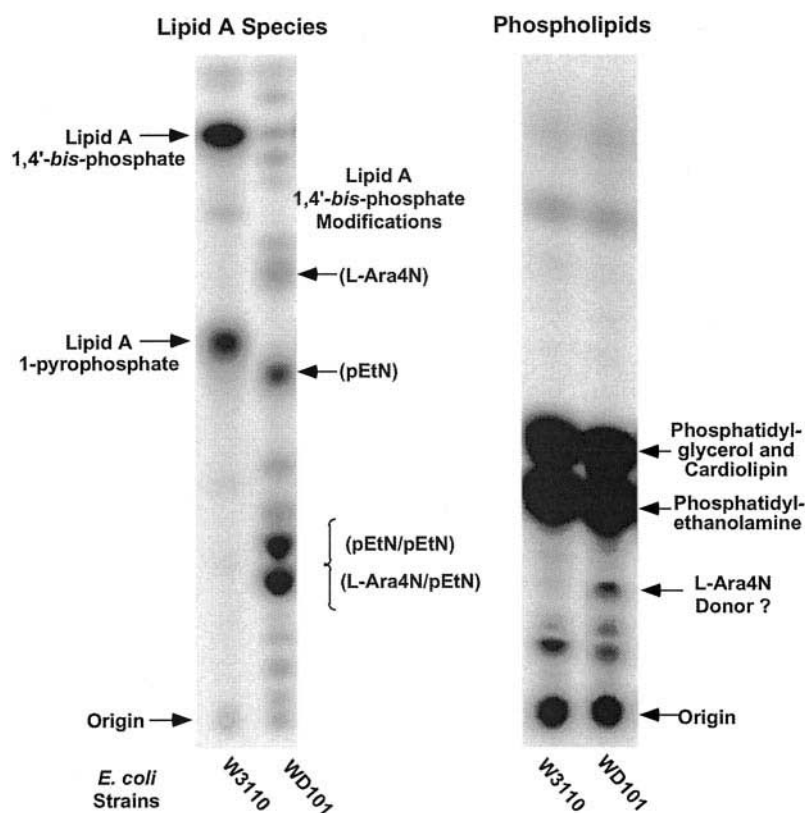
Extraction and Separation of Phospholipids from ³²P_i-Labeled Cells—Log phase cells were uniformly labeled with 5 µCi/ml of ³²P_i in 5 ml of LB broth (or LB broth containing 50 mM MgCl₂ when appropriate) at a starting A₆₀₀ of 0.05. Cells were grown in a rotary shaker at 37 or 30 °C as indicated and harvested when A₆₀₀ reached ~1.0. The cells were collected using a clinical centrifuge and washed with 5 ml of phosphate-buffered saline, pH 7.4. To extract the ³²P-labeled phospholipids, the cell pellet was resuspended in 3 ml of a single-phase Bligh/Dyer mixture (47), consisting of chloroform/methanol/water (1:2:0.8, v/v). After mixing and incubating for 60 min at room temperature, the insoluble material was removed by centrifugation, and the supernatant containing the ³²P-labeled phospholipids was transferred to a clean

glass tube. The supernatant was converted 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 N₂ and redissolved in a small volume of chloroform/methanol (4:1, v/v). The ³²P-labeled phospholipids (100,000 cpm/lane) were separated on Silica Gel 60 TLC plates in the solvent chloroform/methanol/water/NH₄OH (65:25:3.6:0.4, v/v). The plate was dried and exposed to a Phosphor-Imager screen overnight to visualize the ³²P-containing bands.

Extraction of Lipid A from ³²P_i-Labeled Cells—³²P_i-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 ³²P-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 ³²P-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, v/v). The plate was dried and exposed to a PhosphorImager screen overnight to visualize the ³²P-lipid A species.

Large Scale Purification of the Putative Donor Lipid Undecaprenyl Phosphate-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 MgCl₂ to A₆₀₀ of ~2.0. This culture was used to inoculate four 2-liter cultures, each in a 6-liter Erlenmeyer flask, at a starting A₆₀₀ of 0.05. The cells were grown in a rotary shaker at 37 °C until A₆₀₀ 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-

FIG. 2. Accumulation of a novel minor phospholipid in the polymyxin-resistant *E. coli* mutant WD101. 32 P-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), and the phospholipids were separated in chloroform/methanol/water/ NH_4OH (65:25:3.6:0.4, v/v). The position of the putative L-Ara4N donor lipid, seen only in the polymyxin-resistant mutant, is indicated.



sion was divided equally into six 125-ml Corex tubes. The content of each tube was converted into a single phase Bligh/Dyer system by addition of 25 ml of chloroform and 50 ml of methanol. After mixing and 60 min of incubation at room temperature, the insoluble material was removed by centrifugation at $4000 \times g$ for 15 min. The supernatants were combined (570 ml final volume) and then divided equally into eight clean 125-ml Corex centrifuge tubes. The content of each tube was converted to a two-phase Bligh/Dyer system by adding 18.75 ml of chloroform and 18.75 ml of water. After mixing, the phases were separated by centrifugation at $4000 \times g$ for 15 min. The lower phases, containing the phospholipids and the putative L-Ara4N donor lipid, were pooled and dried by rotary evaporation.

The residue was dissolved in 60 ml of chloroform/methanol/water (2:3:1 v/v) and subjected to a 30-s sonic irradiation in a bath apparatus. The sample was then applied to a 20-ml DEAE-cellulose column in the acetate form at room temperature, pre-equilibrated, and washed with 200 ml of chloroform/methanol/water (2:3:1 v/v) as described previously (26, 49, 50). Fractions of 5 ml were collected. After the flow-through, the column was washed with another 60 ml of chloroform/methanol/water (2:3:1 v/v). The wash was followed by four separate 60-ml elution steps, using the 2:3:1 system with the aqueous phases containing either 60, 120, 240, or 500 mM ammonium acetate in ascending order. For each fraction emerging from the column, a 30- μ l sample was spotted onto a TLC plate, and the lipids were separated using chloroform/methanol/water/ NH_4OH (65:25:3.6:0.4, v/v). The presence or absence of the donor lipid and other phospholipids was detected by charring with 10% sulfuric acid in ethanol. The putative donor lipid, which was initially identified by its presence as a minor component among the lipids of a *pmrA*^C mutant and its absence in wild type, emerged in the flow-through and the first 25 ml of the wash fractions, consistent with its net charge of zero (Fig. 1). Fractions containing the donor lipid were pooled (85 ml) and converted to a two-phase Bligh/Dyer system (see above). The phases were separated by low speed centrifugation, and the lower phase, which contained the donor lipid, was dried by rotary evaporation.

Reverse phase C_{18} chromatography was used as the second step of the purification. Solvent A, consisting of acetonitrile/water (1:1, v/v), and solvent B, consisting of isopropanol/water (85:15, v/v), were used in various ratios, and all solutions contained 10 mM tetra-butyl ammonium dihydrogen phosphate. A 4.0-ml C_{18} column was first equilibrated using 40 ml of solvent A/B (1:1, v/v). The DEAE-cellulose purified donor lipid fraction was dissolved in 12 ml of solvent A/B (1:1, v/v), subjected

to sonic irradiation for 60 s, and applied to the C_{18} column. The flow-through was saved, and the column was washed stepwise with 40-ml portions of A/B solvent mixtures as follows: A/B (1:1, v/v), A/B (1:2, v/v), A/B (1:4, v/v), A/B (1:6, v/v), and A/B (1:10, v/v). All fractions were 4 ml, and 30 μ l of each was spotted onto a TLC plate, after which the lipids were separated in the system chloroform/methanol/water/ NH_4OH (65:25:3.6:0.4, v/v). Lipids were detected by charring with 10% sulfuric acid in ethanol. The donor lipid was found to elute in the A/B (1:10, v/v) step. The fractions containing the putative donor lipid were pooled (~10 ml) and dried immediately by rotary evaporation.

An additional fractionation on silicic acid was necessary to obtain pure donor lipid. The above residue was dissolved in 10 ml of chloroform/methanol (95:5, v/v) and loaded onto a 1-ml acid-washed silicic acid column (Bio-SilA from Bio-Rad), equilibrated in chloroform/methanol (95:5, v/v). After the flow-through, the column was washed with 5 ml of chloroform/methanol (95:5, v/v). The lipid was eluted using 5 ml of chloroform/methanol (80:20, v/v), and the appropriate fractions (1 ml each) were dried by rotary evaporation. To remove tetra-butyl ammonium dihydrogen phosphate carried over from the C_{18} column, the solvent was converted into a two-phase Bligh/Dyer system (as described above). The lower phase, containing the donor lipid, was washed 3 times with fresh upper phase. Although a small amount of residual tetra-butyl ammonium dihydrogen phosphate remained behind, as judged by NMR studies (see below), about 98% was removed by a single two-phase partitioning.

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 (Kompact 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 $M_r \sim 2000$. Saturated 6-aza-2-thiothymine in 50% acetonitrile and 10% tribasic ammonium citrate (9:1, v/v) 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 $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{D}_2\text{O}$ (2:3:1, v/v) in a 5-mm NMR tube. NMR spectra were obtained at 25 $^\circ\text{C}$ using Varian Inova 800, Inova 600, or Unity 500 spectrometers, as indicated, equipped with

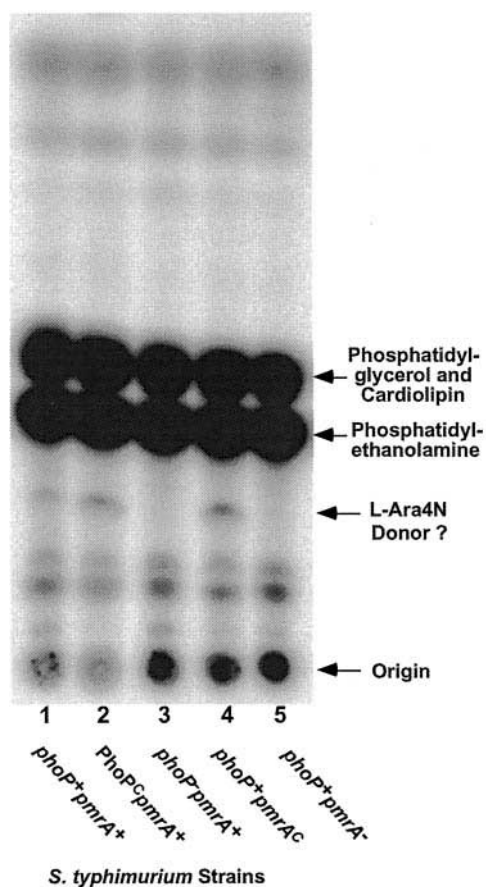


FIG. 3. The presence of the putative L-Ara4N donor lipid is regulated by the PmrA and PhoP/PhoQ systems of *S. typhimurium*. 32 P-labeled phospholipids from each strain of *S. typhimurium* were separated using the system chloroform/methanol/water/ NH_4OH (65:25:3.6:0.4, v/v) and were visualized with a PhosphorImager.

Sun Ultra 10 computers and 5-mm Varian probes. The ^2H signal of CD_3OD was used for a field frequency lock. Use of the $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{D}_2\text{O}$ solvent system introduces four solvent resonances. The signals from CH_3OD (3.3 ppm) and CHCl_3 (7.6 ppm) do not overlap with the donor lipid resonances. The HOD (4.6 ppm) and CD_3OH (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 μs), 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 μs), 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 obtain a digital resolution of 0.20 Hz/point. ^{31}P NMR spectra at 202 MHz (500 MHz field) and selective inverse (^{31}P) 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-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 $\mu\text{g}/\text{ml}$ 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

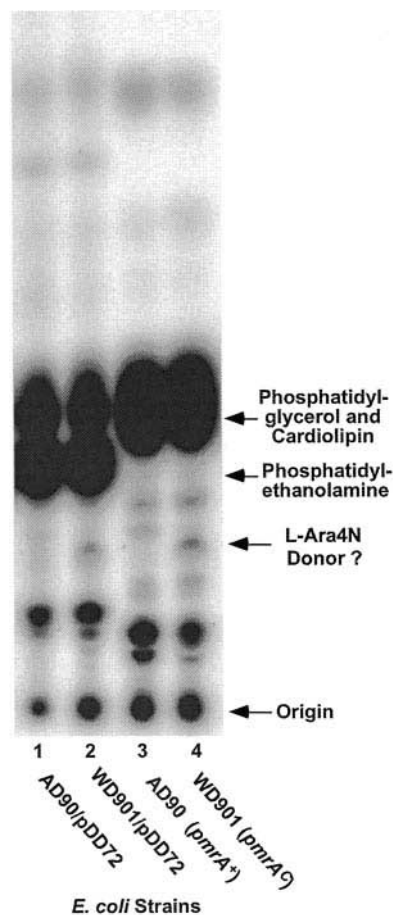


FIG. 4. Presence of the putative L-Ara4N donor lipid in a *pmrA*^C mutant of *E. coli* lacking phosphatidylethanolamine. 32 P-labeled phospholipids were isolated and separated by TLC using the system chloroform/methanol/water/ NH_4OH (65:25:3.6:0.4, v/v) and were visualized with a PhosphorImager.

that two amino acids were altered in the transcription factor PmrA (A42T and G53E). No mutations were present in *pmrB*.

TLC analysis of ^{32}P -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 ^{32}P -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, a minor ^{32}P -labeled substance, designated “L-Ara4N Donor,” accumulates in WD101 to about 0.2–0.4% of the total (Fig. 2, right panel).

The presence or absence of this putative L-Ara4N donor lipid was further investigated using well characterized strains of *S. typhimurium* containing mutations in the *phoP*/*phoQ* or *pmrA*/*pmrB* systems (28, 29, 33, 54). As in WD101, the putative donor lipid accumulates to the highest levels in *S. typhimurium* strains in which PmrA is constitutively active (*pmrA*^C) (Fig. 3, lane 4). However, in contrast to *E. coli* W3110 (Fig. 2), the lipid is also detectable in wild-type *S. typhimurium* (Fig. 3, lane 1).

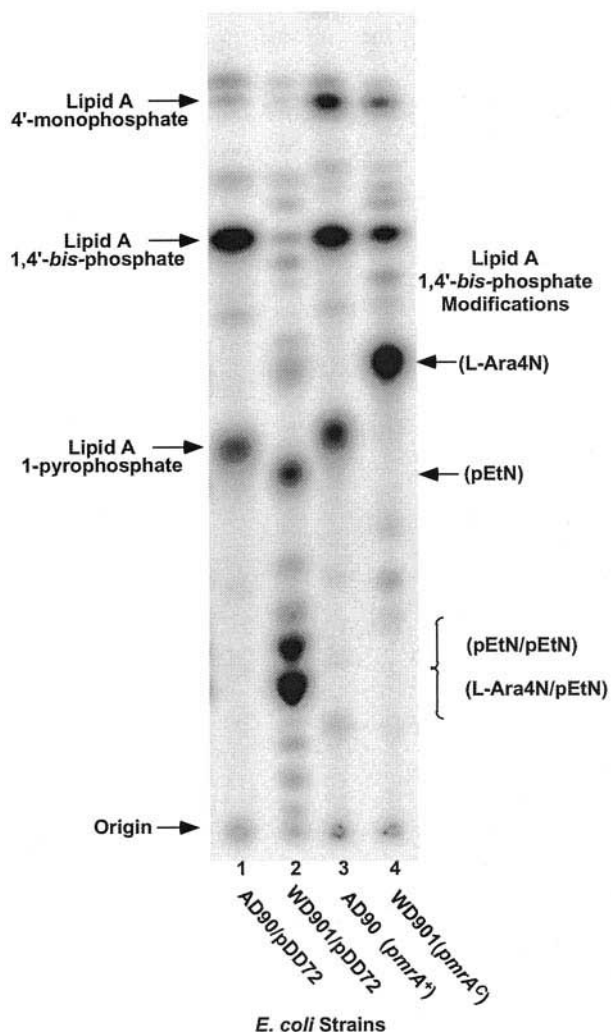


FIG. 5. Formation of pEtN-modified lipid A species requires phosphatidylethanolamine in *E. coli*. The ^{32}P -labeled lipid A species from the indicated strains were isolated as described under "Experimental Procedures," separated by TLC using the solvent chloroform, pyridine, 88% formic acid, water (50:50:16:5, v/v), and visualized with a PhosphorImager. A small amount of lipid A 4'-monophosphate may arise as a decomposition product during pH 4.5 hydrolysis at 100 °C to remove the Kdo moiety.

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 *pmrA*^C mutants (26, 53).

The putative donor lipid was absent in a *S. typhimurium pmrA*⁻ mutant (Fig. 3, lane 5) and was also missing in *pmrA*^C 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 PhoP^C 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*^C *E. coli* Mutant (WD901) Lacking Phosphatidylethanolamine—Purification of the putative donor lipid from WD101 or from a *pmrA*^C mutant of *S. typhimurium* was not feasible because it could not be separated from phosphatidylethanolamine, the major phospholipid of these bacteria (56),

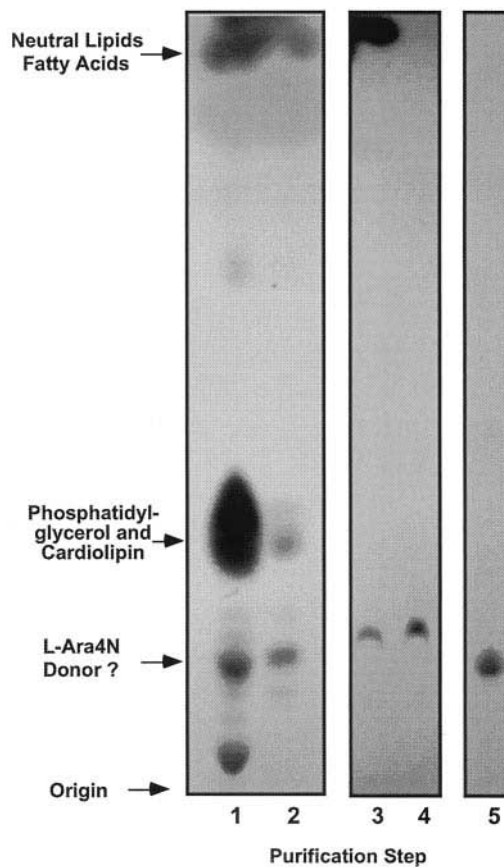


FIG. 6. Purification of the putative L-Ara4N donor lipid from a *pmrA*^C mutant of *E. coli* lacking phosphatidylethanolamine. The lipids at each step of the purification were separated by TLC in the system chloroform/methanol/water/ NH_4OH (65:25:3.6:0.4, v/v) and were visualized by charring after spraying the plate with 10% sulfuric acid in ethanol. Lane 1, unfractionated phospholipids of WD901 (*pmrA*^C*pss*⁻); lane 2, DEAE-cellulose column flow-through; lane 3, after C_{18} reverse phase chromatography; lane 4, after silicic acid chromatography; lane 5, after removal of the residual tetra-butyl dihydrogen ammonium phosphate by two-phase Blich/Dyer partitioning.

in various large scale chromatography steps (data not shown). To solve this problem, the *pmrA*^C allele of WD101 was transferred into *E. coli* AD90/pDD72 (46) by P1vir 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 *pmrA*^C 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 L-Ara4N moiety to lipid A, ^{32}P -labeled lipid A species were prepared from AD90 (*pss*⁻) and WD901 (*pss*⁻*pmrA*^C) 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*⁺*pmrA*^C) (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 L-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 Mg²⁺ (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 zwitterionic character of the donor lipid (Fig. 1). If *pss*⁺ 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/water/isopropyl alcohol system containing tetra-butyl ammonium dihydrogen phosphate, and eluted with increasing amounts of isopropyl alcohol (Fig. 6, lane 3). The final step was chromatography on a 1-ml silicic column, yielding a homogeneous preparation (Fig. 6, lane 4) as judged by TLC analysis. The apparent *R_f* 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]⁻ for the proposed structure (Fig. 1), undecaprenyl phosphate-L-Ara4N (*M_r* = 978.41). No peak was observed at *m/z* 1057.4 atomic mass units, the [M-H]⁻ expected for a hypothetical undecaprenyl diphosphate-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]⁺ (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 α isoprene unit (Figs. 1 and 8A) (18, 19); 3) the major unresolved CH resonances (~5.1 ppm) of the polyisoprene chain (Figs. 8A) (18, 19); and 4) the major partially resolved CH₂ (~2.1 ppm) and CH₃ signals (1.6–1.7 ppm) of the polyisoprene chain (Figs. 9B and 10B, and Fig. 1 in the Supplemental Material) (18, 19).

Mass spectrometry (Fig. 7) indicates the presence of 11 isoprene units in the purified donor lipid. Keeping in mind that the ω isoprene unit contains 2 methyl groups (one *cis* and one *trans* in relation to the ω methine proton), a total of 12 methyl groups are expected (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 \times at the top of Figs. 9B and 10B), the ¹H NMR signals

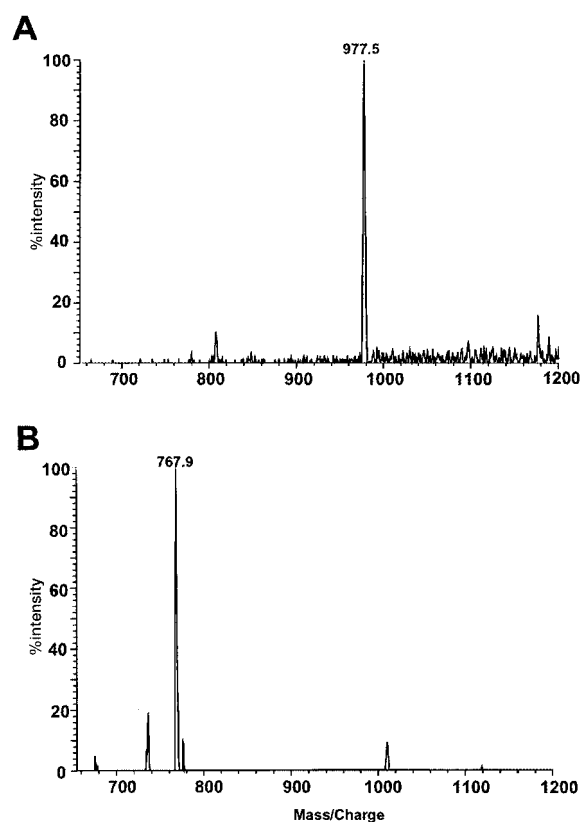


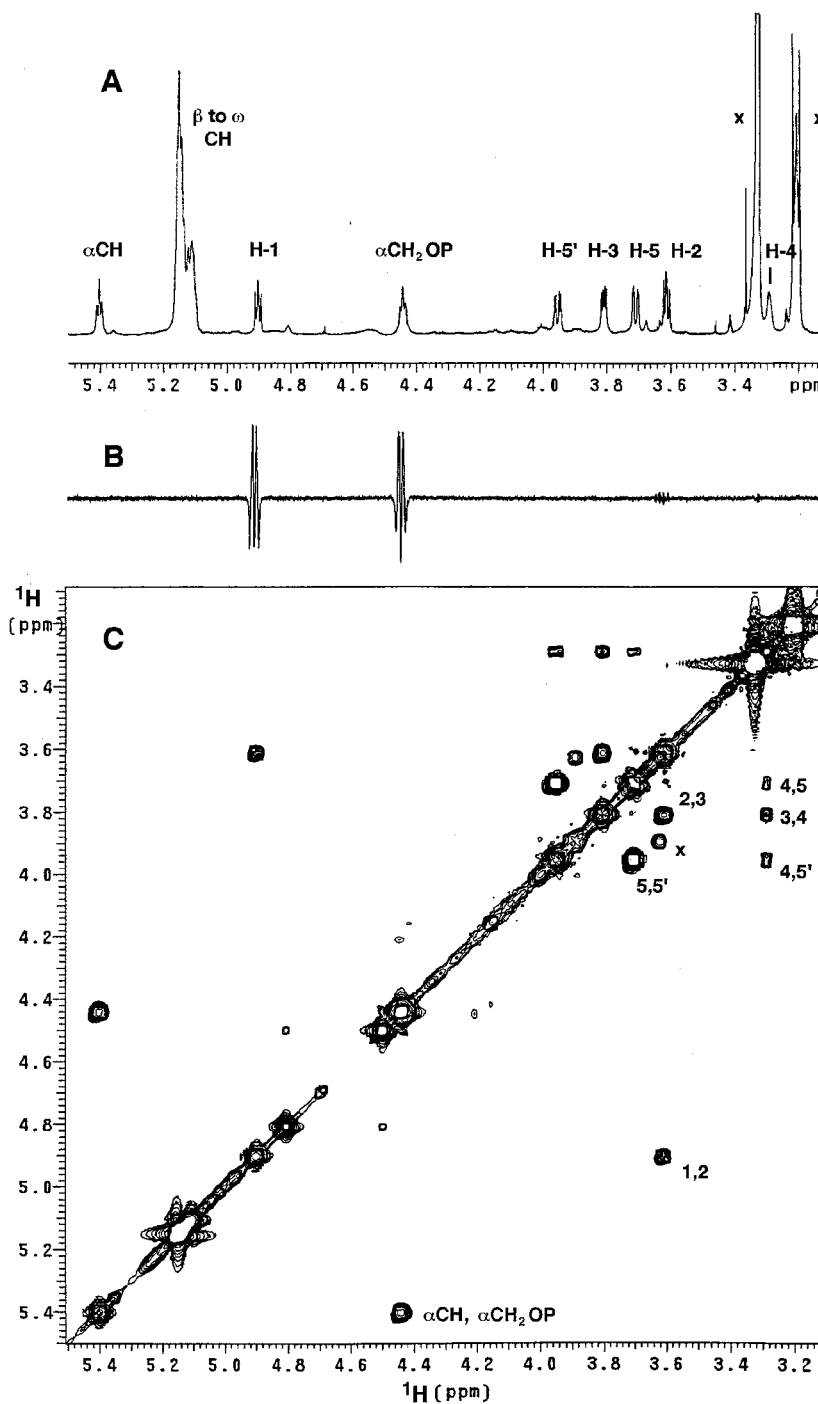
FIG. 7. MALDI/TOF mass spectrometry of the purified donor lipid. The purified material was subjected to mass spectrometry in the negative ion (A) or positive ion (B) modes. The molecular weights of undecaprenyl phosphate-L-Ara4N, undecaprenyl phosphate, and undecaprenol are 978.41, 847.28, and 767.30, respectively.

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 methine 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 ω 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 ω -2, ω -1, and ω 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 CDCl₃/CD₃OD/D₂O (2:3:1, v/v) for weeks and display sharp, well resolved resonances.

The positions of the individual protons of the arabinose sugar were derived from a two-dimensional COSY analysis (Fig. 8C, Table II, and Fig. 1 in the Supplemental Material). The doublet at 4.90 ppm (*J*_{1,2} = 6.4, *J*_{H1,P} = 7.8 Hz) is assigned to the anomeric H-1 proton of the L-Ara4N moiety (57). The COSY cross-peak from H-1 (Fig. 8C) locates the H-2 signal at 3.61 ppm (dd, *J*_{1,2} = 6.4, *J*_{2,3} = 8.0 Hz). A second COSY cross-peak from H-2 connects to H-3 at 3.81 ppm (dd, *J*_{2,3} = 8.0, *J*_{3,4} = 4.3 Hz) which, in turn, is coupled to H-4 at 3.29 ppm (broad

FIG. 8. High resolution ^1H NMR spectra of the purified donor lipid. **A**, the partial one-dimensional 800-MHz ^1H NMR spectrum of the donor lipid in $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{D}_2\text{O}$ (2:3:1, v/v) at 25 °C shows the six resolved L-Ara4N protons (H-1 through H-5'), the methine and proximal methylene protons of the α isoprenyl unit, and the bulk unresolved isoprenyl methine protons. This spectrum was obtained using a presaturation sequence to remove the HOD (4.8 ppm) and CD_3OH (4.5 ppm) resonances, giving a clear sugar region. The large signal at 3.34 ppm (x) arises from residual CD_2HOD , and the multiplet at 3.20 ppm (x) arises from the C-1 methylene protons (the ones closest to the N atom) of the butyl chains of residual tetra-butyl ammonium dihydrogen phosphate carried over from the reverse phase step of the purification. **B**, the selective inverse decoupled difference ^1H NMR spectrum obtained at 500 MHz with on and off resonance ^{31}P decoupling of the -0.44 ppm phosphorus signal shows that the anomeric carbon of L-Ara4N is linked via a phosphodiester to the proximal isoprene unit. **C**, the partial two-dimensional ^1H - ^1H COSY analysis at 800 MHz at 25 °C shows the connectivities between the key sugar and isoprenyl ^1H resonances seen in **A**.



multiplet). Further tracing of the COSY connectivities locate the remaining L-Ara4N protons, *i.e.* H-5 (3.71 ppm; dd, $J_{4,5} = 2.4$, $J_{5',5} = 12.8$ Hz) and H-5' (3.96 ppm; dd, $J_{4,5'} = 4.1$, $J_{5,5'} = 12.8$ 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

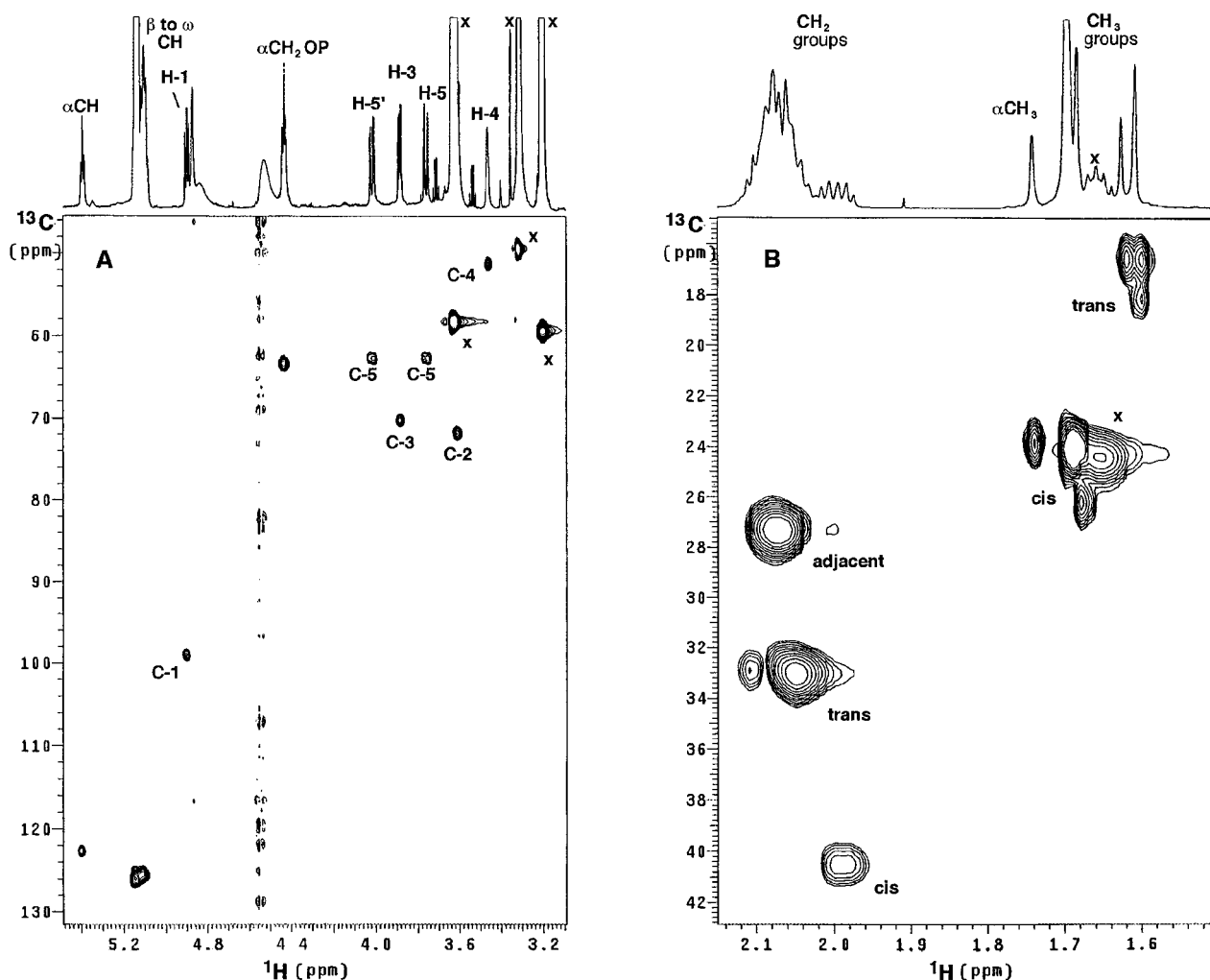


FIG. 9. Partial HMQC spectra of the purified donor lipid. A, single bond ^1H - ^{13}C correlations of the L-Ara4N moiety, part of the proximal isoprene unit, and the unresolved olefinic protons of the undecaprenyl chain are shown. The noise streak at 4.56 ppm arises from the HOD signal. Resonances designated \times are due to residual solvents or other impurities. B, this expansion shows the partially resolved isoprenyl CH_2 and CH_3 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 (\times) at 1.66 ppm partially overlaps with two of the undecaprenyl CH_3 signals of the donor lipid. The donor lipid sample used for the HMQC and HMBC experiments also displayed CH_3 (1.19 ppm) (not shown) and CH_2 (3.63 ppm) signals arising from small amounts of an ethanol impurity in the sample, which resulted in masking of the L-Ara4N H-2 by the ethanol CH_2 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

^1H and ^{13}C NMR assignments and coupling constants (J , Hz) of the L-Ara4N moiety in the purified donor lipid

^1H chemical shifts (ppm from internal TMS) are from one-dimensional ^1H NMR spectra with a digital resolution of 0.2 Hz per point. Coupling constants ($J_{\text{H,H}}$, Hz) and ($J_{1,\text{P}}$, Hz) were obtained from ^{31}P -decoupled ^1H NMR spectra. ^{13}C chemical shifts (ppm from internal TMS) are estimated from 2D HMQC spectra.

Ara4N	H-1 ($J_{1,2}$) ($J_{1,\text{P}}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4	H-5 ($J_{4,5}$) ($J_{5,5'}$)	H-5' ($J_{4,5'}$) ($J_{5,5'}$)
δH	4.902 (6.4) (7.8)	3.611 (8.1)	3.810 (4.3)	3.292	3.709 (2.4) (12.8)	3.955 (4.1) (12.8)
δC	C-1 99.5	C-2 72.4	C-3 70.8	C-4 51.0	C-5 63.0	

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 equatorial H-4 (below plane) showed NOE cross-peaks to H-3 (axial, below plane) and to both H-5 protons but with a stronger NOE cross-peak to H-5 (axial, below plane) and a weaker NOE to H-5' (equatorial, above plane) (see Fig. 2 in the Supplemental Material). The large coupling (12.8 Hz) be-

tween H-5 and H-5' (Table II) is typical of geminal methylene protons within constrained pyranose rings (57).

Selective ^{31}P -Decoupled ^1H Difference Spectroscopy—The linkage between the undecaprenyl chain and the arabinose sugar unit was investigated. One-dimensional ^{31}P NMR spectroscopy revealed a single phosphorus resonance near -0.44 ppm, consistent with a phosphodiester linkage (see Fig. 3 in the

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 CH_2 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 CH_2 carbon of the α isoprene unit of the undecaprenyl chain (Fig. 1).

Evaluation of the Carbon Structure of the Donor Lipid by HMQC Spectroscopy—To confirm the assignments derived from the ^1H NMR analysis, ^{13}C 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 - ^{13}C correlation map (Fig. 9A) reveals six direct ^1H - ^{13}C single-bond correlations in the sugar region (Table II). The L-Ara4N H-1 signal reveals the anomeric carbon resonance at 99.5 ppm (C-1). The H-5 and H-5' multiplets correlate to a single carbon signal at 63.0 ppm (C-5), whereas the H-2 and H-3 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.

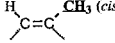
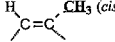
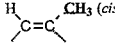
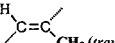
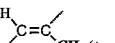
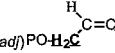
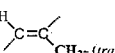
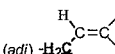
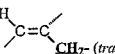
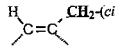
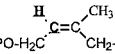
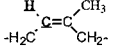
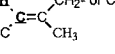
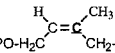
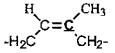
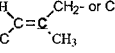
The bulk CH_2 protons of the undecaprenyl chain yield four distinct ^1H - ^{13}C HMQC cross-peaks (Fig. 9B and Table III). Based upon the multibond correlations discussed below, the major carbon peak at 27.2 ppm is assigned to the proximal CH_2 groups of the bulk isoprene units (*i.e.* the ones adjacent to a methine proton as shown in Table III), whereas the 33.0 ppm peak of about equal intensity is assigned to those CH_2 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*- CH_2 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) CH_2 group of the α isoprene unit (Fig. 1 and Table III).

The CH_3 protons yield six distinct HMQC peaks (Fig. 9B). The *cis*- CH_3 protons of the α isoprene unit (1.74 ppm), six of the seven *cis*- CH_3 protons of the interior β to ω -3 isoprene units (1.70 ppm), and the remaining interior *cis*- CH_3 group that overlaps with the *cis*- CH_3 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 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 CH_2OP 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

TABLE III
 ^{13}C and ^1H NMR assignments (ppm from internal TMS) and coupling constants (J , Hz) of the undecaprenyl chain in the donor lipid

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.

Isoprene Unit	Methyl Groups	δC	δH [multiplicity, $J(\text{Hz})$]
α		23.8	1.743 [d, 1.0 Hz]
β to ω -3		24.1	1.698, 6 methyl groups
		24.1	1.686, 1 methyl group
ω		26.3	1.686, 1 methyl group
ω -1, ω -2		16.8	1.629, 1 methyl group
		16.8	1.610, 1 methyl group
ω		18.2	1.610, 1 methyl group
Isoprene Unit	Methylene Groups	δC	δH [multiplicity, $J(\text{Hz})$]
α		63.5	4.446 [dd, 6.8, 6.8**]
α		32.8	2.11
β to ω		27.2	2.07
β to ω -3		33.0	2.05
ω -1 to ω -2		40.5	1.99
Isoprene Unit	Methine Groups	δC	δH [multiplicity, $J(\text{Hz})$]
α		122.8	5.41 [t, 6.8]
β to ω -3		126.8	~ 5.15
		126.0	~ 5.11
ω to ω -2		126.0	~ 5.11
Isoprene Unit	Quaternary Carbons	δC	
α		142.0	
β to ω -3		137.0	
ω to ω -2		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.

carbon peaks at 23.8 ppm (the *cis*- CH_3 group of the α isoprene unit) and at 32.8 ppm (the distal *trans* CH_2 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-

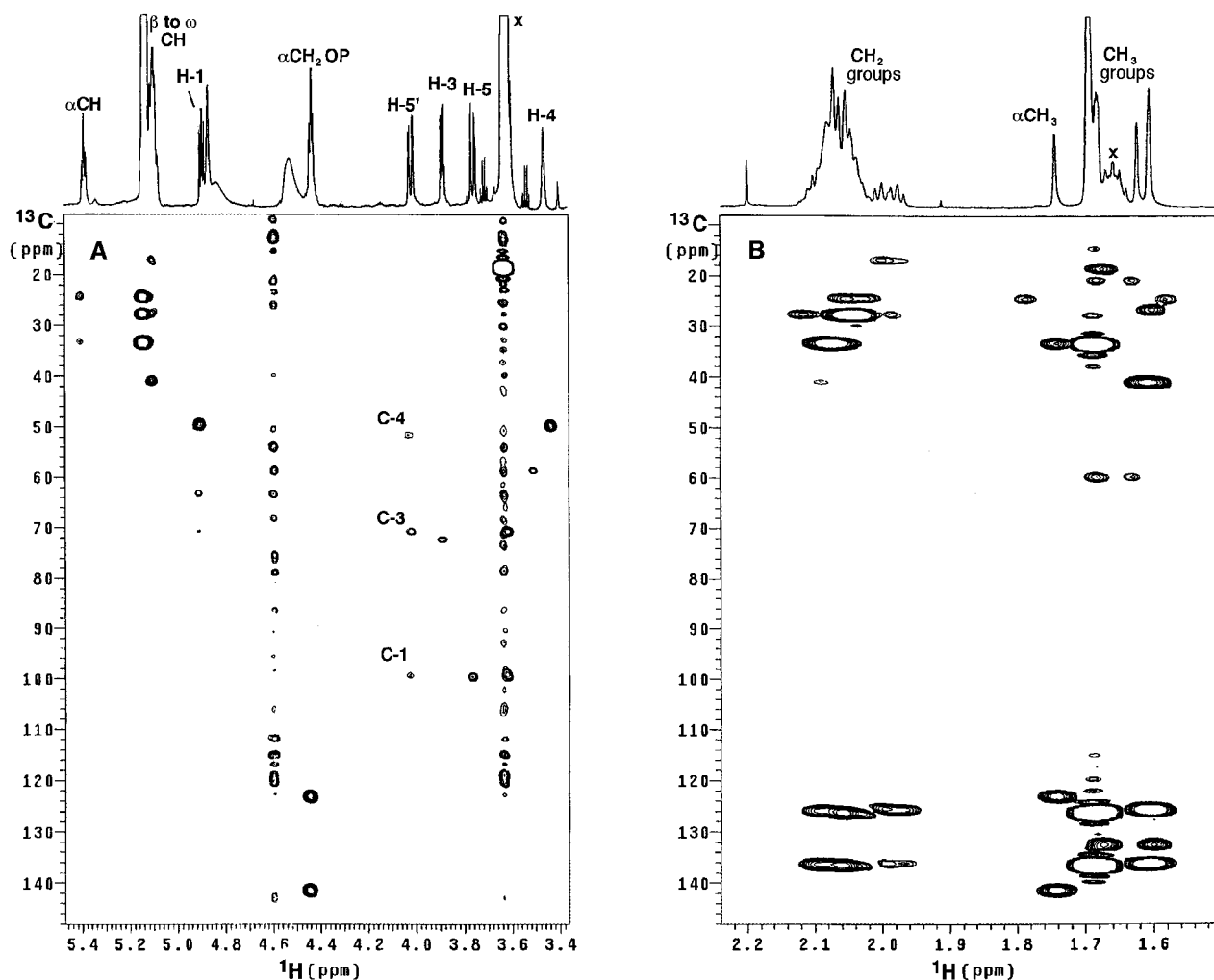


FIG. 10. Partial HMBC spectra of the purified donor lipid. A, the expansion shows multibond ^1H - ^{13}C correlations from the L-Ara4N group and the α isoprenyl methine and proximal methylene unit. Noise streaks arise from HOD and ethanol signals at 4.56 and 3.6 ppm. The major HOD signal has been suppressed with a presaturation pulse. The broad envelope signal at 4.53 ppm is a "probe-hump" artifact from a small residual water magnetization being detected at an off resonance position by the leads of the probe coil. \times indicates residual solvent or impurity resonances. B, this expansion shows the region of the partially resolved bulk isoprenyl CH_2 and CH_3 groups. \times indicates residual solvent or impurity resonances.

ment of the 1.74 ppm signal as the *cis*-methyl group of the α isoprene unit.

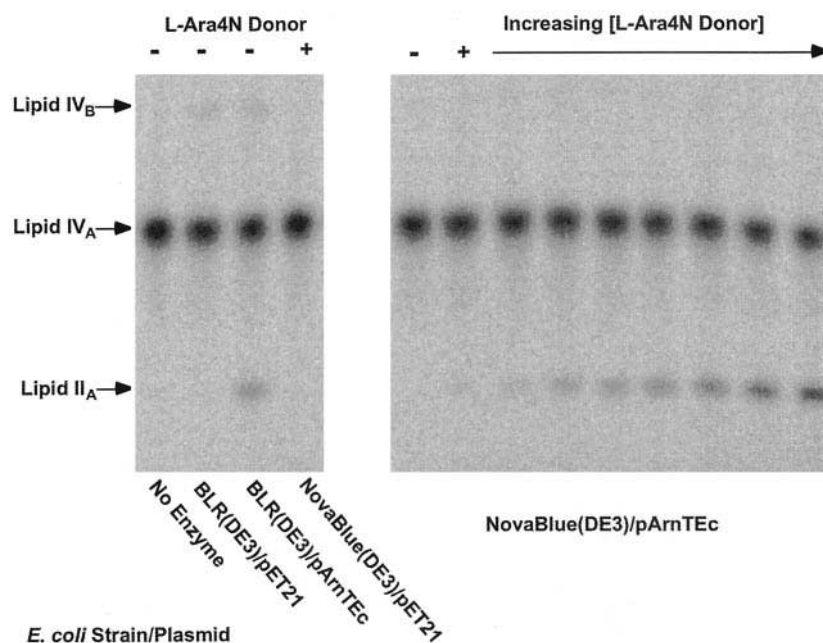
Similarly, the major unresolved CH proton signals (~ 5.1 ppm) of the undecaprenyl chain yield multibond correlations to various CH_3 and CH_2 carbon resonances between 16 and 41 ppm (Fig. 10A). The major CH_2 and CH_3 proton signals show multibond correlations to methine carbon peaks near 126 ppm and to quaternary carbon signals near 136 ppm (Fig. 10B). The *trans*- CH_3 signals of the ω -1 and ω -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 ω -1 and ω -2 units of the undecaprenyl chain. The interior β to ω -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 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 ^1H 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 ω to ω -2 units of the undecaprenyl chain are in excellent agreement with the ^{13}C NMR assignments of farnesol, β -farnesene, β -springene, eleganediol, 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- α -L-Ara4N.

Reconstitution of L-Ara4N Transferase Activity in Vitro with Purified Undecaprenyl Phosphate- α -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- α -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

FIG. 11. Reconstitution *in vitro* of ArnT activity with purified undecaprenyl phosphate- α -L-Ara4N. ArnT was assayed using $10 \mu\text{M}$ [$4'$ - ^{32}P]lipid IV_A as the acceptor substrate under standard assay conditions for 5 min with 0.5 mg/ml of membrane protein, except that the Triton X-100 concentration was increased to 0.7% to dissolve the undecaprenyl phosphate- α -L-Ara4N. The source of the membranes, as indicated, was from the polymyxin-resistant BLR(DE3)/pLysS or the polymyxin-sensitive NovaBlue(DE3)/pLysS *E. coli* host strains, containing either the pET21 vector or the hybrid pArnTEc plasmid (25). The purified donor substrate was added in increasing concentrations ranging from about 1.5 to $150 \mu\text{M}$. In the left panel, $150 \mu\text{M}$ donor was included in the indicated reaction. The products were separated by TLC using the solvent chloroform, pyridine, 88% formic acid, water (50:50:16:5, v/v) and were detected with a PhosphorImager.



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 *pmrA*^C 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'-phosphate groups 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 polyisoprene-linked 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 a 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 *pmrA*^C and PhoP^C mutants. It is absent in *pmrA*⁻ strains (Fig. 3).

The purification to near-homogeneity of the donor lipid required as a first step the construction of a special *pmrA*^C mutant of *E. coli* lacking phosphatidylethanolamine 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 CDCl₃/CDOD/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 HMQC experiment (Fig. 9A). The size and configuration of the polyisoprene chain are confirmed by mass spectrometry (Fig. 7) and by the HMQC/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-IV_A to lipid-II_A (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 mannosyltransferases (23, 68). All the enzymes of this family appear to use polyisoprene-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 (RfbX), 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 diphosphate oligosaccharides (70). Nevertheless, *wzx*-deficient mutants should be tested for their sensitivity to polymyxin. Most importantly, perhaps, *in vitro* systems for measuring flip-flop of undecaprenyl phosphate- α -L-Ara4N across lipid bilayers need to be developed, but first, efficient enzymatic, radiochemical, and chemical methods for making undecaprenyl phosphate- α -L-Ara4N must be devised. A new enzymatic system for the conversion of UDP-glucuronic

acid to UDP-L-Ara4N has recently been identified in extracts of polymyxin-resistant mutants of *E. coli*,³ and should facilitate the preparation of undecaprenyl phosphate- α -L-Ara4N.

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