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Lipid A Modifications Characteristic of *Salmonella typhimurium* Are Induced by NH_4VO_3 in *Escherichia coli* K12*

DETECTION OF 4-AMINO-4-DEOXY-L-ARABINOSE, PHOSPHOETHANOLAMINE AND PALMITATE*

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Two-thirds of the lipid A in wild-type *Escherichia coli* K12 is a hexa-acylated disaccharide of glucosamine in which monophosphate groups are attached at positions 1 and 4′. The remaining lipid A contains a monophosphate substituent at position 4′ and a pyrophosphate moiety at position 1. The biosynthesis of the 1-pyrophosphate unit is unknown. Its presence is associated with lipid A translocation to the outer membrane (Zhou, Z., White, K. A., Polissi, A., Georgopoulos, C., and Raetz, C. R. H. (1998) *J. Biol. Chem.* 273, 12466–12475). To determine if a phosphatase regulates the amount of the lipid A 1-pyrophosphate, we grew cells in broth containing nonspecific phosphatase inhibitors. Na_2WO_4 and sodium fluoride increased the relative amount of the 1-pyrophosphate slightly. Remarkably, NH_4VO_3-treated cells generated almost no 1-pyrophosphate, but made six major new lipid A derivatives (EV1 to EV6). Matrix-assisted laser desorption ionization/time of flight mass spectrometry of purified EV1 to EV6 indicated that these compounds were lipid A species substituted singly or in combination with palmitoyl, phosphoethanolamine, and/or aminodeoxypentose residues. The aminodeoxypentose residue was released by incubation in chloroform/methanol (4:1, v/v) at 25 °C, and was characterized by ^{1}H NMR spectroscopy. The chemical shifts and vicinal coupling constants of the two anomers of the aminodeoxypentose released from EV3 closely resembled those of synthetic 4-amino-4-deoxy-L-arabinose. NH_4VO_3-induced lipid A modification did not require the PhoP/PhoQ two-component regulatory system, and also occurred in *E. coli msbB* or *htrB* mutants. The lipid A variants that accumulate in NH_4VO_3-treated *E. coli* K12 are the same as many of those normally found in untreated *Salmonella typhimurium* and *Salmonella minnesota*, demonstrating that *E. coli* K12 has latent enzyme systems for synthesizing these important derivatives.

Lipopolysaccharide is a major component of the outer leaflet of the outer membranes of Gram-negative bacteria (1–7). The hydrophobic anchor of lipopolysaccharide, termed lipid A (1, 2, 8, 9), is a β(1′-6)-linked disaccharide of glucosamine. In *Escherichia coli* K12, the 2, 3, 2′, and 3′ positions of the disaccharide are acylated with *R*-3-hydroxymyristoyl groups, and the 1 and 4′ positions are phosphorylated (Fig. 1*A*). Two-thirds of the lipid A recovered from wild-type *E. coli* K12 (designated the "*bis*-phosphate") contains monophosphate substituents at positions 1 and 4′ (Fig. 1*A*). The rest (termed the "lipid A 1-pyrophosphate")[1] contains a monophosphate group at 4′ and an unsubstituted pyrophosphate unit at 1 (Fig. 1*A*) (10–12). The 2′ and 3′ *R*-3-hydroxymyristoyl groups are further acylated with laurate and myristate, respectively (1, 2). A nonrepeating oligosaccharide known as the core is attached at the 6′ position of lipid A (1–4). In the proper genetic setting, the outer core sugars may be further glycosylated with polymeric *O*-antigen (1–7).

With the exception of the reaction that generates the 1-pyrophosphate unit (Fig. 1*A*), all the enzymes required for making lipid A in *E. coli* K12 are now known (1, 13, 14). However, in *Salmonella typhimurium* and *Salmonella minnesota*, additional lipid A species derivatized with palmitate, 4-amino-4-deoxy-L-arabinose (L-4-aminoarabinose), *S*-2-hydroxymyristate, and/or phosphoethanolamine are recovered in significant amounts (1, 2, 8), resulting in multiple molecular subtypes (Fig. 1*B*) (15). The enzymes that catalyze the synthesis and attachment of these interesting substituents have not yet been identified, but they appear to be under the control of the PhoP/PhoQ system in *S. typhimurium* (16–18). The PhoP/PhoQ system is activated at low magnesium ion concentrations (16, 19), and it is required for the establishment of animal infections (16, 19). Lipid A species modified with L-4-aminoarabinose are found in many other Gram-negative bacteria, including strains of *Klebsiella*, *Proteus,* and *Chromobacterium* (20–23).

Higher than normal levels of L-4-aminoarabinose are made in polymyxin-resistant mutants of *S. typhimurium*, which harbor lesions in another two component regulatory system, known as PmrA/PmrB (24, 25). The latter is thought to be downstream of and activated by PhoP/PhoQ (16, 19). Polymyxin-resistant mutants of *E. coli* K12 (26, 27) have recently been characterized, and like strains of *Salmonella*, they synthesize significant amounts of lipid A species bearing palmitate, L-4-aminoarabinose, and/or phosphoethanolamine (27). *E. coli* K12 must therefore possess the enzymatic machinery to generate these substitutions, despite their absence in cells grown on nutrient broth.

An operon of PhoP/PhoQ-regulated genes that is required for the maintenance of polymyxin resistance (and possibly for L-4-aminoarabinose biosynthesis) has recently been discovered in both *S. typhimurium* and *E. coli* K12 (18). The regulatory and enzymatic functions of the products encoded by these genes have not yet been elucidated (18). A separate PhoP/PhoQ-

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[1] In a previous communication (12) the 1-pyrophosphate was termed the "lipid A *tris*-phosphate." Since the latter incorrectly implies the presence of three monophosphate substituents, we now prefer the designation "lipid A 1-pyrophosphate."

TABLE I
Strains and plasmids used in this study

Strain/plasmid	Relevant genotype	Source or reference
E. coli K12		
W3110	Wild type, F^-, λ^-	*E. coli* Genetic Stock Center, Yale University
T180A	*recA*$^+$ *srl*::Tn10	39
CF10	*pgpA235*::Kan^r	39
CF20	*pgpB159*::Amp^r	39
CF30	*pgpA235*::Kan^r/*pgpB159*::Amp^r	39
CSH26	F^-, *ara* Δ *(lacpro) thi*	31
CSH26ΔQ	CSH26 with an internal *Sca*I-*Pae*R7I *phoQ* deletion	43
CSH26ΔPQ	CSH26 with an internal *Cla*I-*Pae*R7I *phoP*/*Q* deletion	Waldburger and Sauer, unpublished data
MLK1067	W3110 *msbB1*::ΩCam	33 63
MLK986	W3110 *htrB1*::Tn10/*msbB1*:: ΩCam	63
MLK986/pKW2	MLK986/pKW2 (*msbA*$^+$)	This study
S. typhimurium LT-2		
ATCC14028	Virulent wild type	Salmonella Genetic Stock Center, University of Calgary, Canada
Plasmid		
pKW2	pACYC184 containing *msbA*$^+$	12

regulated gene (*pagP*), which is required for resistance to a subset of the antibacterial polypeptides present in neutrophils (17), may encode the enzyme that incorporates the palmitate residue found in some lipid A molecular species of *S. typhimurium*. However, *pagP* is not part of the L-4-aminoarabinose gene cluster (17).

We now report that six major lipid A variants derivatized with palmitate, L-4-aminoarabinose, and/or phosphoethanolamine (Fig. 1*C*) accumulate in wild-type cells of *E. coli* K12 treated with 25 mM NH_4VO_3, despite their complete absence under ordinary growth conditions (Fig. 1*A*). The lipid A modifications induced by NH_4VO_3 in *E. coli* K12 resemble those seen in untreated strains of *Salmonella* (15, 16, 18, 28, 29), but their induction in *E. coli* is not dependent upon a functional PhoP/PhoQ signaling system, suggesting that NH_4VO_3 acts downstream of PhoP/PhoQ, perhaps on PmrA/PmrB. We have devised methods for isolating milligram quantities of several of the predominant lipid A species found in NH_4VO_3-treated *E. coli*. These substances were released from cells by pH 4.5 hydrolysis at 100 °C in SDS (10, 30), which cleaves the Kdo lipid A linkage, and were separated from each other by ion exchange and thin layer chromatography (28). The compounds were analyzed by MALDI/TOF[2] mass spectrometry and ^1H NMR spectroscopy to validate their structures. Procedures for isolating pure, hexa-, or hepta-acylated lipid A species, substituted with L-4-aminoarabinose and/or phosphoethanolamine, have not been reported previously.

EXPERIMENTAL PROCEDURES

Materials—$^{32}P_i$ was purchased from NEN Life Science Products Inc. Na_2WO_4, NH_4VO_3, sodium fluoride, adenine, and *p*-anisaldehyde were obtained from Sigma. D_2O was from Aldrich. Pyridine, methanol, and 88% formic acid were obtained from Mallinckrodt, and chloroform was purchased from EM Science. Glass backed Silica Gel 60 thin layer chromatography plates (0.25 mm) were from E. Merck, Germany.

Bacterial Strains—The bacterial strains used in this study are described in Table I. Strains CSH26, CSH26ΔQ, and CSH26ΔPQ were kindly provided by Dr. Carey D. Waldburger, Columbia University. Cells were generally grown at 37 °C in LB broth, consisting of 10 g of NaCl, 5 g of yeast extract, and 10 g of Tryptone per liter (31). Antibiotics were added when necessary at final concentrations of 12 μg/ml for tetracycline, 10 μg/ml for chloramphenicol, 100 μg/ml for ampicillin, and 50 μg/ml for kanamycin. Strain CSH26ΔPQ was also supplemented with 75 μg/ml adenine. LB broth containing the nonspecific phosphatase inhibitors, Na_2WO_4, NH_4VO_3, or sodium fluoride, was filter sterilized before use. LB broth containing 25 mM NH_4VO_3 was made by mixing equal volumes of autoclaved (2-fold concentrated) LB medium and filter-sterilized aqueous 50 mM NH_4VO_3.

Analysis of Lipid A Released from $^{32}P_i$ Labeled Cells by Mild Acid Hydrolysis—To label lipid A with ^{32}P, cells were grown and extracted, as described previously (12, 32, 33). Briefly, an overnight culture grown at 37 °C on LB medium was diluted 100-fold into 5 ml of fresh medium. Cells were then labeled by addition of 5 μCi/ml $^{32}P_i$, and allowed to continue growing at 37 or 42 °C (as indicated) for 3 h. The ^{32}P-labeled cells were collected by centrifugation in a glass tube equipped with a Teflon-lined cap, and washed twice with 5.0 ml of phosphate-buffered saline, pH 7.4. Next, they were resuspended in 0.8 ml of phosphate-buffered saline, and a single phase Bligh/Dyer mixture (34) was made by addition of 2 ml of methanol and 1 ml of chloroform. After 60 min at room temperature, the insoluble material was collected by centrifugation in a clinical centrifuge at top speed for 20 min. This pellet was washed once with 5.0 ml of a fresh single-phase Bligh/Dyer mixture, consisting of chloroform/methanol/water (1:2:0.8, v/v). The pellet was then dispersed in a 1.8-ml portion of 12.5 mM sodium acetate, pH 4.5, containing 1% SDS, with sonic irradiation in a bath apparatus. The mixture was incubated at 100 °C for 30 min to cleave the glycosidic linkage between Kdo and lipid A (10, 12, 30, 35). To recover the lipid A, the hydrolyzed material was converted to a two-phase Bligh/Dyer mixture by addition of 2 ml of chloroform and 2 ml of methanol. After centrifugation at low speed, the lower phase was collected and washed twice with 4 ml of the upper phase derived from a fresh neutral two phase Bligh/Dyer mixture, consisting of chloroform/methanol/water (2:2:1.8, v/v). The washed lower phase was dried under N_2. The lipid A sample was re-dissolved in the solvent of chloroform/methanol (4:1, v/v), and several microliters (~1,000 cpm) were applied to the origin of a Silica Gel 60 TLC plate. The plate was developed in the solvent of chloroform/pyridine, 88% formic acid/water (50:50:16:5, v/v). The plate was dried and exposed to a PhosphorImager screen overnight (Molecular Dynamics) to visualize the lipid A species.

Purification of Modified Lipid A Derivatives that Accumulate in NH_4VO_3-treated Cells—A 4-ml overnight culture of *E. coli* W3110 was inoculated into fresh LB broth (4.0 liters) containing 25 mM NH_4VO_3. The cells were grown at 37 °C until A_{600} had reached ~2. Cells were harvested by centrifugation. The cell pellet was washed once with 320 ml of phosphate-buffered saline, pH 7.4, and then was resuspended in 160 ml of the same buffer. A single phase Bligh/Dyer mixture was made by addition of 400 ml of methanol and 200 ml of chloroform. Cells were extracted at room temperature for 60 min. After centrifugation at 5,000 rpm for 15 min in 125-ml Corex bottles, the combined pellet was washed twice with 250-ml portions of a fresh single-phase Bligh/Dyer mixture, consisting of chloroform/methanol/water (1:2:0.8, v/v), followed each time by centrifugation to recover the pellet. The pellet was then dispersed in a 180-ml portion of 12.5 mM sodium acetate, pH 4.5, containing 1% SDS, with the aid of a Branson Sonifier (Model 250) equipped with a micro-tip. The mixture was incubated at 100 °C for 30 min to release the lipid A species (10, 12, 30, 35). The hydrolysis mixture was then converted to a two-phase Bligh/Dyer system by addition of 200 ml each of chloroform and methanol. After thorough mixing, the phases

[2] The abbreviations used are: MALDI/TOF, matrix-assisted laser desorption ionization/time of flight; FAB, fast atom bombardment.

were separated by low speed centrifugation, and the lower and the upper phases were collected. The upper phase was extracted with 80 ml of lower phase derived from a fresh neutral two-phase Bligh/Dyer mixture, consisting of chloroform/methanol/water (2:2:1.8, v/v). The lower phase was extracted with 80 ml of the upper phase from the same fresh neutral two-phase Bligh/Dyer mixture. The lower phases, containing the released lipid A species, were then pooled and dried with a rotary evaporator at room temperature.

The modified lipid A derivatives were first separated by anion exchange chromatography on DEAE cellulose, as described previously (28, 35). A 2-ml DEAE cellulose column (Whatman DE52) in the acetate form was prepared and equilibrated with the solvent of chloroform/methanol/water (2:3:1, v/v). One-fourth of the total dried lipid A sample described above was re-dissolved in 10 ml of chloroform/methanol/water (2:3:1, v/v). The material was centrifuged at low speed to remove insoluble debris, and the supernatant was loaded onto the column at its natural flow rate. The column then was washed with 12 ml of chloroform/methanol/water (2:3:1, v/v). Fractions (2 ml each) were collected. Lipids EV5 and EV6 were eluted with 12 ml of chloroform/methanol/60 mM aqueous ammonium acetate (2:3:1, v/v). Lipids EV2, EV3, and EV4 were eluted with 12 ml of chloroform, methanol, 120 mM ammonium acetate (2:3:1, v/v). The "normal" hexa-acylated lipid A 1,4′-*bis*-phosphate and the hepta-acylated species EV1 were eluted with 12 ml of chloroform/methanol/240 mM ammonium acetate (2:3:1, v/v). Finally, the column was eluted with 12 ml of chloroform/methanol/480 mM ammonium acetate (2:3:1, v/v) to make certain no other lipids were present. To locate the fractions containing the desired lipids, 20 μl of each fraction was spotted onto a 10 × 20-cm Silica Gel 60 TLC plate. The plate was developed in the solvent of chloroform/pyridine/88% formic acid, water (50:50:16:5, v/v). The spots were visualized by charring on a hot plate after spraying the chromatogram with a mixture of ethanol/*p*-anisaldehyde/H_2SO_4/acetic acid (89:2.5:4:1, v/v) (36). The DEAE cellulose fractions containing the lipids of interest were then converted to neutral two-phase Bligh/Dyer mixtures by addition of the necessary amounts of chloroform and water. The lower phases were pooled, as appropriate, dried under N_2, and stored at −20 °C.

With the exception of EV5, which was produced in much lower quantities, the substituted lipid A derivative EV1, EV2, EV3, EV4, and EV6 that accumulated in NH_4VO_3-treated cells (as well as the lipid A 1,4′-*bis*-phosphate from untreated cells) were further purified by preparative thin layer chromatography. Lipid A samples from the dried DEAE cellulose column fractions were re-dissolved in chloroform/methanol (4:1, v/v), and each ~0.5-mg sample was applied in a line to a 20 × 20-cm Silica Gel 60 TLC plate (0.25 mm thickness). The plates were developed in the solvent chloroform/pyridine/88% formic acid, water (50:50:16:5, v/v). While the plates were drying at room temperature, the lipid A bands could be seen transiently as white zones. These were marked with a pencil, and the plates were then allowed to dry completely at room temperature for ~20 min before each marked zone was scraped off the plate with a clean razor blade. Each lipid A derivative was extracted from the silica chips with 24.0 ml of an acidic single-phase Bligh/Dyer mixture, consisting of chloroform/methanol/50 mM aqueous ammonium acetate adjusted to pH 1.5 with HCl (1:2:0.8, v/v). The silica chips were removed by low speed centrifugation, and the supernatant was collected and passed through a layer of glass wool stuffed into a Pasteur pipette in order to remove any residual silica. The filtered material was converted to a two-phase Bligh/Dyer mixture by addition of 6.0 ml each of chloroform and water. The lower phase was collected and neutralized by the addition of 24 drops of pyridine prior to the addition of 30 drops of extra methanol to clear the solution. The lower phase was then dried under a stream of N_2. Finally, prior to mass spectrometry, the TLC-purified lipid A derivatives were subjected to a second DEAE cellulose column chromatography, as described above, to remove contaminating metal ions. The purified lipid A derivatives were stored at −20 °C prior to mass spectrometry and between purification steps.

Mass Spectrometry Analysis of Purified Lipid A Derivatives—Spectra were acquired in the negative linear mode by using a time of flight matrix-assisted laser desorption/ionization (MALDI) Kompact 4 mass spectrometer (Kratos Analytical Manchester, United Kingdom), equipped with a 337-nm nitrogen laser and set at a 20 kV extraction voltage (37). Each spectrum was the average of 50 shots. Two kinds of matrices were used in the present study. One was a saturated solution of 2,5-dihydroxybenzoic acid in 50% acetonitrile for the lipid A 1,4′-*bis*-phosphate from untreated cells, as well as for EV2, EV3, and EV6. The other was a mixture of saturated 6-aza-2-thiothymine in 50% acetonitrile and 10% tribasic ammonium citrate (9:1, v/v) for EV1, EV4, and EV5. Lipid samples were dissolved in a mixture of chloroform/methanol (4:1, v/v) before mixed with a matrix (1:1, v/v) on a slide. The sample mixtures were allowed to dry at room temperature prior to mass analysis.

Mass Spectrometry of the Aminodeoxypentose Released from Purified Lipid EV2—The spectrum was acquired in positive mode on a JEOL JMS-SX-102 high resolution mass spectrometer (Instrument Center, Department of Chemistry, Duke University) at 62.5 °C with a fast atom bombardment (FAB) gun set at 8 kV and a 10 kV acceleration voltage.

^{1}H NMR Spectroscopy of the Aminodeoxypentose Released from Purified Lipid EV3—^{1}H NMR spectra of several hundred micrograms of the putative aminodeoxypentose substituent released from purified lipid EV3 were recorded at 25 °C in D_2O (0.6 ml) at 500 MHz on a Varian Unity 500 spectrometer at the Duke University NMR Center.

RESULTS

Lipid A 1-Pyrophosphate Levels in E. coli Mutants Lacking the Phosphatidylglycerophosphate Phosphatases—In previous studies (12), the molar ratio of the hexa-acylated lipid A 1,4′-*bis*-phosphate to the lipid A 1-pyrophosphate (Fig. 1*A*) was shown to be about 2.5 in wild-type strains of *E. coli* grown on nutrient broth. Mass spectrometry confirmed that the pyrophosphate unit was indeed attached to position 1 of the glucosamine disaccharide (Fig. 1*A*) (12). To exclude the possibility that the two known phosphatidylglycerophosphate phosphatases of *E. coli* (PgpA and PgpB) (38) are involved in regulating the levels of the lipid A 1-pyrophosphate, ^{32}P-labeled lipid A species from the phosphatase-defective strains CF10 (*pgpA*$^-$), CT20 (*pgpB*$^-$), and CF30 (*pgpA*$^-$/*pgpB*$^-$) (39) were prepared and analyzed. The ratio of lipid A 1,4′-*bis*-phosphate to lipid A 1-pyrophosphate was not altered in these mutants when compared with wild-type (data not shown).

Covalent Modifications of Lipid A in E. coli Cells Treated with NH_4VO_3—To examine the possibility that other (as yet uncharacterized) phosphatases might play a role in determining the amount of the lipid A 1-pyrophosphate, *E. coli* cells were treated with Na_2WO_4, NH_4VO_3, and sodium fluoride. These compounds are nonspecific phosphatase inhibitors that have been used to perturb the levels of lipid intermediates in some systems (40). An overnight culture of *E. coli* W3110 was grown on LB broth at 37 °C, and was diluted 100-fold into separate culture tubes each of which contained 5 ml of fresh LB medium supplemented with 5, 10, 25, or 50 mM Na_2WO_4, NH_4VO_3, or sodium fluoride. Next, 5 μCi/ml $^{32}P_i$ was added to each diluted culture. Cells were grown at 42 °C for 3 h. Lipid A from the cells in each ^{32}P-labeled culture was released by pH 4.5 hydrolysis (12) and analyzed by thin layer chromatography (Fig. 2). In the Na_2WO_4 and the sodium fluoride-treated cells, the lipid A profiles were similar to that of untreated cells (Figs. 2 and 3) with only slightly elevated relative levels of the 1-pyrophosphate species.

Unexpectedly, the lipid A 1-pyrophosphate disappeared altogether in cells treated with 25 mM NH_4VO_3 (Figs. 2 and 3). However, at least six new major lipid species were observed, which generally migrated more slowly than the predominant hexa-acylated lipid A 1,4′-*bis*-phosphate found in the untreated cells. Based on their mobility on TLC plates, they were designated EV1 to EV6 (Figs. 2 and 3). In control experiments, in which 25 mM NH_4Cl was included in the LB broth instead of 25 mM NH_4VO_3, no lipid A modifications were observed (not shown), indicating that the VO_3^- anion and/or its oligomers were responsible for the effect. In the presence of 25 mM NH_4VO_3, the cells grew exponentially, but their doubling time was prolonged by ~20% (data not shown).

To obtain preliminary evidence that the NH_4VO_3-induced compounds are indeed lipid A derivatives, several of them (EV3, 4, and 5/6) were isolated from a culture of ^{32}P-labeled *E. coli* W3110 grown on LB broth containing 25 mM NH_4VO_3 by pH 4.5 hydrolysis and TLC. Each compound was then further hydrolyzed in 0.2 M HCl at 100 °C for 90 min to convert any

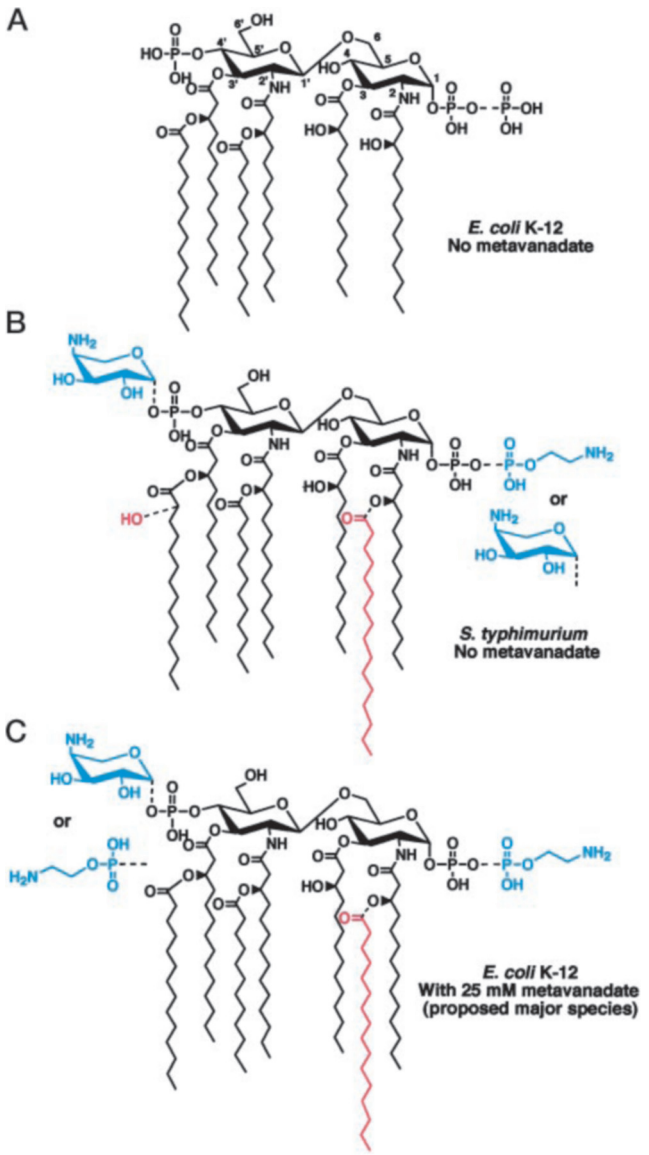

FIG. 1. **Proposed structures of lipid A species found in *E. coli* K12 and *S. typhimurium*.** *A,* under ordinary growth conditions on LB broth in the absence of metavanadate, about two-thirds of the *E. coli* K12 lipid A, which is obtained by hydrolysis of cells at 100 °C in acetate buffer at pH 4.5, is recovered as a hexa-acylated 1,4′-*bis*-phosphate. The rest is a 1-pyrophosphate (*dashed bond*) containing species (10, 12). *B,* when grown on LB broth in the absence of metavanadate, lipid A of *S. typhimurium* is extensively derivatized singly or in combination with the four substituents indicated by the *dashed* bonds (16, 46). The palmitoyl and *S*-2-OH moieties are *red*, whereas the L-4-aminoarabinosyl and phosphoethanolamine substituents are *blue*. The presence of these substituents requires a functioning PhoP/PhoQ system (16). *C,* lipid A modifications resulting from growth of *E. coli* K12 in the presence of NH_4VO_3 are generally the same as those produced in *S. typhimurium*, except that the *S*-2-OH substituent is not detected in *E. coli* grown with NH_4VO_3. The derivatives induced by NH_4VO_3 in *E. coli* K12 are designated *EV1-EV6* (also see Table II). They all contain the usual hexa-acylated lipid A 1,4′-*bis*-phosphate scaffold to which the following are attached as indicated: EV1, palmitate; EV2, palmitate and L-4-aminoarabinose; EV3, L-4-aminoarabinose; EV4, phosphoethanolamine; EV5, two phosphoethanolamine residues, a species that is recovered together with an additional impurity (see text); and EV6, phosphoethanolamine and L-4-aminoarabinose.

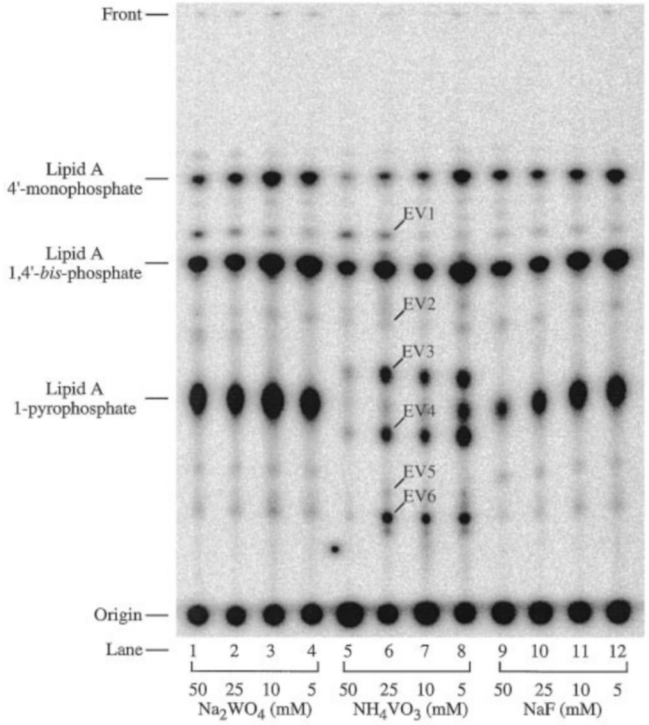

FIG. 2. **The effects of nonspecific phosphatase inhibitors on lipid A molecular species in *E. coli* K12.** An overnight culture of *E. coli* W3110 was diluted 100-fold into 5-ml portions of fresh LB broth containing 50, 25, 10, or 5 mM Na_2WO_4 (*lanes 1–4*), NH_4VO_3 (*lanes 5–8*), or sodium fluoride (*lanes 9–12*), and 5 μCi/ml $^{32}P_i$. Cells were grown at 42 °C for 3 h. Cells were harvested and extracted by the Bligh/Dyer method to remove glycerophospholipids, as described previously (32, 35). Lipid A was then released from the insoluble pellet by hydrolysis in sodium acetate buffer, pH 4.5, at 100 °C for 30 min in the presence of 1% SDS (30, 35). Lipid A species were extracted and separated by thin layer chromatography in the solvent of chloroform/pyridine/88% formic acid, water (50:50:16:5, v/v). The lipid A species were visualized by overnight exposure of the TLC plate to a PhosphorImager screen. The hexa-acylated lipid A 4′-monophosphate, 1,4′-*bis*-phosphate, and 1-pyrophosphate, as well as the 6 novel species (EV1-EV6) that accumulate in NH_4VO_3-treated cells, are indicated. The small amount of the lipid A 4′-monophosphate species seen under all conditions is likely to be a hexa-acylated species that has lost its 1-phosphate residue during hydrolysis, since the 4′-phosphate moiety is more stable than the 1-phosphate.

lipid A species that might be present to its 4′-monophosphate derivative (32, 33). The unknown substances EV3, 4, and 5/6 all yielded the same pattern of lipid A 4′-monophosphates that were obtained by 0.2 M HCl hydrolysis of the hexa-acylated lipid A 1,4′-*bis*-phosphate obtained from wild-type *E. coli* (data not shown). Accordingly, the above compounds from the NH_4VO_3 cells appear to be a family of related lipid A derivatives substituted with acid labile hydrophilic groups. However, compound EV1 is likely to be a hepta-acylated lipid A 1,4′-*bis*-phosphate (Fig. 1*C*), based on its TLC migration (Figs. 2 and 3) and physical characterization (see below), and EV2 also contains a hepta-acylated lipid A moiety (see below). The relative amounts of EV1 to EV6 varied slightly depending upon the growth conditions, the strain, and the protocol for $^{32}P_i$ labeling (Figs. 2 and 3).

Lipid A Modifications in E. coli Cells Treated with NH_4VO_3 Resemble Those Normally Found in Salmonella—To test if NH_4VO_3 had any effect on lipid A biosynthesis in *S. typhimurium*, wild-type cells of strain LT2 were labeled and grown on LB broth in the presence or absence of 25 mM NH_4VO_3. Lipid A species were then analyzed in parallel with lipid A from NH_4VO_3-treated *E. coli* W3110 (Fig. 3). Even in the absence of NH_4VO_3, *S. typhimurium* LT2 produced a complex series of lipid A derivatives, some of which migrated like the species observed in NH_4VO_3–treated *E. coli* (Fig. 3). There was very little lipid A 1-pyrophosphate in *S. typhimurium* LT2 cells under any condition. This TLC analysis suggests that NH_4VO_3 induces *E. coli* K12 to synthesize lipid A modifications that are

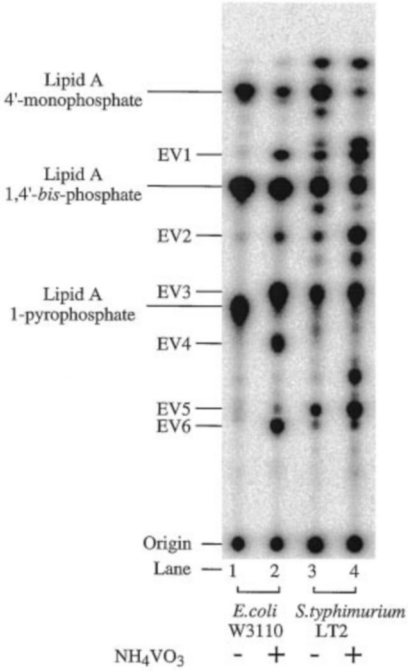

FIG. 3. **Comparison of the lipid A molecular species recovered from *E. coli* K12 *versus S. typhimurium*.** Lipid A species present in *E. coli* K12 and *S. typhimurium* LT-2 wild-type cells were analyzed as described in the legend to Fig. 2. Cells growing on LB broth were labeled with $^{32}P_i$ either in the absence (*lanes 1* and *3*) or presence of 25 mM NH_4VO_3 (*lanes 2* and *4*).

made in the absence of NH_4VO_3 in cells of *Salmonella*, as shown in Fig. 1, *B* and *C*. However, addition of NH_4VO_3 to *S. typhimurium* LT2 does cause several further lipid A modifications (Fig. 3).

Large Scale Purification of the Modified Lipid A Species Found in NH_4VO_3-treated E. coli—Larger amounts of the substituted lipid A derivatives found in NH_4VO_3-treated cells, as well as the unsubstituted hexa-acylated lipid A 1,4′-*bis*-phosphate, were first resolved by anion exchange chromatography on DEAE cellulose in chloroform/methanol/water (2:3:1, v/v) (28, 35). Fig. 4 shows the elution profile of the lipids emerging from the column with increasing salt concentrations. The lipids in each fraction were analyzed by spotting 20-μl portions onto a TLC plate, which was then developed in the solvent of chloroform/pyridine/88% formic acid, water (50:50:16:5, v/v). The lipids were detected by spraying the plate with ethanol/*p*-anisaldehyde/H_2SO_4/acetic acid (89:2.5:4:1, v/v) (36), followed by charring on a hot plate. The slowly migrating unknowns (EV5 and EV6) emerged from the DEAE cellulose column together with the residual, rapidly migrating glycerophospholipids in chloroform/methanol/60 mM ammonium acetate (2:3:1, v/v) (Fig. 4). EV2, 3, and 4 eluted slowly with chloroform/methanol/120 mM ammonium acetate (2:3:1, v/v) (Fig. 4). Based on its charring intensity, EV3 appeared to be the most abundant species. Finally, EV1 and the hexa-acylated lipid A 1,4′-*bis*-phosphate emerged with chloroform/methanol/240 mM ammonium acetate (2:3:1, v/v). The lipid A 1-pyrophosphate species seen in untreated cells was not detectable in the experiment of Fig. 4, consistent with the ^{32}P labeling results (Figs. 2 and 3). Elution of the DEAE cellulose column with chloroform/methanol/480 mM ammonium acetate (2:3:1, v/v) did not yield any additional lipids (not shown). Since EV2-EV6 elute earlier from DEAE cellulose than the unsubstituted lipid A 1,4′-*bis*-phosphate, it appears that the modifications present in these compounds reduce the overall negative charge.

The lipid A derivatives were purified further by preparative thin layer chromatography. To remove residual silica particles and metal ions, as well as any minor breakdown products, all compounds were subjected to chromatography on a second DEAE cellulose column (not shown), as described under "Experimental Procedures." The final samples were dried, and were stored at −20 °C prior to further analysis. The hexa-acylated lipid A 1,4′-*bis*-phosphate, EV3 and EV6 were obtained in milligram quantities. The rest were recovered in microgram amounts.

MALDI/TOF Mass Spectrometry of the Purified Lipids—The structures of the various lipid A species isolated from the NH_4VO_3-treated cells were analyzed using MALDI/TOF mass spectrometry in the negative-ion mode. The hexa-acylated lipid A 1,4′-*bis*-phosphate from *E. coli* K12 W3110 served as the control. The spectrum of the latter (Fig. 5) was characterized by a prominent peak at *m/z* 1796.8, consistent with $[M - H]^-$ for the structure shown in Fig. 1*A* (M_r = 1798.4) and previous reports (11, 15, 35, 41). The small peak at *m/z* 1818.1 (Fig. 5) in the spectrum of the hexa-acylated lipid A 1,4′-*bis*-phosphate was interpreted as $[M + Na\text{-}2H]^-$.

The negative-ion spectrum of EV1 (Fig. 5) demonstrated a major peak at *m/z* 2035.4, consistent with the molecular ion $[M - H]^-$ of a hepta-acylated lipid A 1,4′-*bis*-phosphate species bearing a palmitoyl group, as shown in Fig. 1*C* (M_r = 2036.8). The negative-ion spectrum of EV2 (Fig. 5) showed a major peak at *m/z* 2166.8, consistent with $[M - H]^-$ of the hepta-acylated lipid A *bis*-phosphate species EV1, bearing an additional aminodeoxypentose substituent (Fig. 1*C*, M_r = 2167.9). The smaller peak at *m/z* 2036.3 (Fig. 5) in EV2 was attributed to loss of the aminodeoxypentose moiety (16, 28, 42), which is attached via a rather labile phosphodiester linkage.[3] This fragmentation presumably occurred during mass spectrometry, as the sample migrated like a single pure compound during TLC.

The negative-ion spectrum of EV3 (Fig. 5) revealed a prominent molecular ion $[M - H]^-$ at *m/z* 1928.3, corresponding to a hexa-acylated lipid A *bis*-phosphate species derivatized with one aminodeoxypentose moiety, as shown in Fig. 1*C* (predicted M_r = 1929.5). The smaller peaks at *m/z* 1797.1 and *m/z* 1702.1 were attributed to the loss of the aminodeoxypentose moiety or of an *R*-3-hydroxymyristoyl residue, respectively. The extent of fragmentation during mass analysis varied slightly from sample to sample.

The negative-ion spectrum of EV4 (Fig. 5) demonstrated a major peak at *m/z* 1919.3, consistent with a molecular ion $[M - H]^-$ of hexa-acylated lipid A *bis*-phosphate substituted with an extra phosphoethanolamine moiety, as in Fig. 1C (M_r = 1921.4). The negative-ion spectrum of EV6 (Fig. 5) showed a molecular ion at *m/z* 2051.7, interpreted as $[M - H]^-$ of a hexa-acylated lipid A *bis*-phosphate substituted with both an aminodeoxypentose residue and a phosphoethanolamine moiety, as shown in Fig. 1*C* (M_r = 2052.6). Loss of the aminodeoxypentose residue would account for the peak at *m/z* 1921.4.

EV5 was recovered together with another minor co-migrating lipid, which was not removed because of the low abundance of EV5 (Figs. 3 and 4). The negative-ion spectrum of EV5 (Fig. 5) revealed at least two molecular ions. The minor one at *m/z* 2289.1 was consistent with the $[M - H]^-$ of the hepta-acylated lipid A *bis*-phosphate EV1, further substituted with one aminodeoxypentose residue and one phosphoethanolamine substituent, as shown in Fig. 1*C* (M_r = 2291.0). The more prominent peak at *m/z* 2043.1 was interpreted as the $[M - H]^-$ of a hexa-acylated lipid A *bis*-phosphate further substituted with two phosphoethanolamine moieties (M_r = 2044.5) (Fig. 1*C* and

[3] Z. Zhou, A. A. Ribeiro, and C. R. H. Raetz, manuscript in preparation.

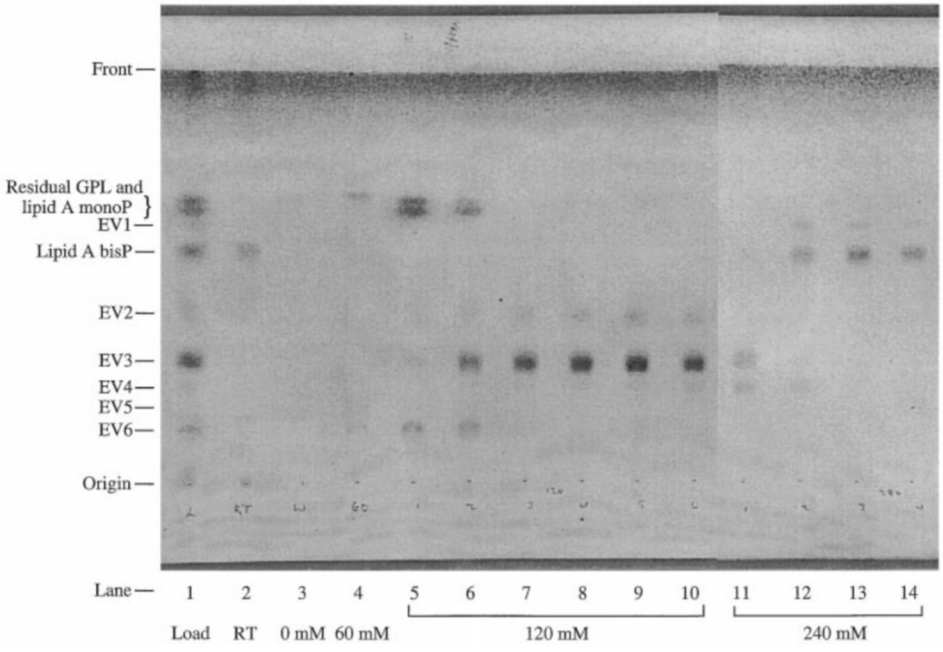

FIG. 4. **Separation and large scale preparation of lipid A derivatives that accumulate in NH_4VO_3-treated cells on a DEAE cellulose column.** Lipid A species released from 1 liter of cells by hydrolysis at pH 4.5 were dissolved in 10 ml of chloroform/methanol/water (2:3:1, v/v). The sample was loaded onto a 2-ml column of DEAE cellulose (Whatman DE52, acetate form) in the same solvent. A 20-μl portion of the initial sample was spotted (*Load, lane 1*). The run-through was collected as a single fraction (*RT, lane 2*). The column was washed with 12 ml of chloroform/methanol/water (2:3:1, v/v) (*0 mM, lane 3*), collected as a single fraction. The column was then eluted with 12 ml of chloroform/methanol/60 mM ammonium acetate in water (2:3:1, v/v) *(lane 4*), 12 ml of chloroform/methanol/120 mM ammonium acetate (2:3:1, v/v) (*lanes 5–10*), 12 ml of chloroform/methanol/240 mM ammonium acetate (2:3:1, v/v) (only the first 4 fractions out of six are shown, *lanes 11–14*), and 12 ml of chloroform/methanol/480 mM ammonium acetate (2:3:1, v/v) (fractions not shown). A 20-μl sample from each fraction was spotted onto a 10 × 20-cm Silica Gel 60 TLC plate. The plate was developed in the solvent of chloroform/pyridine/88% formic acid, water (50:50:16:5, v/v). The lipids on the plates were visualized by sulfuric acid charring.

Table II). The peak at m/z 2158.8 was attributed to the loss of an aminodeoxypentose residue from the species at m/z 2289.1. The peak at 2064.9 was attributed to a sodium adduct of the molecular ion at m/z 2043.1. The peak at m/z 1921.0 might arise by loss of one phosphoethanolamine residue from the species at m/z 2043.1. The peak at m/z 1822.3 could not be assigned.

MALDI/TOF analysis was also conducted in the positive-ion mode (not shown) in an attempt to determine the sites of attachment of the aminodeoxypentose and the phosphoethanolamine residues. Although the positive-ion spectra were entirely consistent with the results shown in Fig. 5, they did not provide any information regarding the site of aminodeoxypentose substitution, since the relevant linkages appear to be too labile under the conditions employed. On the other hand, the phosphoethanolamine substitutions in EV4 and EV6 were stable in both the positive- and negative-ion modes of mass analysis. The positive-ion spectrum of EV4 (not shown) revealed two oxonium ions (41), B_1^+ at m/z 1088.1 and B_2^+ at m/z 1702.2, as well as the molecular ion at m/z 1921.2, corresponding to $[M + H]^+$. The fact that both the B_1^+ and B_2^+ of EV4 were the same as those observed for the unmodified hexa-acylated lipid A 1,4′-*bis*-phosphate (not shown) indicated that the 4′-phosphate was not substituted in EV4. Therefore, the phosphoethanolamine substituent in EV4 is likely to be attached at the 1-position (Fig. 1*C*).

In summary, the substituted lipid A derivatives analyzed in Fig. 5 all appear to contain three kinds of substituents singly or in combination: a palmitoyl group, an aminodeoxypentose residue, and one (or two) phosphoethanolamine moieties (Fig. 1*C* and Table II). The combinations of these substituents account for the micro-heterogeneity of the lipid A species associated with NH_4VO_3 treatment of *E. coli* K12. Interestingly, these and other modifications of lipid A are usually seen in *S. typhimurium* cells grown on nutrient broth without any special treatments (Figs. 1*B* and 3) (16, 18, 28, 29). In *E. coli* K12, they have been reported only in polymyxin-resistant mutants (27).

FAB Mass Spectrometry of the Aminodeoxypentose Substituent—Although stable for days at 25 °C during DEAE cellulose chromatography (Fig. 4) in chloroform/methanol/water (2:3:1, v/v), all samples, including the lipid A 1,4′-*bis*-phosphate, EV2, EV3, and EV6, decomposed within hours when dissolved in $CDCl_3/CD_3OD$ (4:1, v/v). As shown in Fig. 6, the resulting degradation products included several rapidly migrating, partially deacylated lipid A 4′-monophosphates (Fig. 6, *lanes 1–4*), all of which had lost their 1-phosphate substituents during exposure to $CDCl_3/CD_3OD$ (4:1, v/v). The three metavanadate-induced lipid A derivatives (EV2, 3, and 6) generated an additional, slowly migrating substance (Fig. 6, *lanes 2–4*), not seen in the degradation products of the hexa-acylated lipid A 1,4′-*bis*-phosphate (Fig. 6, *lane 1*). This hydrophilic compound, which is readily detected by charring with sulfuric acid, is the aminodeoxypentose moiety of EV2, 3, and 6 (see below). To isolate this material, the lipid A samples that had been exposed to $CDCl_3/CD_3OD$ (4:1, v/v) for 3 days at room temperature were dried under N_2 and were resuspended in a neutral, two-phase Bligh/Dyer system, consisting of chloroform/methanol/water (2:2:1.8, v/v). The rapidly migrating degradation products partitioned into the lower phase, and the slowly migrating material was recovered in the upper phase (Fig. 6, *lanes 5–7*). The upper phase of each sample was washed twice with fresh pre-equilibrated lower phase to remove residual lipids, and the upper phases were then dried. The hydrophilic substances released in this way from EV2, 3, and 6 all stained with ninhydrin (not shown), confirming the presence of an amino group.

The positive-ion FAB mass spectrum of the hydrophilic material released from EV2 showed a prominent molecular ion $[M + H]^+$ at m/z 150.11 (Fig. 7). This is consistent with the

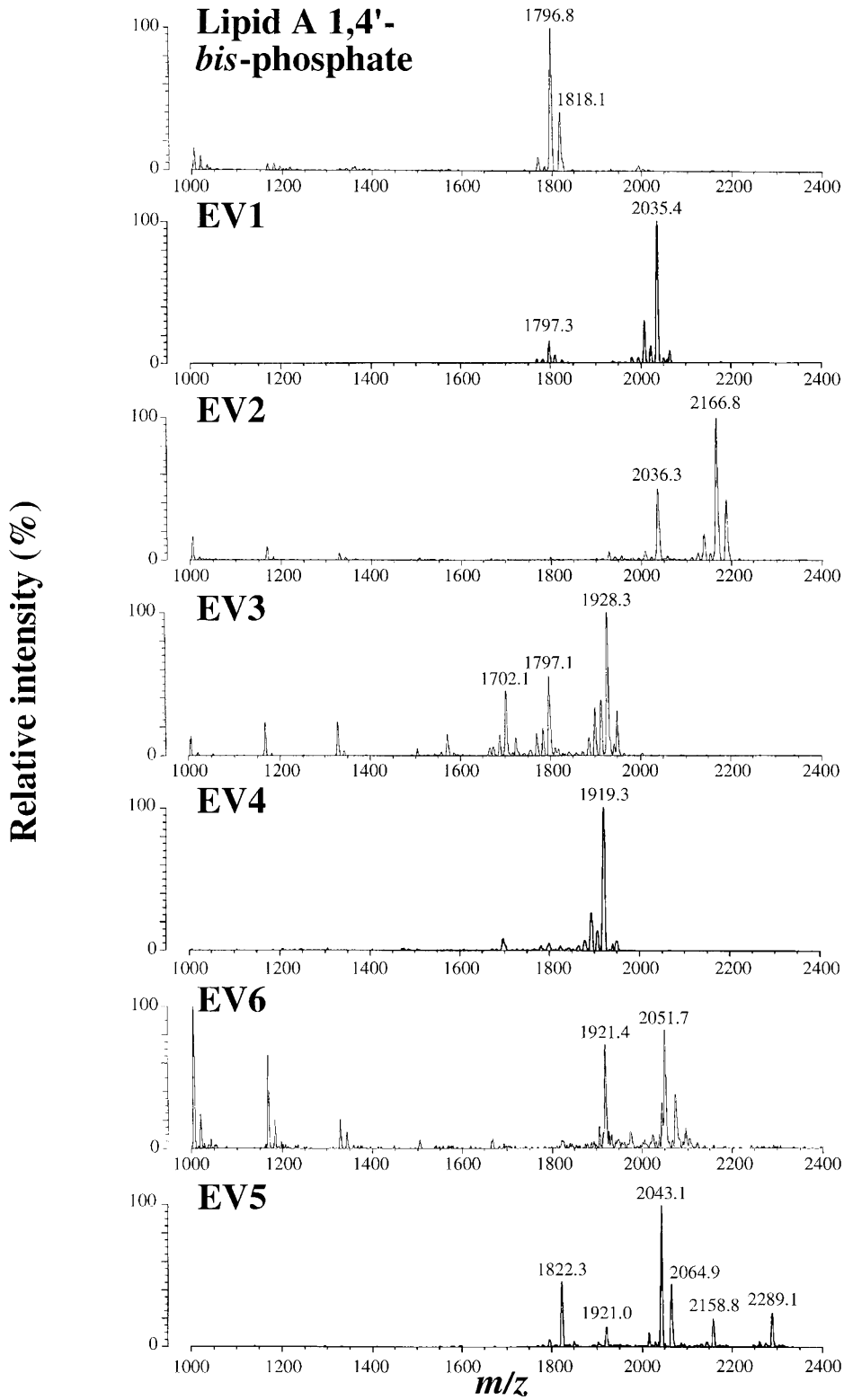

FIG. 5. **Negative ion MALDI/TOF mass spectrometry of lipid A species purified from *E. coli* cells treated with NH_4VO_3.** Hexaacylated lipid A 1,4′-*bis*-phosphate, EV1, EV2, EV3, EV4, EV5, and EV6 were purified by DEAE cellulose chromatography and preparative TLC from strain W3110 grown on LB broth in the presence of 25 mM NH_4VO_3.

elemental composition of an aminodeoxypentose, like 4-amino-4-deoxy-L-arabinose, the molecular weight of which is 149.15 (42).

Analysis of the Aminodeoxypentose Substituent Released from EV3 by ^1H NMR—As shown in Fig. 8 and Table III, ^1H NMR experiments were used to characterize the structure of the putative 4-amino-4-deoxy-L-arabinose released from EV3. Since the anomeric OH of the released sugar is no longer phosphorylated after exposure to $CDCl_3/CD_3OD$ (4:1, v/v), two anomeric forms (designated A and B in Fig. 8) were detected in the spectrum. Based upon the proton connectivities determined by two-dimensional COSY experiments (not shown), the indi-

TABLE II
MALDI/TOF mass analysis of lipid A species purified from E. coli K12 grown on LB broth in the presence of 25 mM NH_4VO_3

The M_r is the calculated molecular weight for the proposed structure (see also Fig. 1*C*). $[M - H]^-$ is the observed molecular ion determined in the experiment shown in Fig. 5.

Lipid A species	$[M - H]^-$	M_r	Modification		
			$C_{16:0}$	L-Ara4N	pEtN
1,4′-*bis*-Phosphate	1796.8	1798.4	0	0	0
EV1	2035.4	2036.8	1	0	0
EV2	2166.8	2167.9	1	1	0
EV3	1928.3	1929.5	0	1	0
EV4	1919.3	1921.4	0	0	1
EV5	2043.1	2044.5	0	0	2
	2289.1	2291.0	1	1	1
EV6	2051.7	2052.6	0	1	1

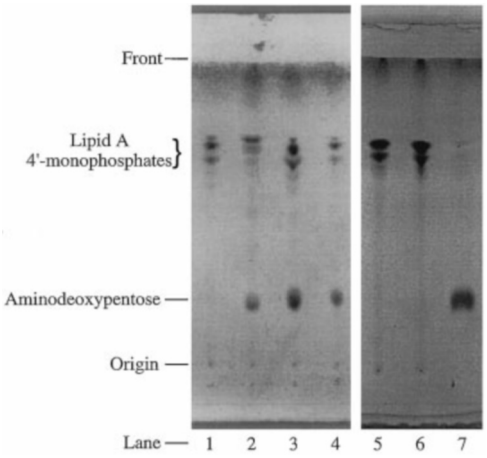

FIG. 6. **TLC analysis of the decomposition products formed from lipid A derivatives incubated in $CDCl_3/CD_3OD$.** Lipid A samples like those in Fig. 5 were dissolved in $CDCl_3/CD_3OD$ (4:1, v/v) for NMR spectroscopy. Under these conditions all the samples unexpectedly decomposed over the course of 0.5 to 3 days at room temperature. To evaluate this problem, 1 μl of each sample was spotted onto a TLC plate (*lanes 1–4*, hexa-acylated lipid A 1,4′-*bis*-phosphate, EV2, 3, and 6, respectively). The rest of the samples were dried under a stream of N_2. Each sample was then resuspended in 4.0 ml of a fresh two phase Bligh/Dyer mixture, consisting of chloroform/methanol/water (2:2:1.8, v/v). Next, 5 μl of the lower and 5 μl of the upper phase was spotted onto another TLC plate (*lane 5*, the lower phase from hexa-acylated lipid A 1,4′-*bis*-phosphate; *lanes 6* and *7*, the lower and upper phase, respectively, from EV3). The plates were developed in the solvent of chloroform/pyridine/88% formic acid, water (50:50:16:5, v/v). The lipids on the plates were visualized by charring.

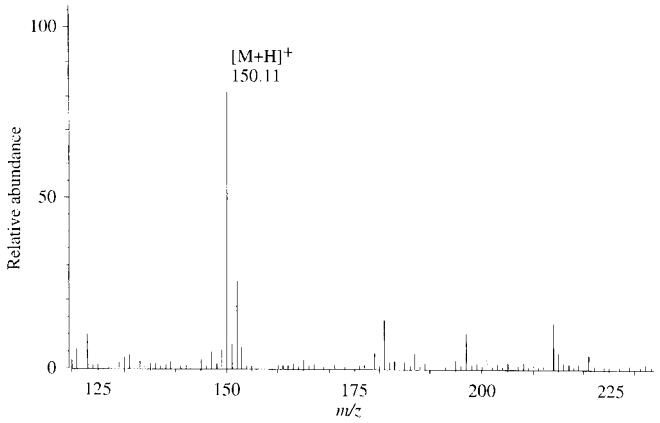

FIG. 7. **Positive mode FAB mass spectrum of the aminodeoxypentose released from EV2.** The upper phase of EV2, prepared as described in the legend to Fig. 6, was collected and extracted twice with several milliliters of a lower phase from a fresh two phase Bligh/Dyer system. The washed upper phase was dried by lyophilization, and the water-soluble compound released from EV2 was analyzed by FAB mass spectrometry. The spectrum was the average of 7 scans.

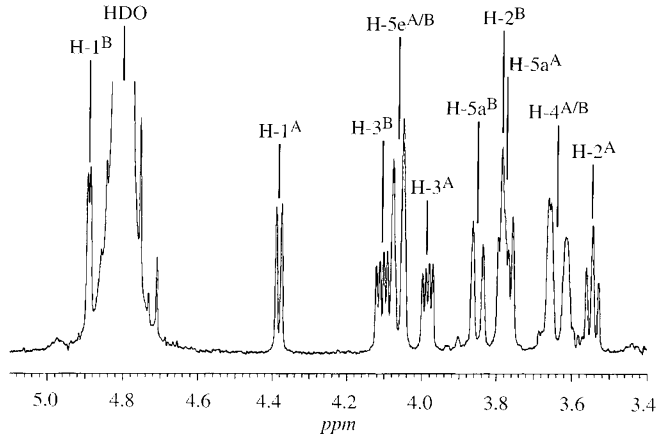

FIG. 8. **500 MHz ^{1}H NMR spectrum of the aminodeoxypentose released from EV3.** The aminodeoxypentose, as isolated by the protocols described in the legends to Figs. 6 and 7, was dried and redissolved in 0.6 ml of D_2O. The 500 MHz ^{1}H NMR spectrum shown was recorded at 25 °C with the HDO signal referenced to 4.80 ppm. Sugar resonances were assigned by two-dimensional COSY analysis (not shown). The sugar resonances corresponding to the two anomeric forms of the aminodeoxypentose released from EV3 are designated *A* and *B*. The equatorial and axial protons at the 5 position are designated *H-5e* and *H-5a*.

vidual proton resonances of the two anomeric species could be assigned. Their chemical shifts (*ppm*) and vicinal coupling constants ($J_{H,H}$, Hz) were measured directly from the one-dimensional ^{1}H NMR spectrum (Table III). The chemical shifts were referenced to the internal HDO signal at 4.80 *ppm* and were compared with the data previously reported for chemically synthesized 4-amino-4-deoxy-L-arabinose (42) (Table III). The shapes of the individual proton resonances in the spectrum of the A- and B-forms derived from EV3 and the vicinal coupling constants ($J_{H,H}$, Hz) (Fig. 8 and Table III) are indeed very similar to those of the α- and β-anomers of the standard (42). The only exceptions are the chemical shifts of the H-1 signals, which differ from the standard by 0.2–0.3 ppm. However, differences in the solvent acidity of the EV-3-derived sample and the previously reported standard (42) might account for these minor discrepancies, since the pD was not carefully controlled. Taken together with the previous work (23, 28, 29, 42) on 4-amino-4-deoxy-L-arabinose-modified lipid A species in *Salmonella*, it seems very likely that the aminodeoxypentose substituent present in the *E. coli* lipid A derivatives EV2, 3, and 6 is 4-amino-4-deoxy-L-arabinose. Detailed side by side comparisons of the ^{1}H NMR spectra of intact purified EV3 *versus* lipid II_A (a precursor isolated from Kdo-deficient mutants of *S. typhimurium* that is known to contain 4-amino-4-deoxy-L-arabinose) (28, 29, 42) further confirm the above assignments.[3]

NH_4VO_3 Induces Lipid A Substitutions in phoQ and phoP/Q Deletion Mutants—To determine if NH_4VO_3 induction of lipid A modifications in *E. coli* requires the PhoP/PhoQ system, as is the case for *S. typhimurium* grown in the absence of NH_4VO_3 (16, 18), the *E. coli phoQ* and *phoP* deletion mutants CSH26ΔQ and CSH26ΔPQ (43)[4] were grown in the presence of 25 mM NH_4VO_3 and $^{32}P_i$. The lipid A species were then released from the cells by pH 4.5 hydrolysis, and were analyzed by thin layer chromatography and PhosphorImager analysis (Fig. 9). Untreated cells of all strains contained the usual hexa-acylated lipid A 1,4′-*bis*-phosphate and the lipid A 1-pyrophosphate. NH_4VO_3-treated CSH26ΔQ and CSH26ΔPQ cells generated a

[4] C. Waldburger, unpublished data.

TABLE III
Comparison of the aminodeoxypentose released from EV3 with synthetic 4-amino-4-deoxy-L-arabinose (L-Ara4N) anomers

	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5e ($J_{5e,5a}$)	H-5a
A-species[a]	4.383	3.545	3.985	3.616	4.065	3.772
from EV3	(7.1)	(8.9)	(4.7)		(14.7)	
α-anomer[b]	4.57	3.41	3.94	3.6	4.02	3.80
(L-Ara4N)	(7.7)	(9.1)	(4.6)	(1.4,1.2)	(13.8)	
B-species[a]	4.890	3.786	4.107	3.658	4.065	3.850
from EV3	(3.3)	(10.0)	(4.7)		(13.4)	
β-Anomer[b]	5.21	3.69	4.10	3.6	4.16	3.71
(L-Ara4N)	(3.3)	(9.1)	(4.6)	(2.3,3.0)	(13.3)	

[a] ^{1}H NMR spectra for the isolated aminodeoxypentose isomers released from EV3 were recorded at 500 MHz and 25 °C with the HDO signal referenced to 4.80 ppm, as described by Naleway *et al.* (42). The 500 MHz spectra were obtained with a digital resolution of 0.3 Hz/point. At the actual digital resolution, chemical shifts are accurate to ~0.001 ppm, and coupling constants (J) have an uncertainty of 0.3 Hz.
[b] ^{1}H NMR spectra for the synthetic L-Ara4N anomers were recorded at 270 MHz with the HDO signal referenced to 4.80 ppm by Naleway *et al.* (42).

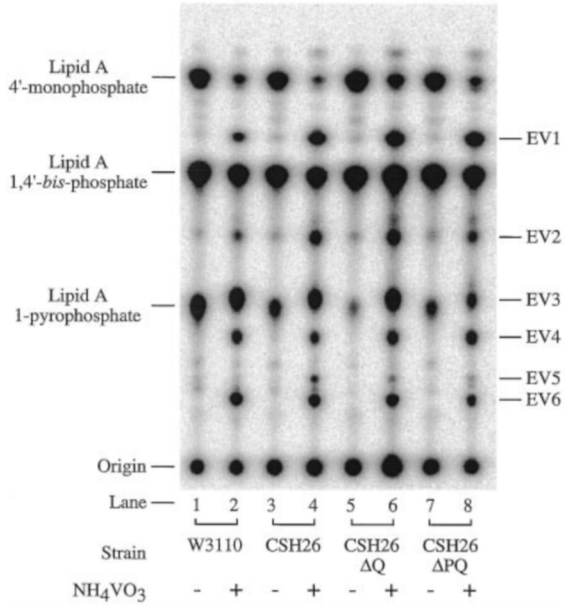

FIG. 9. **Lipid A modifications are induced by NH_4VO_3 in *phoP* and *phoQ* deletion mutants of *E. coli*.** Strains were grown at 37 °C, labeled and analyzed, as described in the legend to Fig. 2, in the presence or absence of 25 mM NH_4VO_3.

similar pattern of substituted lipid A species as observed in the parental strain CSH26 (Fig. 9) or in the wild-type *E. coli* W3110 (Fig. 3) grown in the presence of NH_4VO_3. These results clearly demonstrate that NH_4VO_3 induction does not require the PhoP/PhoQ system, perhaps because NH_4VO_3 acts downstream of PhoP/PhoQ.

When *S. typhimurium* cells are grown at low Mg^{2+} concentrations or at low pH, their lipid A is derivatized with higher levels of 4-amino-4-deoxy-L-arabinose (16) than under ordinary growth conditions. In our strains of *E. coli* K12, however, low Mg^{2+} and low pH did not trigger aminodeoxypentose modification of lipid A (data not shown).

The NH_4VO_3 Effect Is Independent of Acyloxyacyl Group Formation—To determine whether or not acyloxyacyl residues need to be present on lipid A for the attachment of the NH_4VO_3-induced modifications, *E. coli* W3110, MLK1067 (*msbB*$^-$), and MLK986 (*htrB*$^-$/*msbB*$^-$)/pKW2 (*msbA*$^+$) (12, 33) were grown at 42 °C on LB broth in the presence or absence of 25 mM NH_4VO_3. HtrB and MsbB are late acyltransferases that incorporate the laurate and myristate residues, respectively, of *E. coli* lipid A (33, 44). Accordingly, the wild-type and mutant cells were labeled with $^{32}P_i$ for 3 h. The temperature-sensitive growth phenotype of MLK986 (*htrB*$^-$/*msbB*$^-$) was suppressed by a plasmid carrying msbA$^+$, which encodes an essential ABC family transporter required for lipopolysaccharide export (12, 45). Lipid A species were released from the ^{32}P-labeled cells by pH 4.5 hydrolysis, and were analyzed by thin layer chromatography and PhosphorImager analysis (Fig. 10). When grown on LB broth in the absence of NH_4VO_3, these three strains produced mainly hexa-, penta-, or tetra-acylated lipid A moieties, consistent with their genotypes (Fig. 10, *lanes 1, 3*, and *5*). In each mutant, the expected 4′-monophosphate, 1,4′-*bis*-phosphate, and 1-pyrophosphate variants were also present. When treated with NH_4VO_3, all three strains generated a more complex series of slowly migrating lipid A derivatives at the expense of the 1-pyrophosphate species (Fig. 10, *lanes 2, 4*, and *6*), consistent with the modifications shown in Fig. 1*C*. These findings indicate that the acyloxyacyl moieties of lipid A are not needed for the proper functioning of the enzymes that attach the NH_4VO_3-induced modifications.

DISCUSSION

The enzymes that generate the hexa-acylated lipid A 1,4′-*bis*-phosphate (Fig. 1*A*) found in *E. coli* K12 and other Gram-negative bacteria are well characterized (1, 13). However, many additional covalent modifications of lipid A have been reported. In *S. typhimurium*, for instance, lipid A derivatives exist that are modified with 4-amino-4-deoxy-L-arabinose, phosphoethanolamine, palmitate, and/or *S*-2-hydroxymyristate (Fig. 1*B*) (15, 28, 46). Structural diversity and partial substitution (Fig. 1*B*) give rise to a large number of distinct molecular species. While the existence of such lipid A modifications has been recognized for a long time (47, 48), the enzymes that generate them are still largely unknown.

Although not required for the growth under laboratory conditions, the modified lipid A species of *S. typhimurium* (Figs. 1*B* and 3) are interesting from the perspective of pathogenesis (16–18). Extensive modification of lipid A with L-4-aminoarabinose is associated with resistance to polymyxin and other cationic antibacterial peptides (16–19, 24, 25). In *S. typhimurium*, formation of modified lipid A derivatives is under the control of the PhoP/PhoQ system, a global regulatory network that controls over 40 genes and is essential for pathogenesis (16, 19). In the case of the lipid A modifications, the PhoP/PhoQ system generally functions by activating PmrA/PmrB, a separate two-component system that may directly activate the transcription of the genes encoding some of the relevant enzymes (18).

In the present study, we have discovered that 25 mM NH_4VO_3 induces three kinds of covalent modifications of *E. coli* K12 lipid A, which resemble those normally found in *S. typhimurium* (Fig. 1, *B versus C*), resulting in the accumulation of six major species (Figs. 1–5). Of these, EV1, 2, 3, 4, and 6 have been purified to apparent homogeneity as judged by mass spectrometry (Fig. 5) and TLC analysis. Techniques for isolating such modified lipid A species in a pure form had not been

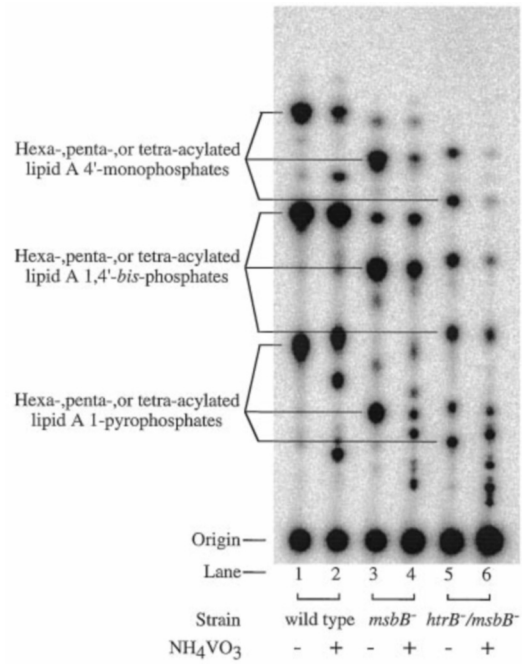

FIG. 10. **Lipid A modifications are induced by NH_4VO_3 in *htrB* and *msbB* mutants of *E. coli* lacking acyloxyacyl moieties.** Strains were grown at 42 °C, labeled, and analyzed, as described in the legend to Fig. 2, in the presence or absence of 25 mM NH_4VO_3. *Lanes 1* and *2*, W3110 (wild-type); *lanes 3* and *4*, MLK1067 (*msbB*$^-$); *lanes 5* and *6*, MLK986 (*htrB*$^-$/*msbB*$^-$)/pKW2 (*msbA*$^+$).

described prior to the present investigation, as all previous structural investigations of lipid A modifications have been based on the use of mixtures (25, 27). EV2, 3, and 6 all contain an aminodeoxypentose group, very likely to be 4-amino-4-deoxy-L-arabinose, as judged by NMR spectroscopy (Fig. 8 and Tables II and III) of the substituent released from EV3. EV1 and EV2 (Fig. 1*C* and Table II) are characterized by the presence of hepta-acylated lipid A moieties. EV4, EV5, and EV6 (Fig. 1*C* and Table II) contain phosphoethanolamine substituents. Although the NH_4VO_3 effect does not require a functional PhoP/PhoQ system (Fig. 9), it may be that metavanadate (or one of its oligomers) activates PmrA/PmrB by blocking the action of a key regulatory phosphatase (49). Interestingly, NH_4VO_3 has no effect on the composition of lipid A in *pmrA*-deficient mutants of *S. typhimurium*.[5] Whatever its mechanism, the NH_4VO_3 effect opens the possibility of investigating the enzymology of lipid A modifications in diverse strains of *E. coli* K12, the organism in which most studies of lipid A biosynthesis have been conducted (1, 8, 9). In this context, it is already clear that NH_4VO_3-induced lipid A modifications do not require the presence of the acyloxyacyl groups (Fig. 10).

Mass spectrometry of the lipid A derivatives isolated from NH_4VO_3-treated *E. coli* failed to show the presence of *S*-2-hydroxymyristate, which is easily detected in *S. typhimurium* lipid A under conditions of PhoP/PhoQ activation (16).[6] It may be that *E. coli* can generate only a subset of the lipid A modifications that are found in *S. typhimurium*, suggesting the existence of additional biosynthetic enzymes in the latter organism. Alternatively, NH_4VO_3 treatment of *E. coli* may not activate the entire enzymatic system that is involved in lipid A modification.

It has not yet been demonstrated unequivocally that the 4-amino-4-deoxy-L-arabinose moiety is always attached to the 4′-phosphate and that the phosphoethanolamine residue is predominantly found at the 1-phosphate of lipid A in NH_4VO_3-treated *E. coli*, as suggested in Fig. 1*C*. In the lipid A precursors that accumulate in Kdo-deficient mutants of *S. typhimurium*, the 4-amino-4-deoxy-L-arabinose is attached to the 1-phosphate and the phosphoethanolamine is on the 4′-phosphate (28, 29). Further characterization of EV2, 3, 4, and 6 by ^1H and ^{31}P NMR spectroscopy is in progress and should establish the sites at which these modifications are attached. The locations of the 4-amino-4-deoxy-L-arabinose and phosphoethanolamine substitutions also need to be reinvestigated in the mature lipid A of wild-type *S. typhimurium* (Figs. 1*B* and 3). Purification of homogeneous molecular species based on the new procedures described above should greatly facilitate this effort.

It has been suggested that the 4-amino-4-deoxy-L-arabinose-substituted lipid A species seen in polymyxin-resistant mutants of *S typhimurium* and *E. coli* reduce the overall negative charge of the lipopolysaccharide, thereby reducing the binding of polycationic antibiotics (50, 51). An attempt to show that NH_4VO_3-treated cells are polymyxin-resistant was unsuccessful, because polymyxin precipitated in the presence of 5 mM NH_4VO_3.[5]

The enzymes that catalyze the lipid A modifications shown in Fig. 1, *B* and *C,* remain to be characterized. The ethanolamine phosphate groups found in EV4, EV5, and EV6 might be derived from phosphatidylethanolamine (52), but so far, no *in vitro* systems have been developed. A membrane-bound palmitoyl transferase that uses glycerophospholipids as the palmitate donor was previously shown to convert the diacylated monosaccharide lipid X to lipid Y in *E. coli* extracts (1, 53). This unusual acyltransferase has recently been shown to incorporate the palmitate moieties found in EV1 and *S. typhimurium* lipid A (Fig. 1, *B* and *C*) (54). Genetic and enzymatic studies have revealed that the *pagP* gene (17), which is present in both *S. typhimurium* and *E. coli*, encodes the palmitoyltransferase (54).

Although the enzymes that generate L-4-aminoarabinose are obscure, a hypothetical pathway can now be proposed (Fig. 11). The important studies of Gunn *et al.* (18) have recently revealed the existence of several genes in *S. typhimurium* and *E. coli* required for the maintenance of polymyxin resistance. For instance, mutations in the *ugd*/*pmrE* or in the *pmrF* genes render *S. typhimurium* polymyxin-sensitive and incapable of making aminoarabinose under conditions of PhoP/PhoQ activation (18). We therefore suggest (Fig. 11) that the UDP-glucose dehydrogenase (Ugd/PmrE) could initiate the L-4-aminoarabinose pathway, in analogy to the role of this enzyme in the biosynthesis of UDP-xylose in plants (55, 56). Orf3, which is encoded by one of the genes of unknown function found in the *pmrF* cluster (18), might then catalyze the oxidation of the 4-position (Fig. 11). Orf3 shows a high degree of similarity to enzymes that oxidize the 4-OH of pyranoses, such as UDP-galactose 4-epimerase. Decarboxylation of the intermediate generated by Orf3 might be spontaneous, and could be followed by a transamination catalyzed by Orf1 of the *pmrF* cluster (18), which is related to a large family of transaminases. The product of Orf1 would be the novel sugar nucleotide, UDP-L-4-aminoarabinose (designated L-Ara4N in Fig. 11).

The least obvious feature of the proposed pathway is the involvement of a bactoprenol-linked intermediate (Fig. 11), a possibility that is suggested by the sequence of the *pmrF* gene product (18). The latter shows significant similarity to dolichol-phosphomannose synthase of yeast (57), an enzyme that generates a key substrate required for protein glycosylation. The sequence similarity of *pmrF* to dolichol-phosphomannose syn-

[5] Z. Zhou and C. R. H. Raetz, unpublished data.
[6] Z. Zhou and C. R. H. Raetz, manuscript in preparation.

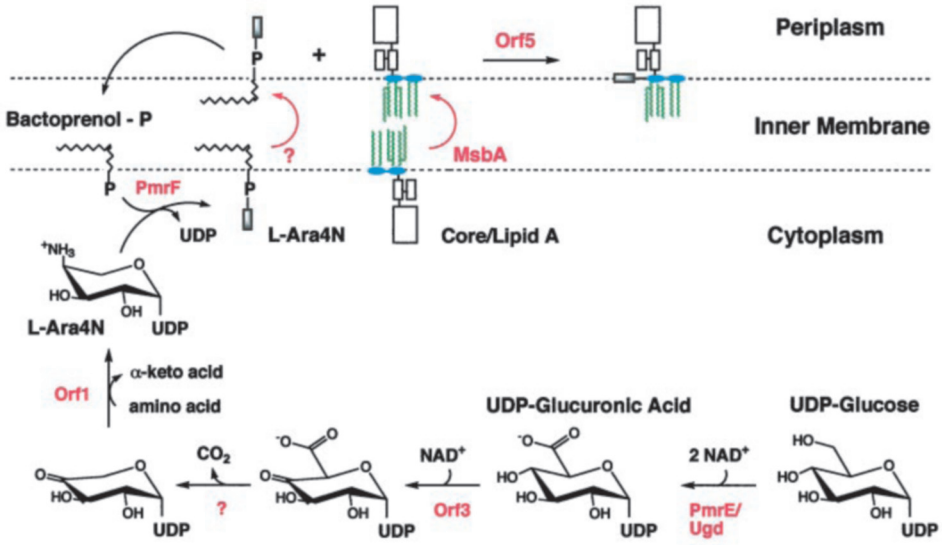

FIG. 11. **A hypothetical pathway for the biosynthesis and attachment of L-4-aminoarabinose to lipid A in *E. coli*.** Orf1, 3, and 5 are encoded by genes that are clustered together with *pmrF* on the chromosomes of *E. coli* and *S. typhimurium* (18). Other open reading frames in this cluster (18) cannot yet be assigned a specific function. Presumably, many or all of these genes are activated by exposure of *E. coli* cells to NH_4VO_3, but they are expressed (under the control of the *phoP/Q* and *pmrA/B* systems) in the absence of NH_4VO_3 in *Salmonella*. An intriguing observation is the fact (not shown) that Orf3 (18) contains what appears to be a second catalytic domain with similarity to formyl transferases. While the function of a formyl transferase in the above scheme is not immediately apparent, the possibility that a formyl moiety might be attached, perhaps transiently, to the amino group of L-4-aminoarabinose (designated L-Ara4N) deserves consideration.

thase could reflect a related catalytic reaction mechanism. In this scenario, PmrF might function to transfer L-4-aminoarabinose to bactoprenol phosphate with release of UDP, as shown in Fig. 11, in analogy to dolichol-phosphomannose synthase, which condenses GDP-mannose and dolichol phosphate with the release of GDP (57).

The proposed involvement of the novel intermediate bactoprenol phosphoaminoarabinose in lipid A modification raises the interesting possibility that L-4-aminoarabinose transfer to lipid A occurs on the periplasmic surface of the inner membrane (Fig. 11). Many enzymatic systems that utilize bactoprenol phosphate derivatives, such as some *O*-antigen polymerases and certain peptidoglycan glycosyltransferases, function in the periplasm (1, 58, 59). We have not yet identified a gene that might encode the putative bactoprenol phosphoaminoarabinose flippase (Fig. 11), but we suggest that Orf5 of the *pmrF* cluster (18) might be the glycosyltransferase that attaches the L-4-aminoarabinose to lipid A (Fig. 11). This possibility is based on the observation that Orf5 displays distant sequence similarity to mannosyl transferases and that Orf5 is an integral membrane protein.

An additional reason for transferring L-4-aminoarabinose to the 4′-position of lipid A on the periplasmic surface of the inner membrane may be related to the substrate specificity of the Kdo transferase (KdtA) (60, 61). The latter requires the presence of an unsubstituted 4′-monophosphate moiety for catalysis (60, 61). Premature transfer of L-4-aminoarabinose to the 4′-phosphate on the cytoplasmic surface of the inner membrane would prevent Kdo addition, since EV3 (unlike lipid IV_A or lipid A) is not a substrate for the Kdo transferase.[7]

Proof of the hypothetical pathway shown in Fig. 11 will require synthesis of the relevant substrates, demonstration of the proposed enzymes capable of utilizing them, and analysis of the precursors that accumulate in specific mutants lacking the enzymes. These criteria have all been applied to the lipid A and the glycerophospholipid pathways in *E. coli* (13, 38, 60, 61). The recent completion of the genome sequence of *E. coli* K12 (62) should greatly facilitate such studies.

[7] K. A. White, Z. Zhou, and C. R. H. Raetz, unpublished data.

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