THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 46, Issue of November 16, pp. 43122–43131, 2001
Printed in U.S.A.

An Inner Membrane Enzyme in *Salmonella* and *Escherichia coli* That Transfers 4-Amino-4-deoxy-L-arabinose to Lipid A

INDUCTION IN POLYMYXIN-RESISTANT MUTANTS AND ROLE OF A NOVEL LIPID-LINKED DONOR*[S]

Received for publication, July 23, 2001, and in revised form, August 27, 2001
Published, JBC Papers in Press, September 4, 2001, DOI 10.1074/jbc.M106961200

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Attachment of the cationic sugar 4-amino-4-deoxy-L-arabinose (L-Ara4N) to lipid A is required for the maintenance of polymyxin resistance in *Escherichia coli* and *Salmonella typhimurium*. The enzymes that synthesize L-Ara4N and transfer it to lipid A have not been identified. We now report an inner membrane enzyme, expressed in polymyxin-resistant mutants, that adds one or two L-Ara4N moieties to lipid A or its immediate precursors. No soluble factors are required. A gene located near minute 51 on the *S. typhimurium* and *E. coli* chromosomes (previously termed *orf5*, *pmrK*, or *yfbI*) encodes the L-Ara4N transferase. The enzyme, renamed ArnT, consists of 548 amino acid residues in *S. typhimurium* with 12 possible membrane-spanning regions. ArnT displays distant similarity to yeast protein mannosyltransferases. ArnT adds two L-Ara4N units to lipid A precursors containing a Kdo disaccharide. However, as shown by mass spectrometry and NMR spectroscopy, it transfers only a single L-Ara4N residue to the 1-phosphate moiety of lipid IV_A, a precursor lacking Kdo. Proteins with full-length sequence similarity to ArnT are present in genomes of other bacteria thought to synthesize L-Ara4N-modified lipid A, including *Pseudomonas aeruginosa* and *Yersinia pestis*. As shown in the following article (Trent, M. S., Ribeiro, A. A., Doerrler, W. T., Lin, S., Cotter, R. J., and Raetz, C. R. H. (2001) *J. Biol. Chem.* 276, 43132–43144), ArnT utilizes the novel lipid undecaprenyl phosphate-α-L-Ara4N as its sugar donor, suggesting that L-Ara4N transfer to lipid A occurs on the periplasmic side of the inner membrane.

Although the overall structure of the lipid A component of lipopolysaccharide is relatively conserved in diverse Gram-negative bacteria, sub-stoichiometric covalent modifications of lipid A have been identified in many organisms (Fig. 1) (1–4). Some of these appendages may be important for bacterial pathogenesis and survival within animal hosts (5, 6). Covalent substituents that can be attached to lipid A in both *Escherichia coli* and *Salmonella typhimurium* include 4-amino-4-deoxy-L-arabinose (L-Ara4N)[1] (7–11), phosphoethanolamine (pEtN) (8, 9, 11), and palmitate (5, 9, 12) (Fig. 1). *S. typhimurium* lipid A may, in addition, contain *S*-2-hydroxymyristate in place of myristate as the secondary acyl chain at position 3′ (Fig. 1) (13, 14). During animal infections, lipid A activates the innate immune system by interacting with the pattern recognition receptor TLR-4, which is present on macrophages and endothelial cells (15–18). An important component of the host response to infection is the production of amphipathic, cationic peptides that possess anti-microbial activity (19, 20). Bacteria may acquire resistance to such peptides by adding L-Ara4N units to the phosphate groups of their lipid A, reducing its net negative charge, and lowering its affinity for these peptides (21–24).

Polymyxin is a cationic lipopeptide antibiotic produced by Gram-positive bacteria (21). It binds to lipid A and kills Gram-negative bacteria in a manner that shares some common features with the cationic antibacterial peptides of the innate immune system (21). Substitution of lipid A with L-Ara4N units is greatly elevated in polymyxin-resistant mutants of *S. typhimurium* (22, 25) and *E. coli* K-12 (23). The polymyxin resistance phenotype is usually due to mutation(s) in the *pmrA* locus (26), which encodes a transcription factor that is activated during growth under mildly acidic conditions (pH < 6), in a PhoP/PhoQ-dependent manner during Mg^{2+} limitation (10 μM), or by exposure to ferric ions (20, 27, 28). The first two of these conditions exist within the phagolysosomes of macrophages, which engulf *S. typhimurium* during the course of an infection (19, 27, 29).

PmrA activation by environmental stimuli or by appropriate point mutations within *pmrA* induces the expression of genes needed for polymyxin resistance and covalent modification of lipid A with L-Ara4N (24, 27, 30, 31). A cluster of genes mapping near minute 51 in *S. typhimurium* and *E. coli* (24) have been proposed to encode a set of enzymes required for the biosynthesis of the L-Ara4N moiety and its attachment to lipid A (Fig. 2) (10). However, *in vitro* assays have not yet been developed to validate the functions of these putative enzymes.

We now report a novel enzyme, present in inner membranes of polymyxin-resistant mutants of *S. typhimurium*, that can transfer one or two L-Ara4N moieties to lipid A and certain lipid

* This work was supported in part by National Institutes of Health Grants GM-51310 (to C. R. H. R.) and GM54882 (to R. J. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at *http://www.jbc.org*) contains Fig. 1.

§ Supported by National Institute of Health Grant 1 F32 AI1056-02.

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[1] The abbreviations used are: L-Ara4N, 4-amino-4-deoxy-L-arabinose; MES, 2-[*N*-morpholino]ethanesulfonic acid; Kdo, 3-deoxy-D-*manno*-octulosonic acid; PCR, polymerase chain reaction; pEtN, phosphoethanolamine; MALDI/TOF mass spectrometry, matrix assisted laser desorption-ionization/time of flight mass spectrometry.

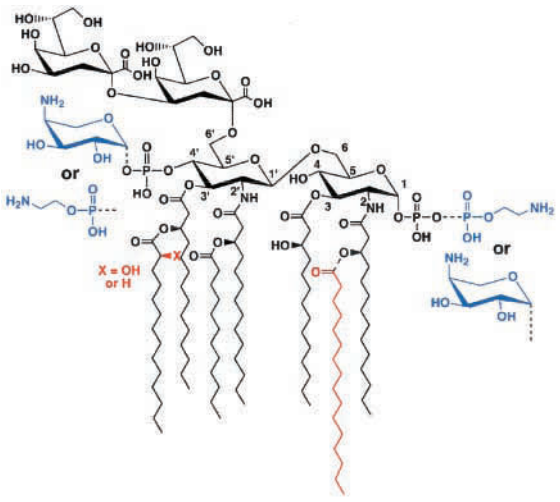

FIG. 1. **Structures of Kdo_2-lipid A and its covalent appendages.** The phosphate residues and acyl chains of lipid A in *S. typhimurium* may be modified in a regulated fashion (30), as indicated by the *dashed bonds*. The phosphates moieties may be substituted with L-Ara4N and/or pEtN groups, both of which are under PmrA control (*blue* substituents) (11, 22, 24). Minor species are present in which the locations of the L-Ara4N and pEtN groups are reversed or in which both phosphates are modified with the same substituent (11). Addition of the palmitoyl chain is catalyzed by the outer membrane enzyme PagP (12), and formation of 2-hydroxymyristate (×) requires a novel hydroxylase homologue, encoded by *lpxO* (14). Substituents that are incorporated in a PhoP/PhoQ-dependent manner are shown in *red*.

A precursors. Transferase activity is dependent upon activation of the *pmrA* gene, either directly by mutation or secondarily by growth under appropriate conditions. The enzyme adds a single L-Ara4N unit to the 1-phosphate moiety of the tetraacylated lipid A precursor, lipid IV_A, which lacks Kdo (Fig. 3). However, lipid A molecules containing a Kdo disaccharide are modified with two L-Ara4N units, indicating that the addition of L-Ara4N to the 4′-position is Kdo-dependent. Transferase activity is greatly elevated when the *orf5*(*pmrK*) gene of *S. typhimurium* (6, 24), now renamed *arnT* (Fig. 2), is expressed behind a T7*lac* promoter in *E. coli* BLR(DE3), which is itself shown here to be a polymyxin-resistant strain containing L-Ara4N modified lipid A. The L-Ara4N transferase is not dependent upon added soluble factors. As shown in the following article (32), ArnT utilizes the novel lipid, undecaprenyl phosphate-α-L-Ara4N, as its donor substrate (Fig. 2).

EXPERIMENTAL PROCEDURES

Materials—$^{32}P_i$ and [γ-^{32}P]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. $CDCl_3$, CD_3OD, and D_2O were purchased from Aldrich. All other chemicals were reagent grade and were purchased from either Sigma or Mallinckrodt.

Bacterial Strains—Bacterial strains are described in Table I. Typically, the bacteria were grown at 37 °C in LB broth, which contains 10 g of NaCl, 10 g of tryptone, and 5 g of yeast extract per liter (33). For experiments requiring Mg^{2+} limitation or exposure to low pH, cells were grown as described previously on a defined medium (34). When required for selection of plasmids, cells were grown in the presence of 100 μg/ml ampicillin, 12 μg/ml tetracycline, 30 μg/ml chloramphenicol, or 30 μg/ml kanamycin.

Recombinant DNA Techniques—Plasmids were prepared using the Qiagen Spin Prep kit. DNA fragments were isolated from agarose gels using the Qiaex II gel extraction kit. T4 DNA ligase (Life Technologies, Inc.), restriction endonucleases (New England Biolabs), and shrimp alkaline phosphatase (U. S. Biochemical Corp.) were used according to the manufacturer's instructions.

Overxpression of the L-Ara4N Lipid A Transferase (ArnT) Behind a T7lac Promoter—The *arnT*(*orf5*) gene of *S. typhimurium* (Fig. 2) was cloned into pET21 (Novagen) behind the T7*lac* promoter. The gene was amplified by PCR using *S. typhimurium* 14028 genomic DNA as the template. The forward primer contained a clamp region, an *Nde*I site (*underlined*), and the *arnT* coding region with its start codon. The reverse primer contained a clamp region, a *Bam*HI site (*underlined*), and the coding region with its stop codon. Sequences of the *S. typhimurium* forward and reverse primer were 5′-GCGCGCCATATGATGATGAAATCGATA-3′, and 5′-GCGCGCGGATCCTCATTTAGGCCGATA-3′, respectively. The PCR contained 140 ng of genomic DNA template, 0.15 μg of each primer, 200 μM each of dNTP, 100 mM Tris-HCl, pH 8.8, 35 mM $MgCl_2$, 250 mM KCl, and 2.5 units of *Pfu* DNA polymerase (Stratagene) in a reaction volume of 0.05 ml. The reaction mixture was subjected to a 2-min denaturation at 95 °C followed by 25 cycles of 95 °C for 45 s, 60 °C for 45 s, 72 °C for 120 s and a final extension for 10 min at 72 °C, using the PerkinElmer Life Sciences GeneAmp PCR system 2400. The PCR product and the vector were digested with *Nde*I and *Bam*HI, ligated together, and transformed into Xl-1 Blue cells (Stratagene) for propagation of the plasmid, designated pArnTSt. The plasmid was then transformed into BLR(DE3) or Nova-Blue(DE3) (Table I) with or without the pLysS plasmid for overexpression of the protein. First, a single colony of *E. coli* containing pArnTSt was inoculated into 20 ml of LB broth and grown in a rotary shaker at 37 °C to A_{600} = 0.8. The culture was then used to inoculate 1 liter of fresh LB medium, and when A_{600} reached ~0.6, the culture was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h. Crude extracts, membrane-free cytosol, and washed membranes were prepared as described below.

Preparation of Cell-free Extracts and Membranes—Typically, 100-ml cultures of bacteria were grown to A_{600} = 1.0 at 37 °C and harvested by centrifugation at 7,000 × *g* for 15 min. All steps were carried out at 0–4 °C. Cells were resuspended in 50 mM Hepes, pH 7.5, at a protein concentration of 5–10 mg/ml, and broken by one passage through a French pressure cell at 18,000 pounds/square inch. The crude lysate was centrifuged at 7,000 × *g* for 15 min to remove unbroken cells. Membranes were prepared by two successive centrifugations at 149,000 × *g* for 60 min, with a washing step inbetween to remove residual soluble components. The final membrane pellet was resuspended in 50 mM Hepes, pH 7.5, at a protein concentration of 5–10 mg/ml. Cytosol from the first 149,000 × *g* centrifugation step was subjected to a second centrifugation to remove any remaining contaminating membranes. All membrane and cytosol preparations were stored in aliquots at −80 °C, and protein concentrations were determined with bicinchoninic acid (35), using bovine serum albumin as the standard.

Isolation and Analysis of Lipid A Species from $^{32}P_i$-Labeled Cells—Cells were labeled uniformly with 5 μCi/ml $^{32}P_i$ in LB broth, starting at an initial A_{600} of ~0.05. Cells were then grown at 37 °C for several hours, as indicated, and harvested when A_{600} reached ~1.0. The ^{32}P-labeled cells were collected using a clinical centrifuge and washed with 5 ml of phosphate-buffered saline, pH 7.4 (36). The final cell pellet was resuspended in 3 ml of a single-phase Bligh/Dyer mixture (37), consisting of chloroform/methanol/water (1:2:0.8, v/v). After 60 min, the insoluble material, which still contains the ^{32}P-labeled lipid A covalently linked to the lipopolysaccharide core via its Kdo residues, was released by hydrolysis at 100 °C in the presence of 1% SDS at pH 4.5, as described previously (10, 38). The ^{32}P-labeled lipid A species were recovered by two-phase Bligh/Dyer extraction (10, 38) and spotted onto a Silica Gel 60 TLC plate (~10,000 cpm/lane). The plate 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 resolved ^{32}P-lipid A species.

Preparation of Radiolabeled Substrates—The intermediate [4′-^{32}P]-lipid IV_A was prepared using 100 μCi of [γ-^{32}P]ATP, tetraacyldisaccharide 1-phosphate acceptor, and membranes from an *E. coli* strain that overexpresses the lipid 4′-kinase (39), as described previously (34, 40). To prepare Kdo_2[4′-^{32}P]lipid IV_A, purified *E. coli* Kdo transferase was used in tandem with the 4′-kinase (40, 41). Kdo_2[^{32}P]lipid A was prepared by labeling the heptose-deficient mutant WBB06 (42) with $^{32}P_i$ and purifying its Kdo_2[^{32}P]lipid A as reported previously (12).

Assay Conditions for Detecting L-Ara4N Transferase Activity—The L-Ara4N transferase was assayed under optimized conditions in a 10-μl reaction containing 50 mM MES, pH 6.5, 0.2% Triton X-100 and either 10 μM [4′-^{32}P]lipid IV_A or Kdo_2[4′-^{32}P]lipid IV_A (each at 20,000 cpm/nmol) as the acceptor substrate. Washed membranes (0.5–1.0 mg/ml) were employed as the source of enzyme and L-Ara4N donor, as indicated. Reaction mixtures were incubated at 30 °C for various times. Enzymatic reactions were stopped by spotting 5-μl portions of the mixtures onto Silica Gel 60 thin layer chromatography plates. In most

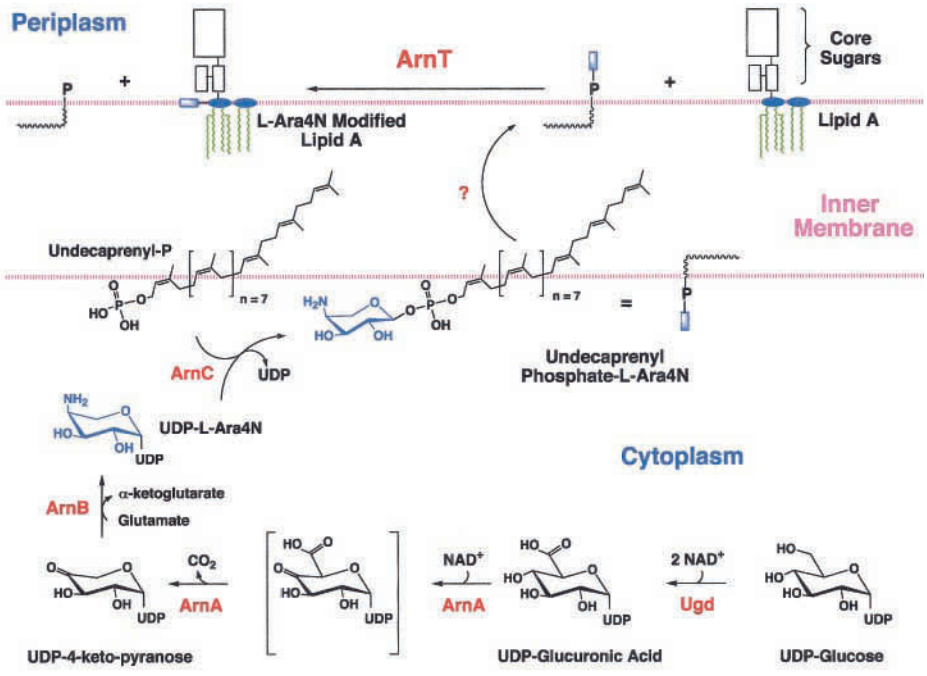

FIG. 2. **Pathway for L-Ara4N biosynthesis and mechanism of polymyxin resistance in *E. coli* and *S. typhimurium*.** In accordance with the proposal of Reeves *et al*. (58), we have renamed the genes of the polymyxin resistance operon "*arn*," given their function in the biosynthesis of the L-Ara4N moiety and its transfer to lipid A (10). The proposed pathway starts with the conversion of UDP-glucose to UDP-glucuronic acid by the well characterized dehydrogenase, Ugd. Next, ArnA (previously Orf3 or PmrI) (6, 24) catalyzes the oxidative decarboxylation of UDP-glucuronic acid to generate a novel UDP-4-keto-pyranose intermediate, which can be isolated in mg quantities using ArnA (S. Breazeale, A. A. Ribeiro, and C. R. H. Raetz, manuscript in preparation). In contrast to the proposal of Baker *et al*. (53), a separate enzyme to catalyze the decarboxylation step is not necessary in our scheme (10). ArnB (previously Orf1 or PmrH) (6, 10, 24) then catalyzes a further transamination to form UDP-L-Ara4N (S. Breazeale, A. A. Ribeiro and C. R. H. Raetz, manuscript in preparation). Based upon its homology to dolichyl phosphate-mannose synthase of yeast, we propose that ArnC (PmrF) (6, 10, 24) transfers the L-Ara4N moiety to undecaprenyl phosphate, forming the novel compound undecaprenyl phosphate-α-L-Ara4N, the existence of which is demonstrated in the following article (32). After translocation to the outer surface of the inner membrane by unknown mechanisms (10), ArnT (previously Orf5, PmrK, or YfbI) (6, 24) transfers the L-Ara4N unit to lipid A. Other genes of the polymyxin operon (*pmrJ*, *pmrL*, and *pmrM*), as well as the adjacent *pmrG* gene (6,24), cannot yet be assigned specific enzymatic or transport functions in our scheme. The ArnA protein has a second catalytic domain (reaction not shown) that can transfer a formyl group from *N*-10-formyltetrahydrofolate to UDP-L-Ara4N (S. Breazeale, A. A. Ribeiro, and C. R. H. Raetz, manuscript in preparation), but the significance of this modification is unclear. Addition of the L-Ara4N moiety to lipid A reduces the affinity of lipid A for polymyxin and other cationic anti-microbial peptides.

of the initial experiments, the PmrA^C *S. typhimurium* strain JSG435 (Table I) was used for the characterization of the assay conditions, because of its apparently high levels of endogenous L-Ara4N transferase activity and the presence of the donor substrate.

When [4′-^{32}P]lipid IV$_A$ was employed as the acceptor, the substrate and reaction products were separated using the solvent chloroform, pyridine, 88% formic acid, water (50:50:16:5, v/v). For assays containing Kdo$_2$[4′-^{32}P]lipid IV$_A$ as the acceptor substrate, plates were developed in chloroform, pyridine, 88% formic acid, water (30:70:16:10, v/v). Following chromatography, the plates were dried and analyzed using a Molecular Dynamics PhosphorImager equipped with ImageQuant software. The enzyme activity was calculated by determining the percentage of the substrate converted to product. The apparent specific activities were expressed in units of nmol/min/mg, recognizing that the membranes supply not only the enzyme but also the L-Ara4N donor substrate.

Separation of Inner and Outer Membranes—Membranes isolated from the PmrA^C *S. typhimurium* strain JSG435 (Table I) were separated by isopycnic sucrose gradient centrifugation as described previously (34). First, washed membranes were prepared as described above and then were resuspended at a concentration of ~5 mg/ml in 10 mM Hepes, pH 7.0, containing 0.05 mM EDTA. Membranes (2.2 ml) were applied to a 9.6-ml, seven-step sucrose gradient (43), and subjected to ultracentrifugation at 35,000 rpm in a Beckman SW40.1 rotor for 19 h at 3 °C. The gradient was collected in ~0.5 ml fractions by piercing the bottom of the tube. Each fraction was assayed for NADH oxidase as the inner membrane marker and for phospholipase A as the outer membrane marker (38). The amount of protein in each fraction was determined using the bicinchoninic acid assay (35). Finally, each fraction was assayed for L-Ara4N transferase activity, using the optimized assay conditions described above.

Purification of the L-Ara4N-modified Reaction Products Generated in Vitro from Lipid IV$_A$—The lipid IV$_A$ reaction products, generated *in vitro* with membranes of strain JSG435, were purified by preparative thin layer chromatography. A 68-ml L-Ara4N transferase reaction mixture (see above), containing 100 μM lipid IV$_A$ and 1 mg/ml JSG435 membranes, was incubated overnight at 30 °C. The reaction mixture was then converted into a two-phase acidic Bligh/Dyer system, consisting of chloroform, methanol, 0.1 M HCl (2:2:1.8, v/v), by the addition of 76 ml of chloroform, 76 ml of methanol, and 0.6 ml of 12 M HCl. After mixing, the phases were separated by centrifugation at 5,000 × *g* for 15 min. The lower phase was removed, and the upper phase was extracted a second time by the addition of 76 ml of a fresh lower phase derived from a two-phase Bligh/Dyer mixture of chloroform, methanol, and 0.1 M HCl. The lower phases, containing the residual substrate and the modified lipid IV$_A$ species, were pooled. Next, 4 ml of pyridine was added to neutralize remaining HCl carried over during the extraction process. The sample was dried by rotary evaporation, dissolved in 8 ml of chloroform/methanol (4:1, v/v), and spotted as lines onto the origins of 14 individual 20 × 20-cm Silica Gel 60 TLC plates (0.25-mm thickness) (~0.57 ml per plate). The lipids were separated using the solvent system chloroform, pyridine, 88% formic acid, water (50:50:16:5, v/v). While the plates were drying at room temperature, the bands of residual lipid IV$_A$ and its modified products could be seen transiently as white zones. The latter was marked with a pencil, and the plates were dried for an additional 30 min with a cold air stream. Regions containing the putative L-Ara4N-modified lipid IV$_A$ derivatives (*i.e.* presumed to be lipids II$_A$ and II$_B$ based on their migration) (Fig. 4) were removed separately with clean razor blades and then stored in separate thick-walled glass tubes at −80 °C (10).

Silica chips from four separate thin layer plates containing bands with the same R_f were processed together. The lipids were extracted from the chips with 6 ml of an acidic single-phase Bligh/Dyer mixture, consisting of chloroform, methanol, 0.1 M HCl (1:2:0.8, v/v). Following removal of the chips by low speed centrifugation, the supernatant was converted to a two-phase Bligh/Dyer system by adding 1.58 ml of both

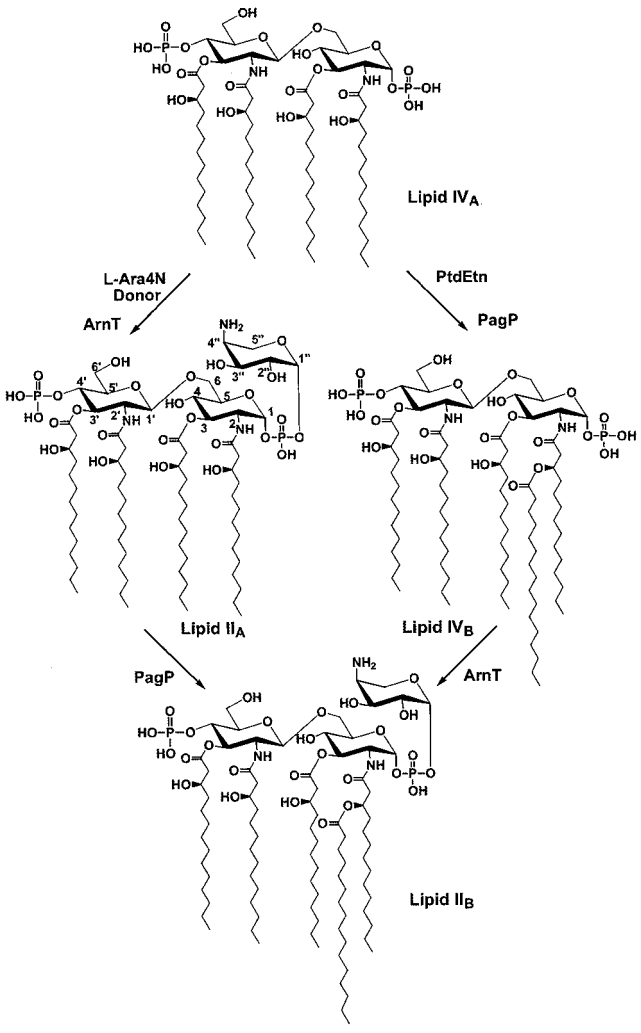

FIG. 3. **Structure of lipid IV$_A$ and the products generated from it by membranes of *pmrA^C S. typhimurium*.** Lipid IV$_A$ and lipid II$_A$ are tetra-acylated species with the latter containing the L-Ara4N moiety attached to the 1-phosphate group (9, 46, 48). The numbering scheme shown for lipid II$_A$ is used for the NMR analysis. Lipids IV$_B$ and II$_B$ are further modified with a palmitoyl group at the 2-position, which is incorporated by the outer membrane enzyme PagP (12).

chloroform and water. The lower phase, which contained the desired lipid, was withdrawn, and the upper phase was re-extracted once more with an equivalent volume of fresh lower phase. The lower phases were pooled, and 6 drops of pyridine were added. The sample was dried under a stream of N_2.

To remove minor breakdown products and contaminating silica particles, the individual lipids recovered from the TLC plates were subjected to anion exchange chromatography on small DEAE-cellulose columns (10). Each lipid sample was re-dissolved in 3 ml of chloroform/methanol/water (2:3:1, v/v) and subjected to sonic irradiation for 30 s in a bath apparatus before application to a 1-ml DEAE-cellulose column, suspended in the same solvent mixture, and equilibrated with acetate as the counter ion (10, 44). After application of the sample, the column was washed with 4 bed volumes of chloroform/methanol/water (2:3:1, v/v). The products were eluted with the same solvent system but with the aqueous portion consisting of 60, 120, 240, or 500 mM ammonium acetate in ascending order. For each elution step, four 1-ml fractions were collected. The L-Ara4N-modified species, lipids II$_A$ and II$_B$, eluted with chloroform, methanol, 120 mM ammonium acetate (2:3:1, v/v), whereas the substrate lipid IV$_A$ eluted with 240–500 mM ammonium acetate. Fractions from the DEAE columns containing the desired lipid products, which were detected by charring on a TLC plate with 10% sulfuric acid in ethanol, were pooled and converted to a two-phase Bligh/Dyer mixture by addition of appropriate amounts of chloroform and water. The upper phases were extracted once with fresh lower phases to maximize the recovery of each lipid. The appropriate combined lower phases for each lipid product were then dried under a stream of N_2 and stored at −80 °C.

Mass Spectrometry of the L-Ara4N-modified Reaction Products—Spectra of the purified lipids were acquired in the negative and positive linear modes, using a matrix-assisted laser desorption-ionization/time of flight (MALDI/TOF) instrument (Kompact MALDI 4, Kratos Analytical, Manchester, UK), equipped with a nitrogen laser (337 nm) (10). By using 20-kV extraction voltage and time-delayed extraction, each spectrum represented the average of 50 laser shots. The instrument was operated at a resolution of about ±1 atomic mass units for compounds with M_r ~2000. Saturated 6-aza-2-thiothymine in 50% acetonitrile and 10% tribasic ammonium citrate (9:1, v/v) served as the matrix in negative and positive ion modes. The samples were dissolved in chloroform/methanol (4:1, v/v) and mixed with an equal portion of matrix. The sample was dried at 25 °C prior to mass analysis.

Nuclear Magnetic Resonance Spectroscopy of the L-Ara4N-modified Reaction Products—Approximately 1.5 and 1 mg, respectively, of the putative lipid II$_A$ and lipid II$_B$, generated *in vitro* and purified by TLC as described above, were dissolved in 0.6 ml of $CDCl_3/CD_3OD/D_2O$ (2:3:1, v/v) and transferred into 5-mm NMR tubes. The NMR spectra were recorded at 25 °C using a Varian Unity 500 spectrometer equipped with a Sun Ultra 5 data system and a 5-mm Varian inverse probe. The ^2H signal of CD_3OD was used for a field frequency lock. Both the homo- and heteronuclear NMR experiments were performed as described previously (11, 45, 46).

RESULTS

A Possible L-Ara4N Transferase in Membranes of PhoP and PmrA Constitutive Mutants of S. typhimurium—Although the L-Ara4N moiety of *S. typhimurium* lipid A was first reported in 1970 (7), its enzymatic synthesis has remained obscure until recently. *S. typhimurium* mutants harboring constitutively active forms of the PhoP or PmrA transcription factors synthesize large amounts of L-Ara4N-modified lipid A (22, 30). Accordingly, an *in vitro* assay was developed with membranes from such mutants, using the precursor lipid IV$_A$ (Fig. 3) as a possible acceptor for L-Ara4N transfer. Membranes of *S. typhimurium* CS022 (47), in which PhoP is constitutively active (PhoP^C), converted a measurable portion of the [4′-^{32}P]lipid IV$_A$ probe to a more hydrophilic product migrating like lipid II$_A$ (Fig. 4, *lane 3*), a well characterized substance that accumulates in Kdo-deficient mutant of *S. typhimurium* (9, 46, 48). As shown by the structural formulas in Fig. 3, lipid II$_A$ has the same the tetra-acylated glucosamine disaccharide backbone as does lipid IV$_A$, but it is modified with the L-Ara4N unit on its 1-phosphate moiety (9, 46, 48).

Membranes of wild-type *S. typhimurium* (Fig. 4, *lane 2*) generated very little lipid II$_A$ under these assay conditions. However, both wild-type and PhoP^C membranes produced large amounts of lipid IV$_B$ (Fig. 4, *lanes 2* and *3*), a penta-acylated product containing a palmitoyl moiety (Fig. 3), which is incorporated by the outer membrane enzyme PagP (12). PagP has a very high specific activity under these assay conditions.

A further increase in the rate and extent of lipid II$_A$ formation was seen with membranes of the PmrA^C *S. typhimurium* strain JSG435 (Fig. 4, *lane 6*), which displayed an apparent specific activity of ~0.06 nmol/min/mg at 1 mg/ml membrane protein. A substance migrating somewhat faster than the 4′-^{32}P-lipid II$_A$ product, designated lipid II$_B$ (9, 48) (Fig. 4, *lanes 3* and *6*), likely arises from the PagP-catalyzed addition of palmitate to lipid II$_A$ or, alternatively, by L-Ara4N transfer to lipid IV$_B$ (Fig. 3). As predicted, lipids IV$_B$ and II$_B$ largely disappear when membranes of a PhoP^C strain that also harbors a *pagP* insertion mutation were used as the enzyme source (Fig. 4, *lane 5*). The putative L-Ara4N transferase was entirely absent in membranes of either *phoP* or *pmrA* knockout mutants (Fig. 4, *lanes 4* and *7*), consistent with the absence of L-Ara4N-modified lipid A in these strains (6, 30, 49).

Membranes of wild-type *S. typhimurium* grown either in LB

TABLE I
Relevant bacterial strains and plasmids

Strain or plasmid	Description	Source or Ref.
S. typhimurium		
ATCC 14028s	Wild type	ATCC
CS022	*pho-24* (PhoP-constitutive)	62
CS015	CSO22, *phoP102*::Tn*10d-cam*	47
CS330	CSO22, *pagP*::Tn*phoA*	49
JSG435	*pmrA*505 *zjd*::Tn*10d-cam* (PmrA-constitutive)	27
JSG421	*pmrA*::Tn*10d* (PmrA$^-$)	27
E. coli		
W3110	Wild type, F$^-$, λ^-	*E. coli* Genetic Stock Center, Yale University
XL-1 Blue-MR	Δ*mcrABC, recA1, lac*	Stratagene
BLR(DE3)	Δ(*srl-recA*)306::Tn*10*(DE3), Tet^r	Novagen
NovaBlue(DE3)	Δ(*srl-recA*)306::Tn*10*(DE3), Tet^r	Novagen
Plasmids		
pET21a(+)	Vector containing a T7*lac* promoter, Amp^r	Novagen
pArnTSt	pET21a containing *S. typhimurium arnT*	This work
pLysS	pACYC184 containing T7 lysozyme, Cam^r	Novagen

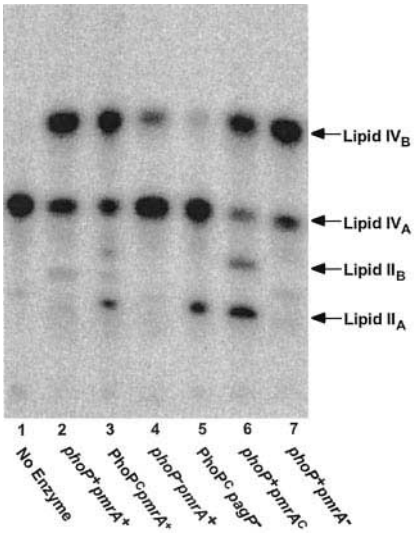

FIG. 4. **Detection of a novel L-Ara4N transferase in *S. typhimurium* membranes.** Membranes from the indicated strains of *S. typhimurium* (Table I) were assayed for transfer of L-Ara4N moieties from endogenous sources to the acceptor substrate 4′-^{32}P-lipid IV$_A$. The protein concentration was 1 mg/ml. Assays were carried out for 6 h at 30 °C with 10 μM 4′-^{32}P-lipid IV$_A$ (20,000 cpm/nmol). The products were separated by TLC and detected with a PhosphorImager.

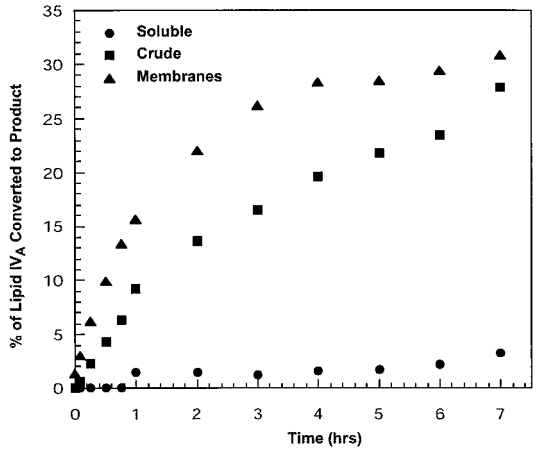

FIG. 5. **Membrane association of the L-Ara4N transferase of *S. typhimurium*.** The L-Ara4N transferase of the *pmrA^C* strain JSG435 was assayed under standard conditions using 1 mg/ml protein and 10 μM 4′-^{32}P-lipid IV$_A$. Crude extract, cytosol, or membranes served as the enzyme source. At each time, reaction products were separated by TLC and visualized with a PhosphorImager.

broth or in minimal medium at pH 7.4 supplemented with 10 mM Mg^{2+} (conditions under which the PhoP and PmrA systems are shut off) showed little or no L-Ara4N transferase activity (data not shown). However, in accordance with the well characterized behavior of PhoP and PmrA, membranes from bacteria grown in minimal medium in the presence of 10 μM Mg^{2+} or at pH 5.8 (conditions leading to activation of PmrA) (20, 27, 29) displayed transferase activity comparable with membranes of *phoP^C* or *pmrA^C* mutants grown on LB broth at pH 7.4 (data not shown).

Catalytic Properties and Inner Membrane Localization of the L-Ara4N Transferase—Transfer of the L-Ara4N unit from its putative isoprenoid carrier, undecaprenyl phosphate-L-Ara4N (Fig. 2), to the radiolabeled acceptor [4′-^{32}P]lipid IV$_A$ (Fig. 3) was dependent upon the presence of the nonionic detergent Triton X-100, with maximal activity observed at 0.2% in the assay system. The pH optimum was 6.5, and the enzyme did not require any soluble cytoplasmic components (Fig. 5). $CaCl_2$ and $MgCl_2$ were inhibitory above 1 mM. Product formation was dependent upon protein concentration up to 1 mg/ml (data not shown) and was linear for about 60 min (Fig. 5) using 10 μM [4′-^{32}P]lipid IV$_A$ as the acceptor with 1 mg/ml membrane protein from strain JSG435 (*pmrA^C*) as the source of enzyme and L-Ara4N donor.

Separation of inner and outer membranes by isopycnic sucrose gradient centrifugation showed that the transferase is located mainly in the inner membrane (Fig. 6). NADH oxidase served as the inner membrane marker. The peak of NADH oxidase and L-Ara4N transferase activity coincided at fraction 17 (Fig. 6). Phospholipase A (50) was used as the marker for outer membranes (Fig. 6). Supplementation of the assay with purified undecaprenyl phosphate-L-Ara4N (32) did not alter the distribution of L-Ara4N transferase activity (data not shown).

MALDI/TOF Mass Spectrometry of the L-Ara4N Transferase Products Formed with Lipid IV$_A$ as the Acceptor—To confirm the presence of the L-Ara4N moiety in the *in vitro* products migrating with lipids II$_A$ and II$_B$ (Fig. 4), 1–1.5 mg of each was purified by ion exchange and thin layer chromatography, as described under "Experimental Procedures." MALDI/TOF mass spectrometry in the negative-ion mode of the product migrating with the lipid II$_A$ standard (Fig. 4) revealed a major peak, interpreted as $[M - H]^-$, at m/z 1535.3 atomic mass units (Fig. 7*A*), consistent with the molecular weight of authentic lipid II$_A$ (M_r = 1536.84) (46, 48). The smaller peak at m/z 1404.6 atomic mass units (Fig. 7*A*) arises from the loss of the L-Ara4N moiety from the parent ion during mass spectrometry and represents $[M\text{-}H\text{-}Ara4N+H]^-$, which is the same as the $[M - H]^-$ of lipid IV$_A$ (M_r = 1405.71) (48). Fragmentation of the labile phosphodiester linkage of lipid II$_A$ during mass spec-

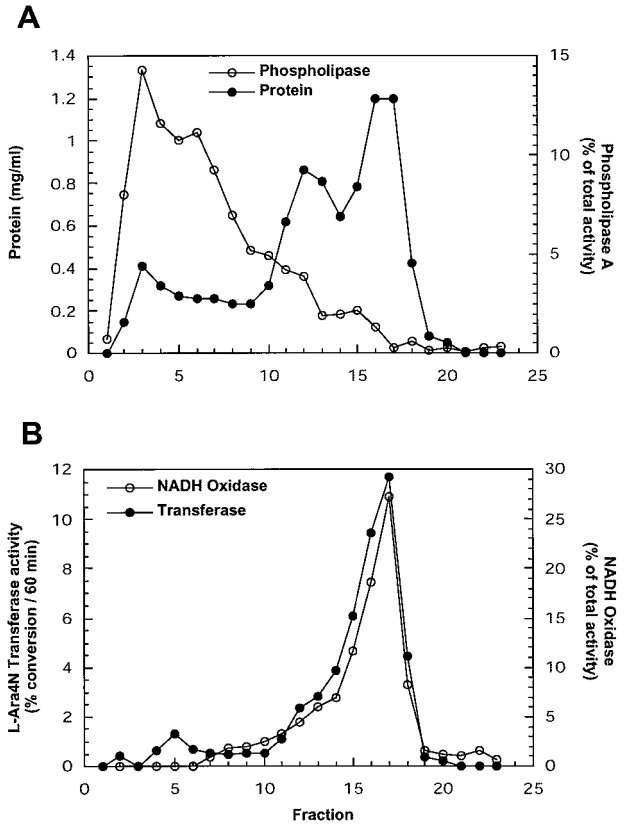

FIG. 6. **Inner membrane localization of the L-Ara4N transferase of *S. typhimurium*.** Membranes isolated from the *pmrA*^{C} strain JSG435 were separated by isopycnic sucrose density gradient centrifugation, and ~0.5-ml fractions were collected (34). *A,* the outer membrane marker phospholipase A (% of total activity) and the protein concentration (mg/ml) were assayed for each fraction. *B,* the inner membrane marker NADH oxidase (% of total activity) and the L-Ara4N transferase activity displayed similar distributions. The L-Ara4N transferase profile was not changed significantly by addition of exogenous undecaprenyl phosphate-L-Arar4N (data not shown).

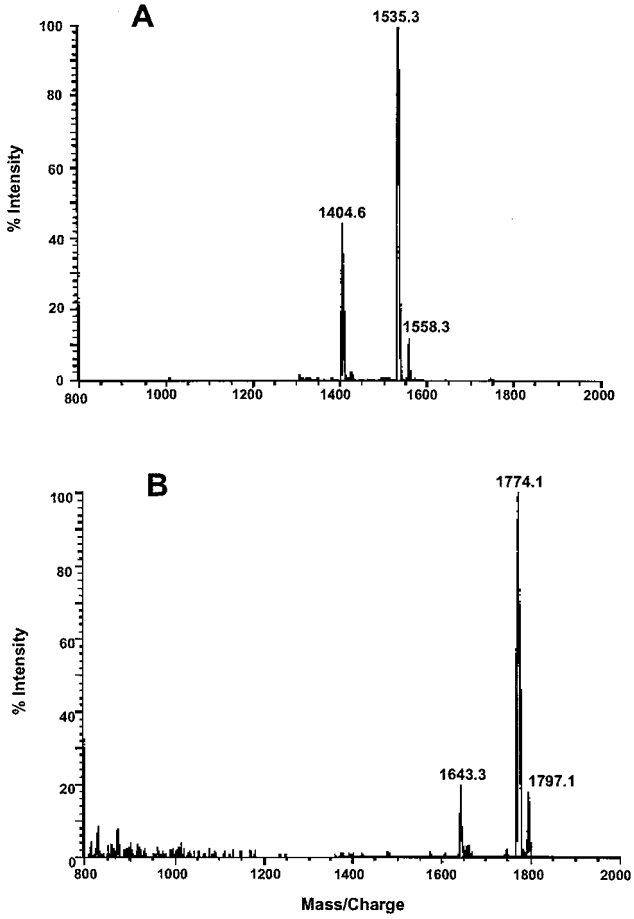

FIG. 7. **Negative ion MALDI/TOF mass spectrometry of the L-Ara4N reaction products generated with lipid IV$_A$ as acceptor.** Reaction products generated by the L-Ara4N transferase migrating with lipid II$_A$ (*A*) or lipid II$_B$ (*B*) were purified by preparative TLC. Spectra were acquired in the negative mode. The molecular weights of authentic lipids II$_A$ and II$_B$ are 1536.84 and 1775.25, respectively (48).

trometry has been documented previously (10, 11). The small peak at *m*/*z* 1558.3 atomic mass units (Fig. 7*A*) is attributed to the sodium adduct $[M + Na - 2H]^-$.

MALDI/TOF mass spectrometry of the additional reaction product, migrating with the lipid II$_B$ standard (Fig. 4), yielded a major ion at *m*/*z* 1774.1 atomic mass units (Fig. 7*B*), consistent with $[M\text{-}H]^-$ of authentic lipid II$_B$ (M_r = 1775.25) (48), which contains one palmitoyl and one L-Ara4N residue attached to the lipid IV$_A$ skeleton. A smaller ion was observed at *m*/*z* 1643.3 atomic mass units, consistent with the loss of the labile L-Ara4N moiety during mass spectrometry and interpreted as $[M\text{-}H\text{-}LAra4N+H]^-$. The small peak at *m*/*z* 1797.1 atomic mass units (Fig. 7*B*) was accounted for by the sodium adduct $[M + Na - 2H]^-$.

Positive-ion mode MALDI/TOF mass spectrometry (not shown) failed to reveal the site of attachment of the L-Ara4N substituent in the *in vitro* products, given the labile nature of the L-Ara4N phosphodiester linkage compared with the β,1′-6 linkage of the glucosamine disaccharide. However, the positive-ion mode spectra (not shown) confirmed the location of the palmitate residue on the proximal glucosamine unit in the lipid II$_B$-like product (9, 48) (Fig. 3).

In summary, the mass spectrometry strongly supports the structural assignments, consistent with the initial TLC analysis (Fig. 4), as representing the *in vitro* formation of lipids II$_A$ and II$_B$ from lipid IV$_A$. An enzyme that adds L-Ara4N units to lipid A or its precursors had not been reported previously. Evidence that the membrane bound donor of the L-Ara4N residue (Fig. 2) is in fact undecaprenyl phosphate-L-Ara4N is presented in the following article (32).

Analysis of the L-Ara4N Attachment Site by Difference ^1H NMR Spectroscopy with Selective Inverse (^{31}P) Decoupling—As discussed previously (9, 46), the precursor lipid II$_A$ that accumulates in Kdo-deficient *S. typhimurium* mutants is modified with a single L-Ara4N moiety via a phosphodiester linkage at the 1-phosphate position, as shown in Fig. 3. In contrast, most of the L-Ara4N attached to mature lipid A in wild-type cells is attached via a phosphdiester linkage to the 4′-phosphate (Fig. 1) (11, 46). As shown in the preceding article (11), however, a portion of the lipid A of *S. typhimurium* does contain two L-Ara4N moieties, implying that the attachment of the L-Ara4N unit to the 4′-position may be Kdo-dependent.

To establish the site of L-Ara4N attachment in the lipid II$_A$-like substance generated *in vitro* by membranes of JSG435, the ^{31}P NMR spectrum of this substance was recorded at 202 MHz in $CDCl_3/CD_3OD/D_2O$ (2:3:1, v/v). As shown in Fig. 8*A*, well resolved ^{31}P resonances were detected at 2.14 and −2.55 ppm. A ^1H NMR spectrum of the same sample was recorded at 500 MHz in the absence of ^{31}P-continuous wave decoupling, and the sugar proton region is shown at the bottom of Fig. 8*B*. The *tracing* in Fig. 8*B* labeled *2.14 ppm* shows the difference spectrum that resulted from subtracting the ^1H NMR spectra of the sample with selective on- and off-resonance decoupling of the 2.14 ppm ^{31}P resonance (Fig. 8*A*). This difference spectrum shows a "triplet" near 4.2 ppm, which can be assigned to the

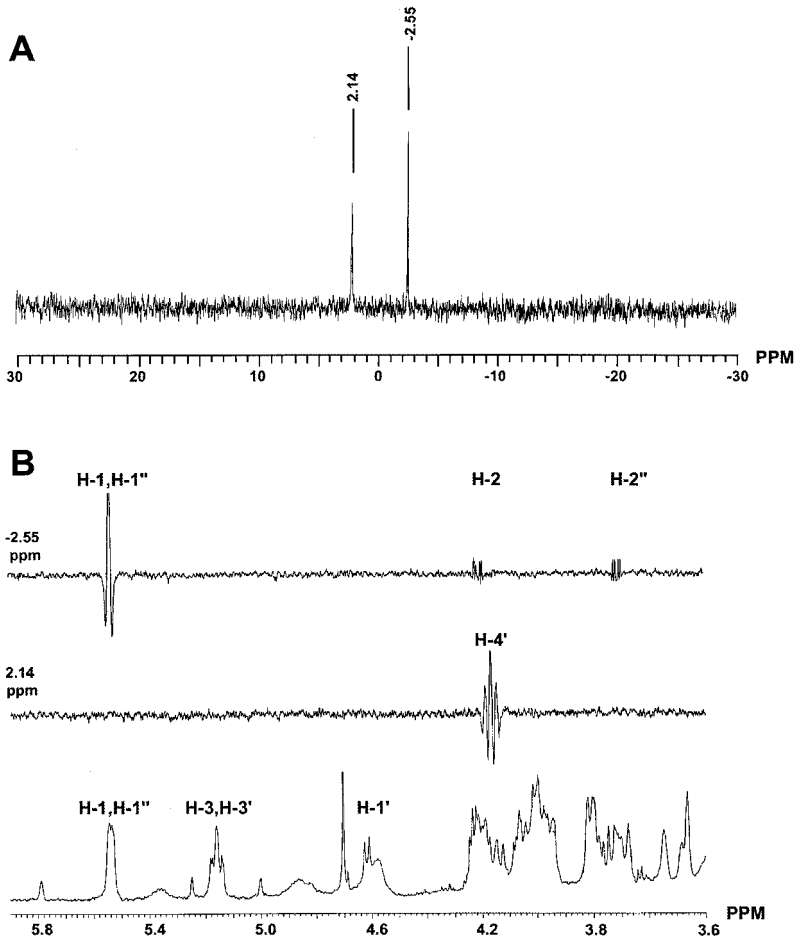

FIG. 8. **^{31}P NMR spectrum of lipid II_A synthesized *in vitro* and selective inverse ^{31}P-decoupled ^{1}H-detected difference spectroscopy.** *A,* the ^{31}P NMR spectrum of the lipid II_A-like material synthesized *in vitro* is consistent with the presence of one monophospho-monoester (2.14 ppm) and one monophosphodiester moiety (−2.55 ppm). *B,* the *bottom tracing* is a reference ^{1}H NMR spectrum, showing the relevant sugar protons from 3.6 to 5.8 ppm. The complete COSY analysis, which is the basis for assignment of key resonances as indicated, is shown in Fig. 1 of the Supplemental Material. The atom numbering is shown in Fig. 3. The *middle tracing* is a difference spectrum obtained with on and off resonance decoupling of the ^{31}P signal at 2.14 ppm. The only observed signal is a triplet near 4.2 ppm assigned to H-4′, demonstrating that the 4′-phosphate is not substituted with the L-Ara4N moiety. The top difference spectrum was obtained by selective on and off resonance decoupling of the ^{31}P signal at −2.55 ppm. It reveals two overlapping proton doublets near 5.5 ppm (H-1 and H-1″) and two smaller doublet of doublets at 4.22 and 3.7 ppm (H-2 and H-2″, respectively). This pattern confirms that the L-Ara4N unit is attached at position 1, as in authentic lipid II_A (46).

H-4′-glucosamine proton (Fig. 3) based upon a complete ^{1}H-^{1}H COSY analysis of the II_A-like product generated *in vitro* (see Fig. 1 in the Supplemental Material), which is the same as that of authentic lipid II_A (46). This outcome clearly establishes the lower field 2.14 ppm phosphate resonance as arising from a monophosphomonoester linked to the C-4′ of the distal glucosamine unit (Fig. 3), exactly as in authentic lipid II_A isolated from Kdo-deficient *S. typhimurium* cells (46).

The *top tracing* in Fig. 8*B*, labeled *−2.55 ppm*, is the difference spectrum obtained upon subtracting ^{1}H NMR spectra with selective on- and off-resonance decoupling of the −2.55 ppm ^{31}P resonance (Fig. 8*A*). This spectrum reveals two overlapping proton "doublet" signals near 5.5 ppm (assigned to H-1 and H-1″ by COSY analysis), and two smaller "doublet of doublets" at 4.22 and 3.70 ppm that are assigned to H-2 and H-2″, respectively (see Fig. 3 for the numbering scheme). These findings establish the upfield −2.55 ppm phosphorus resonance as arising from the bridging monophosphodiester group between C-1 of the proximal glucosamine and C-1″ of the L-Ara4N ring in the *in vitro* synthesized material, further establishing its identity as lipid II_A. Small differences in the actual ^{31}P chemical shifts between the *in vitro* product (Fig. 8*A*) and the lipid II_A previously isolated from cells (2.14 *versus* 1.56 ppm for P-4′ and −2.55 *versus* −2.62 ppm for P-1) (46) are attributed to slight differences in the pH, which was not rigorously controlled.

Difference spectra like those shown in Fig. 8*B* were also acquired for the lipid II_B product generated *in vitro*, again demonstrating that the 1-phosphate is the sole site of L-Ara4N attachment under these assay conditions (data not shown).

Overexpression of the S. typhimurium arnT Gene in a Polymyxin-resistant Strain of E. coli—Based on a PSI-BLAST analysis (51), *arnT* of *S. typhimurium* (Fig. 2) is proposed to encode the L-Ara4N transferase, since ArnT displays distant similarity to the yeast protein mannosyltransferases, which utilize dolichyl phosphate-mannose as their donor substrate (52). The predicted ArnT protein is 548 amino acids long and has 12 membrane-spanning regions (24). To determine whether *arnT* encodes a functional L-Ara4N transferase, the gene was amplified by PCR from *S. typhimurium* genomic DNA and was subcloned behind the T7*lac* promoter in the expression vector pET21. The resulting hybrid plasmid was designated pArnTSt.

Prior to expression studies with pArnTSt, we were surprised to find that *E. coli* B derivatives, such as the BLR(DE3) host strains from Novagen, contain lipid A modifications similar to those found in *pmrA^{C} S. typhimurium* (Fig. 9, *lane 1*). In contrast, *E. coli* K-12 strains do not contain any modified lipid A species (2, 10) when grown on LB broth (Fig. 9, *lanes 2* and *4*). Separation of the ^{32}P-labeled lipid A species from BLR(DE3)pLysS (Fig. 9, *lane 3*) clearly demonstrated the presence of L-Ara4N- and/or pEtN-modified lipid A species. These lipid A modifications in the host strains were not dependent upon the presence of the DE3 lysogen or on the plasmid pLysS that encodes the T7 lysozyme (data not shown). Furthermore, the BLR strains of *E. coli* are polymyxin-resistant when grown in the presence of 2 μg/ml polymyxin B sulfate on LB agar plates (data not shown). Accordingly, a source of L-Ara4N donor substrate (*i.e.* the putative undecaprenyl phosphate-L-Ara4N) should be available in membranes prepared from these *E. coli* B derivatives, commonly used for overexpression of proteins.

Membranes isolated from strains of BLR(DE3)pLysS, containing either the vector control pET21 or pArnTSt, were assayed for L-Ara4N transferase activity using [4′-^{32}P]lipid IV_A as the acceptor (Fig. 10). Massive overproduction of the

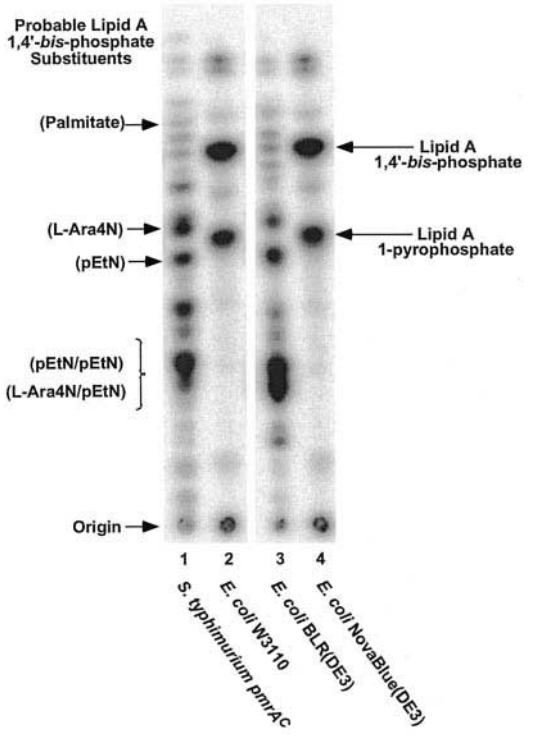

FIG. 9. ***E. coli* BLR(DE3) contains pEtN and L-Ara4N modified lipid A species.** Analysis of ^{32}P-labeled lipid A species present in *E. coli* BLR(DE3) shows this expression host strain modifies its lipid A like a *pmrA* constitutive mutant of *S. typhimurium* (11), consistent with the unexpected observation that *E. coli* BLR(DE3) is in fact polymyxin-resistant. The labeled lipid A species were separated by TLC and visualized by PhosphorImager analysis. Based upon previous studies by Zhou *et al.* (10, 11), the proposed lipid A modifications are indicated. *E. coli* NovaBlue(DE3), which is a K-12 derivative and is polymyxin-sensitive, and its lipid A are unmodified as in *E. coli* W3110 (10).

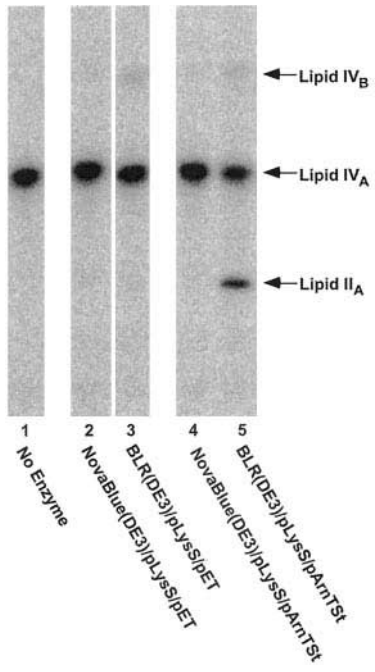

FIG. 10. **High levels of L-Ara4N transferase activity in membranes of *E. coli* BLR(DE3) but not *E. coli* NovaBlue(DE3).** The plasmids, pArnTSt and pET21, were transformed either into the *E. coli* K-12 derivative, NovaBlue(DE3)pLysS, or into the polymyxin-resistant *E. coli* B strain, BLR(DE3)pLysS. The cloned *S. typhimurium arnT* gene was induced with isopropyl-1-thio-β-D-galactopyranoside in mid log phase, and membranes from each construct (0.5 mg/ml) were assayed for L-Ara4N transferase for only 10 min using 10 μM 4′-^{32}P-lipid IV$_A$ as the acceptor. Products were separated by TLC and visualized with a PhosphorImager.

L-Ara4N transferase activity was seen in membranes from cells containing pArnTSt (Fig. 10, *lane 5*) as compared with the vector control (Fig. 10, *lane 3*). The membranes containing the overexpressed ArnT had a specific activity of 12 nmol/min/mg, an ~200-fold increase compared with membranes of the *pmrA*^{C} *S. typhimurium* mutant JSG435 (Fig. 4), when assayed at a protein concentration of 0.5 mg/ml. However, membranes isolated from *arnT* overexpressed in the *E. coli* K12 strain NovaBlue(DE3)pLysS did not catalyze any transfer of the L-Ara4N moiety to [4′-^{32}P]lipid IV$_A$ (Fig. 10, *lane 4*), presumably because of the absence of an endogenous L-Ara4N donor substrate. As expected, the lipid A species isolated from NovaBlue(DE3)pLysS contained no L-Ara4N or pEtN substituents (Fig. 9, *lane 4*). The combined data strongly support the view that *arnT* (Fig. 2) is the structural gene for the L-Ara4N transferase.

Assay of ArnT with Kdo$_2$[4′-^{32}P]Lipid IV$_A$ or Kdo$_2$[^{32}P]Lipid A as the Acceptor—To determine whether modification of the 4′-phosphate with the L-Ara4N moiety is dependent upon the Kdo disaccharide, membranes from cells containing the overexpressed *arnT* gene were assayed using the precursor, Kdo$_2$-[4′-^{32}P]lipid IV$_A$ (Fig. 11). The rate at which the L-Ara4N modification reaction occurred was comparable with that seen with [4′-^{32}P]lipid IV$_A$ as the acceptor, but a second more hydrophilic substance, designated *Product C* in Fig. 11, was observed, consistent with the incorporation of a second L-Ara4N unit. When assayed at various times, Product C appeared to be dependent upon the prior formation of Product B, a species proposed to contain only one L-Ara4N-derivatized phosphate. Unfortunately, it is impossible to determine from these experiments which phosphate residue of Kdo$_2$[4′-^{32}P]lipid IV$_A$ is

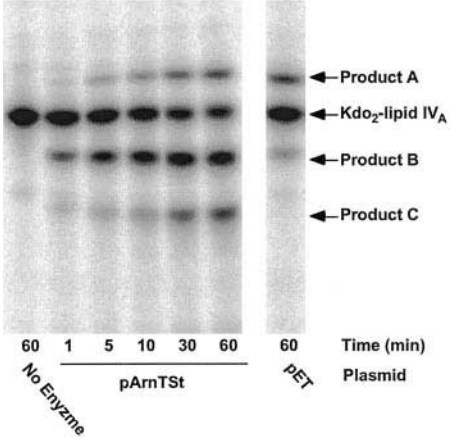

FIG. 11. **ArnT-catalyzed transfer of two L-Ara4N moieties to Kdo$_2$-4′-^{32}P-lipid IV$_A$.** Membranes from BLR(DE3)pLysS cells expressing either pET21 or pArnTSt were assayed for L-Ara4N transferase under standard conditions at 0.5 mg/ml protein and 10 μM Kdo$_2$-4′-^{32}P-lipid IV$_A$ for the indicated times. Products were separated by TLC and visualized by PhosphorImager analysis. The migration of Products B and C is consistent with the addition of one or two L-Ara4N moieties, respectively. Product A is formed by incorporation of a palmitoyl group, catalyzed by PagP (12). The small amount of transferase in the vector control is due to chromosomal *E. coli* ArnT.

modified first with the L-Ara4N moiety. In fact, Product B is likely to be a mixture of singly modified species. The results do strongly suggest, however, that *S. typhimurium* ArnT is a bifunctional glycosyltransferase in that it can incorporate two L-Ara4N units. Product A presumably results from the PagP-dependent palmitoylation (12) of the Kdo$_2$[4′-^{32}P]lipid IV$_A$ acceptor (Fig. 3). Additional studies will be required to determine

the exact structures of the products generated by the action of ArnT on Kdo_2[4′-^{32}P]lipid IV_A.

In a separate set of assays, Kdo_2[^{32}P]lipid A (*i.e.* the hexaacylated material) was used as the acceptor. As seen with Kdo_2[4′-^{32}P]lipid IV_A (Fig. 11), two hydrophilic products were generated by membranes that overexpressed ArnT, confirming that one of the L-Ara4N units is incorporated in a Kdo-dependent manner (data not shown).

DISCUSSION

The L-Ara4N substituent of *S. typhimurium* lipid A was discovered in 1970 (7), but its biosynthesis and function have remained elusive until recently. Increased substitution of lipid A with L-Ara4N moieties occurs in polymyxin-resistant mutants (22, 23, 25), suggesting a charge neutralization function that might protect cells against cationic antimicrobial peptides. Polymyxin resistance is caused by mutations in the transcription factor PmrA, which is rendered constitutively active in resistant strains (26). An important breakthrough was made in 1998 by Gunn *et al*. (24), who discovered an operon near minute 51 required for maintenance of polymyxin resistance in *pmrA* constitutive organisms. Sequence analysis of the protein products of this operon with the PSI-BLAST tool suggested a plausible hypothesis for the enzymatic synthesis of L-Ara4N from UDP-glucuronic acid, as shown in Fig. 2 (10, 53).

We have now developed the first *in vitro* system for L-Ara4N transfer to lipid A (Figs. 4, 10, and 11). Both the transferase (ArnT) and its L-Ara4N donor substrate are associated with the inner membrane of *S. typhimurium* (Figs. 5 and 6). This is consistent with the hydropathy profile of the transferase, which contains 12 membrane-spanning regions and with the proposed role of undecaprenyl phosphate-L-Ara4N as the donor (Fig. 2). Robust L-Ara4N transferase activity is seen only in membranes of cells in which PmrA is activated and is completely absent in membranes of *pmrA*$^-$ mutants (Fig. 4).

Overexpression of *S. typhimurium arnT* behind the T7*lac* promoter in *E. coli* BLR/DE3 results in a further 200-fold elevation of L-Ara4N transferase-specific activity, when compared with membranes of *pmrA*^{C} *S. typhimurium*, supporting the assignment of *arnT* as the structural gene for the transferase (Fig. 2). Unexpectedly, however, the T7 expression strain BLR(DE3) is itself polymyxin-resistant and contains L-Ara4N-modified lipid A (Fig. 9), whereas no L-Ara4N-modified lipid A is made in the polymyxin-sensitive *E. coli* K-12 expression host, NovaBlue(DE3) (Fig. 9). No L-Ara4N transferase activity is seen in membranes of NovaBlue(DE3) cells that overexpress *S. typhimurium arnT* (Fig. 10), because the L-Ara4N donor substrate is absent. Addition of purified undecaprenyl phosphate-L-Ara4N to such membranes reconstitutes full transferase activity (32).

ArnT of *E. coli* K-12 is 550 amino acid residues long, and it displays 71% identity and 82% similarity to the 548-residue *S. typhimurium* ArnT. *E. coli* K-12 furthermore contains all the other genes of the *S. typhimurium* operon required for polymyxin resistance (24). The regulation of these genes in *E. coli* is probably similar to that reported for the better characterized *S. typhimurium* system, but there are some differences. Unlike *S. typhimurium*, lipid A of *E. coli* K-12 does not contain the L-Ara4N moiety when grown in LB broth at pH 6.8 (10, 11). Selection of polymyxin-resistant mutants (23) or treatment of wild-type cells with ammonium metavanadate (10) is required to induce L-Ara4N biosynthesis in *E. coli* K-12.

As demonstrated in the following article (32), the L-Ara4N transferase utilizes the novel carrier lipid, undecaprenyl phosphate-L-Ara4N, as its donor substrate (Fig. 2). The utilization of this substrate by ArnT suggests that the transferase active site faces the periplasm, given that peptidoglycan (54) and *O*-antigen (2, 55–57) precursors are likewise assembled on undecaprenyl carriers. Modification of the lipid A 4′-phosphate moiety with L-Ara4N on the periplasmic surface of the inner membrane would avoid any possibility of interference with the Kdo transferase. The latter is located on the cytoplasmic side of the inner membrane and requires an unsubstituted 4′-phosphate moiety for activity (10, 41).

In vitro, ArnT modifies only the 1-phosphate of lipid IV_A (Figs. 3, 7, and 8), which lacks the Kdo disaccharide. However, ArnT appears to modify both the 1- and the 4′-phosphate groups of Kdo_2[4′-^{32}P]lipid IV_A (Fig. 11) and Kdo_2-[^{32}P]lipid A (not shown), as judged by TLC analysis, suggesting that L-Ara4N transfer to the 4′-position is Kdo-dependent. These findings provide an explanation for the peculiar observation that lipid A precursors isolated from Kdo-deficient strains are modified with L-Ara4N exclusively at the 1-position (9, 46), whereas mature lipid A prepared from wild-type cells is modified with L-Ara4N predominantly at position 4′ (11, 46). However, lipid A of wild-type *S. typhimurium* does in fact contain a minor component in which L-Ara4N is attached both to the 1- and to the 4′-phosphate groups (11), consistent with the ability of ArnT to modify both positions *in vitro*, depending upon the acceptor.

ArnT contains 12 membrane-spanning regions, much like the inner membrane protein Wzx, a proposed undecaprenyl diphosphate-oligosaccharide flippase (57, 58). It may be that ArnT functions not only as a glycosyltransferase but also as a flippase for undecaprenyl phosphate-L-Ara4N. However, other membrane-associated proteins of unknown function present in the polymyxin resistance operon, such as Orf6 and Orf7 (6, 24), could also play a role in the transport process. Isolation of mutants that are able to make undecaprenyl phosphate-L-Ara4N, but are unable to transfer it to lipid A when extra copies of wild-type *arnT* are provided in *trans*, might lead to the identification of genes required for undecaprenyl phosphate-L-Ara4N transport. Very little is known about dolichyl phosphate-sugar transport in eucaryotic systems (59).

Full-length homologues of ArnT are present only in those organisms that make the L-Ara4N moiety, including *E. coli*, *S. typhimurium*, *Pseudomonas aeruginosa,* and *Yersinia pestis.* However, many additional homologues of the N-terminal portion of ArnT are encoded within other bacterial and eucaryotic genomes, as judged by BLASTp and PSI-BLAST analysis (51). These evolutionarily distant ArnT homologues, which include the protein mannosyltransferases of yeast and animal cells (52, 60), may all utilize polyisoprenyl phosphate-sugars as donors. The N-terminal portion of these proteins may contain the region for binding the polyisoprenyl phosphate-sugar substrate or the catalytic site. The less conserved C-terminal half of ArnT may play a more specific role, for instance in binding the lipid A acceptor substrate. To our knowledge, ArnT is unusual among glycosyltransferases of this kind in that it catalyzes the transfer of a sugar moiety from one phosphate residue to another (Fig. 1 in Ref. 32).

Dissection of the molecular mechanisms used by Gram-negative bacteria to protect themselves from antibacterial agents is crucial to understanding pathogenesis. PmrA constitutive mutants of *S. typhimurium* with increased levels of L-Ara4N and pEtN substituents survive longer inside neutrophils (61) than do wild-type bacteria. Conversely, *pmrA*-deficient mutants of *S. typhimurium* show reduced virulence compared with wild-type strains after oral administration to BALB/c mice (6). Inhibitors of the enzymes responsible for L-Ara4N biosynthesis (Fig. 2) might be useful in combination with traditional antibiotics by exposing the lipid A phosphate groups during endo-

cytosis and sensitizing the bacteria to attack by the innate immune system.

Acknowledgments—The Duke NMR Center was supported in part by NCI Grant P30-CA-14236 from the National Institutes of Health. NMR instrumentation was funded by the National Science Foundation, the National Institutes of Health, the North Carolina Biotechnology Center, and Duke University.

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