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Lipid A Modifications in Polymyxin-resistant *Salmonella typhimurium*

PMRA-DEPENDENT 4-AMINO-4-DEOXY-L-ARABINOSE, AND PHOSPHOETHANOLAMINE INCORPORATION*

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Lipid A of *Salmonella typhimurium* can be resolved into multiple molecular species. Many of these substances are more polar than the predominant hexa-acylated lipid A 1,4′-bisphosphate of *Escherichia coli* K-12. By using new isolation methods, we have purified six lipid A subtypes (St1 to St6) from wild type *S. typhimurium*. We demonstrate that these lipid A variants are covalently modified with one or two 4-amino-4-deoxy-L-arabinose (L-Ara4N) moieties. Each lipid A species with a defined set of polar modifications can be further derivatized with a palmitoyl moiety and/or a 2-hydroxymyristoyl residue in place of the secondary myristoyl chain at position 3′. The unexpected finding that St5 and St6 contain two L-Ara4N residues accounts for the anomalous structures of lipid A precursors seen in *S. typhimurium* mutants defective in 3-deoxy-D-*manno*-octulosonic acid biosynthesis in which only the 1-phosphate group is modified with the L-Ara4N moiety (Strain, S. M., Armitage, I. M., Anderson, L., Takayama, K., Quershi, N., and Raetz, C. R. H. (1985) *J. Biol. Chem.* 260, 16089–16098). Phosphoethanolamine (pEtN)-modified lipid A species are much less abundant than L-Ara4N containing forms in wild type *S. typhimurium* grown in broth but accumulate to high levels when L-Ara4N synthesis is blocked in *pmrA*^{C}*pmrE*$^{-}$ and *pmrA*^{C}*pmrF*$^{-}$ mutants. Purification and analysis of selected compounds demonstrate that one or two pEtN moieties may be present. Our findings show that *S. typhimurium* contains versatile enzymes capable of modifying both the 1- and 4′-phosphates of lipid A with L-Ara4N and/or pEtN groups. PmrA null mutants of *S. typhimurium* produce lipid A species without any pEtN or L-Ara4N substituents. However, PmrA is not needed for the incorporation of 2-hydroxymyristate or palmitate.

Salmonella typhimurium and related organisms synthesize lipid A by the same pathway as *Escherichia coli* K-12 (1, 2), but they usually modify the final product with additional covalent appendages (Fig. 1*A*), such as 4-amino-4-deoxy-L-arabinose (L-Ara4N)[1] (3–7), phosphoethanolamine (pEtN) (4–6), (*S*)-2-hydroxymyristate (8, 9), and palmitate (5, 6, 10–12). Different combinations of these substituents account for the remarkable heterogeneity of lipid A molecules found in *S. typhimurium*.

The biosynthesis of lipid A modifications is under the control of the PhoP/PhoQ and the PmrA/PmrB two-component signaling systems (13–15). Addition of the L-Ara4N unit is required for resistance to polymyxin (16–18). Incorporation of the palmitoyl chain confers resistance to certain cationic anti-microbial peptides (11). Modification of lipid A with L-Ara4N, pEtN, and/or palmitate may also occur in *E. coli* K-12, but only under special circumstances, as in polymyxin-resistant (*pmrA* constitutive) mutants (17) or in wild type cells exposed to ammonium metavanadate (7, 19).

With the exception of PagP, the outer membrane enzyme that incorporates palmitate (12, 20), the enzymes responsible for the covalent modifications of lipid A have not been identified. The L-Ara4N residue is attached primarily to the 4′-phosphate group of lipid A in wild type *S. typhimurium* or in metavanadate-treated *E. coli*, whereas pEtN is usually attached to the 1-phosphate (19). However, in *S. typhimurium* mutants defective in Kdo biosynthesis, lipid A precursors accumulate in which L-Ara4N is linked exclusively to the 1-phosphate, and pEtN is attached mainly to the 4′-phosphate (5, 6, 19). The enzymatic pathways that account for these interesting and complex structural anomalies are unknown.

An important clue to the origin of the L-Ara4N moiety has emerged from the discovery of the *pmrE* and *pmrF* genes, which are required for the maintenance of polymyxin resistance and the biosynthesis of L-Ara4N-modified lipid A (18). The *pmrE* (*ugd*) gene encodes UDP-glucose dehydrogenase (18), suggesting that L-Ara4N is derived from UDP-glucuronic acid. The *pmrF* gene encodes a homologue of yeast dolichyl phosphate-mannose synthase and is part of an operon (18) that includes additional open reading frames hypothesized to encode other putative enzymes required for L-Ara4N biosynthesis and attachment to lipid A (7, 21). The operon is regulated directly by PmrA (18), which in turn may be activated by PhoP/PhoQ, low pH, or ferric ions (14, 22, 23). So far, no *in vitro* assays have been developed to validate the functions of the proteins encoded by the *pmrF* operon.

Because of its heterogeneity (Fig. 1*A*), *S. typhimurium* lipid

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

TABLE I
Strains used in this study

Strain	Relevant genotype	Source or Ref.
E. coli K-12		
W3110	Wild type, F^-, λ^-	E. coli Genetic Stock Center, Yale University
S. typhimurium		
ATCC14028	Virulent wild type 14028	Salmonella Genetic Stock Center, University of Calgary, Canada
CS022	*pho*-24 (phoP-constitutive)	38
JSG435	*pmrA505 zjd* :: *Tn 10d-cam* (pmrA-constitutive)	22
JSG485	JSG435 *pmrF1* :: *Tn 10d*	18
JSG486	JSG435 *pmrE1* :: *Tn 10d*	18
JSG421	*pmrA* :: *Tn 10d*	22

TABLE II
MALDI/TOF mass spectrometry of lipid A species purified from S. typhimurium wild type cells grown on LB broth

The predicted M_r is calculated based on the number of additional covalent moieties attached to the standard hexa-acylated lipid A 1,4′-bisphosphate (Fig. 1). $[M-H]^-$ is the observed molecular ion determined by MALDI/TOF mass spectrometry in the negative mode (Fig. 3).

Lipid A species	$[M-H]^-$	M_r	Type and number of covalent modifications		
			$C_{16:0}$	Oxygen atom	L-Ara4N
St1	2166.8	2167.9	1	0	1
St2	2182.9	2183.9	1	1	1
St3	1927.8	1929.5	0	0	1
St4	1944.0	1945.5	0	1	1
St5	2058.8	2060.6	0	0	2
St6	2074.9	2076.6	0	1	2

A has generally been prepared as a mixture of related species (4, 16). The structural details of lipid A substitution patterns could not be deduced unequivocally from such preparations. Recent improvements in lipid A purification methods, in conjunction with new procedures for high resolution NMR spectroscopy and mass spectrometry (7, 19, 24), now provide an opportunity to analyze the lipid A modifications of *S. typhimurium* in a more definitive manner. This effort is essential as a prelude to enzymatic studies of lipid A modifications.

We have now re-evaluated the predominant lipid A species that occur in *S. typhimurium* wild type cells, as well as in $pmrA^C pmrE^-$ and $pmrA^C pmrF^-$ mutants (18), initially using ^{32}P labeling (7) to profile the lipid A modifications. Five or six lipid A components were then purified from each strain. Structures were deduced using MALDI/TOF mass spectrometry and NMR spectroscopy. Novel lipid A subtypes were detected in which two L-Ara4N or two pEtN substituents were present in addition to the predominant, singly substituted species (Fig. 1). In the $pmrA^C pmrE^-$ and $pmrA^C pmrF^-$ mutants (18), which are unable to generate lipid A modified with the L-Ara4N residue, the extent of lipid A modification with pEtN moieties is greatly enhanced (Fig. 1*B*). The pEtN-containing species were not reported in previous analyses of these mutants (18), because lipid A was isolated by different methods and was not separated into its individual components. Analysis of lipid A subtypes from $pmrA^-$ mutants (Fig. 1*C*) confirmed the absolute PmrA requirement for lipid A modification with both the L-Ara4N and the pEtN moieties.

In the accompanying articles (36, 37), we report the first *in vitro* system for L-Ara4N transfer to lipid A and the characterization of a novel undecaprenyl phosphate-L-Ara4N donor substrate.

EXPERIMENTAL PROCEDURES

Materials—NH_4VO_3, (*RS*)-2-hydroxymyristic acid methyl ester, and *p*-anisaldehyde (4-methoxybenzaldehyde) were obtained from Sigma. $^{32}P_i$ was purchased from PerkinElmer Life Sciences. Pyridine, methanol, and 88% formic acid were obtained from Mallinckrodt, and chloroform was purchased from EM Science. $CDCl_3$, CD_3OD, and D_2O, were purchased from Aldrich. Glass-backed Silica Gel 60 thin layer chromatography plates (0.25 mm) were from Merck.

Bacterial Strains—Table I shows the strains used in this study. 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/liter (25). Antibiotics were added when necessary at final concentrations of 12 μg/ml for tetracycline, 25 μg/ml for chloramphenicol. 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 (7).

Rapid Profiling of Lipid A Species Released by Mild Acid Hydrolysis of ^{32}P-Labeled Cells—Cells were grown in LB broth, $^{32}P_i$-labeled, and extracted with chloroform/methanol mixtures as described previously (7). Lipid A species were then released from the cell residue by mild acid (pH 4.5) hydrolysis in the presence of SDS (7, 26–28). The species were resolved by TLC and detected with a PhosphorImager (7).

Large Scale Purification of Selected Lipid A Species Produced by S. typhimurium—Overnight cultures (4 ml) of *S. typhimurium* strain ATCC14028 (wild type), JSG485 ($pmrA^C/pmrF^-$), JSG486 ($pmrA^C/pmrE^-$), or JSG421 ($pmrA^-$) were inoculated into fresh LB broth (4.0 liters). Tetracycline was added to the cultures of all three *pmr* mutants at a final concentration of 12 μg/ml. The cells were grown at 37 °C and until A_{600} had reached ~3. Cells were harvested, washed, and extracted with a single phase Bligh/Dyer mixture (7), after which the lipid A species were released from the cell residue by hydrolysis at pH 4.5 (100 °C) in the presence of SDS (7). The released lipid A species were recovered, subjected to two-phase partitioning, and dried as described previously (7).

The lipid A species from *S. typhimurium* wild type strain 14028 were separated by anion exchange chromatography on a DEAE-cellulose column (acetate form) prepared in chloroform/methanol/water (2:3:1, v/v) (5, 7, 29). Compounds St5 and -6, which contain two L-Ara4N residues, have no net negative charge and therefore do not bind to the column. St-1 to St-4 elute with 60–120 mM ammonium acetate in the aqueous component. DEAE-cellulose-purified St1–6 were further purified by TLC (100–500 μg/plate) (7). Prior to mass spectrometry and NMR spectroscopy, the TLC-purified lipid A samples were subjected to a second DEAE-cellulose column chromatography to remove any contaminating metal ions and silica particles (7).

Lipid A species recovered by pH 4.5 hydrolysis from *S. typhimurium* mutants JSG486 ($pmrA^C/pmrE^-$) or JSG485 ($pmrA^C/pmrF^-$) were purified directly by preparative TLC (7) and were then subjected to DEAE-cellulose column chromatography to remove metals and silica particles.

The lipid A species from the *S. typhimurium* $pmrA^-$ mutant JSG421 were purified only by DEAE-cellulose column chromatography (7). In this case, the hexa- and hepta-acylated lipid A 1,4′-bisphosphates, and their corresponding 1-pyrophosphates, were eluted with chloroform, methanol, 240 mM ammonium acetate (2:3:1, v/v). Resolved fractions containing either the lipid A 1,4′-bisphosphates (designated the StA1 mixture) or the lipid A 1-pyrophosphates (the StA2 mixture) were pooled. The StA1 and StA2 fractions were then converted to neutral two-phase Bligh/Dyer mixtures (30) by addition of the necessary amounts of chloroform and water. The lower phases of StA1 and StA2 fractions were dried under N_2 and stored at −20 °C. All other purified lipid A components were also stored dry at −20 °C prior to further analysis.

Mass Spectrometry of Purified Lipid A Species—Most of the spectra were acquired in the negative linear mode by using a time of flight matrix-assisted laser desorption-ionization/time of flight (MALDI/TOF) Kompact 4 mass spectrometer (Kratos Analytical Manchester, UK), equipped with a 337 nm nitrogen laser and set at a 20-kV extraction voltage (7, 31). Each spectrum was the average of 50 shots. The instrument was operated at a resolution of about ±1 atomic mass units for

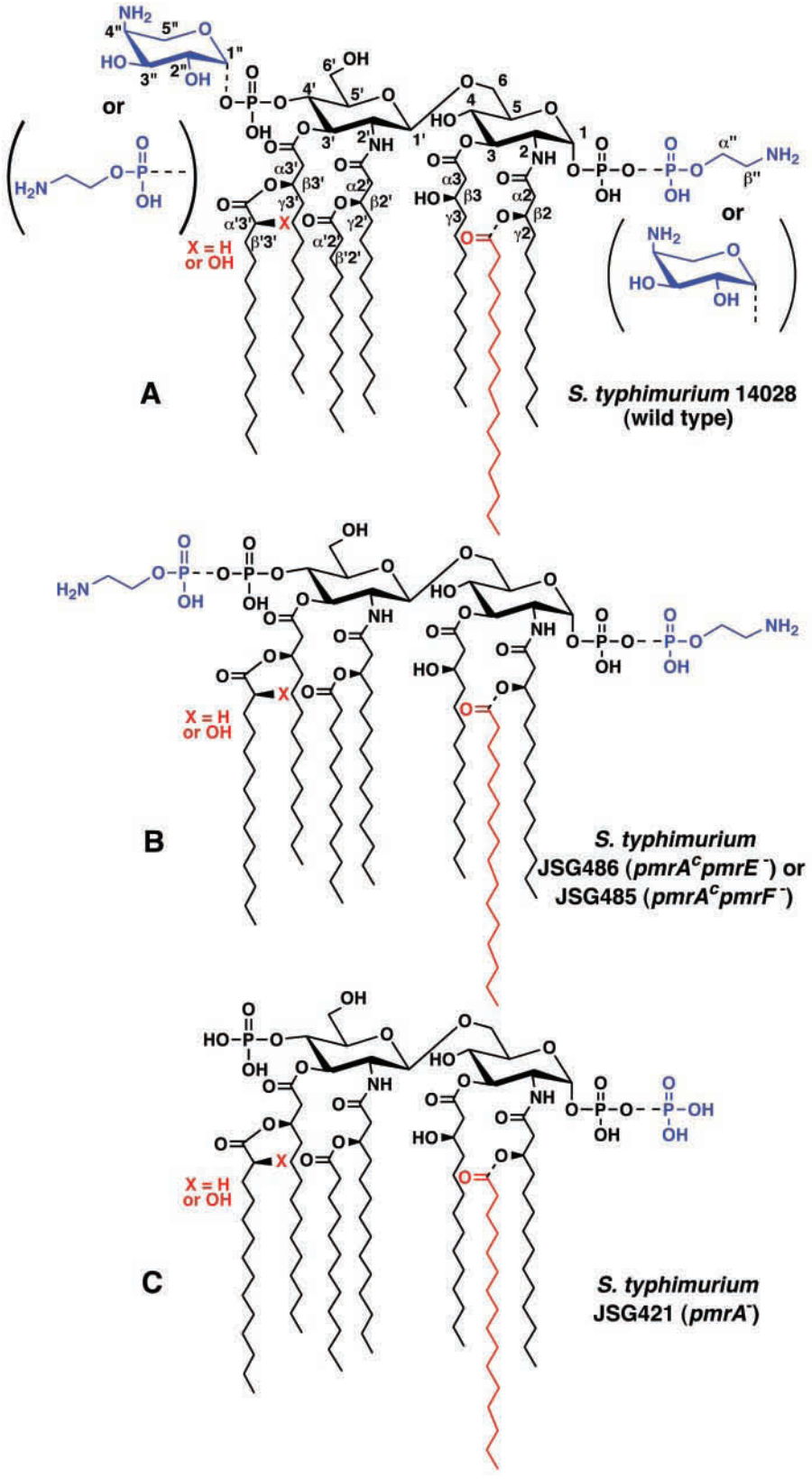

FIG. 1. **Combinations of covalent lipid A modifications found in *S. typhimurium*.** The covalent modifications present in each strain are indicated by the substituents with the *dashed bonds.* Modifications under the control of PmrA are *blue*, whereas modifications primarily under the control of PhoP are *red*. *A*, wild type *S. typhimurium* 14028 lipid A, which contains relatively little pEtN when cells are grown on LB broth at pH 6.8; *B*, *S. typhimurium* JSG486 ($pmrA^C/pmrE^-$) or *S. typhimurium* JSG485 ($pmrA^C pmrF^-$) lipid A in which pEtN-modified species are present at high levels, but there is no L-Ara4N; and *C, S. typhimurium* JSG421 $pmrA^-$ lipid A, which contains neither L-Ara4N nor pEtN moieties. The numbering shown in *A* is used to identify key protons for the NMR analysis. Lipid A species containing the L-Ara4N moiety at the 1-position are generally much less abundant than those derivatized with L-Ara4N at the 4′-position, except when Kdo biosynthesis is blocked (6,19).

compounds with M_r ~2000. Two kinds of matrices were used in the present study. One was a saturated solution of 2,5-dihydroxybenzoic acid in 50% acetonitrile for compounds St1–St6. The other was a mixture of saturated 6-aza-2-thiothymine in 50% acetonitrile and 10% tribasic ammonium citrate (9:1, v/v) for compounds StE1–StE5, for compounds StF1–StF5, and for compounds StA1 and StA2. Lipid samples were dissolved in a mixture of chloroform/methanol (4:1, v/v) before being mixed with the matrix (1:1, v/v) on a slide. The sample mixtures were allowed to dry at room temperature prior to mass analysis.

NMR Spectroscopy of Lipid A Species St3 and St4—Lipid samples (about 4 mg each) were dissolved in 0.6 ml of $CDCl_3/CD_3OD/D_2O$ (2:3:1, v/v) in 5-mm NMR tubes. NMR spectra were obtained at 25 °C using a Varian Unity 500 spectrometer equipped with a Sun Sparc 2 data system and a 5-mm Varian inverse probe. The ^2H signal of

TABLE III

Summary of ^1H chemical shifts (δ) and vicinal coupling constants (Hz) of key sugar and acyl chain protons in St3 and St4

The atom numbering scheme is shown in Fig. 1*A*. The abbreviations used are: $GlcN_1$, the proximal glucosamine; $GlcN_2$, the distal glucosamine; L-Ara4N, 4-amino-4-deoxy-L-arabinose; 2-OH-C:14-Me, (*R,S*)-2-hydroxymyristic acid methyl ester commercial standard. Lipids were dissolved in 0.6 ml of $CDCl_3/CD_3OD/D_2O$ (2:3:1, v/v). Chemical shifts (ppm relative to an internal TMS standard) were obtained from one-dimensional ^1H and two-dimensional ^1H-^1H COSY spectra recorded at 500 MHz (25 °C). The digital resolution for the one-dimensional spectra was 0.3 Hz per point. Some of the coupling constants (Hz) were derived from selectively ^{31}P decoupled ^1H spectra (data not shown).

Lipid	$GlcN_1$							$GlcN_2$						
	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5 ($J_{5,6a}$)	H-6a ($J_{6a,6b}$)	H-6b	H-1′ ($J_{1',2'}$)	H-2′	H-3′ ($J_{3',4'}$)	H-4′ ($J_{4',5'}$)	H-5′	H-6′a	H-6′b
St3	5.46 (3.3)	4.16 (10.7)	5.23	3.67	4.06	4.09	3.86	4.72 (8.2)	3.76	5.24 (~9.5)	4.23 (~9.5)	3.50	3.88	3.88
St4	5.46 (3.5)	4.15 (10.2)	5.22 (10.2)	3.66	4.05	4.10	3.86	4.75 (8.2)	3.72 (9.8)	5.29 (9.8)	4.23 (~9.8)	3.51	3.88	3.88

Lipid	Fatty acyl chains							L-Ara4N					
	H-β2	H-β3	H-β2′	H-β3′	H-α′2′	H-α′3′	H-α/H-β	H-1″ ($J_{1'',2''}$)	H-2″ ($J_{2'',3''}$)	H-3″	H-4″	H-5″eq	H-5″ax
St3	3.95	4.01	5.22	5.23	2.35	2.35		5.49 (3.4)	3.67 (9.7)	4.08	3.59	4.22	3.74
St4	3.95	4.02	5.21	5.35	2.35	4.26		5.48 (3.5)	3.68 (9.6)	4.09	3.58	4.22	3.74
2-OH-C:14-Me									4.19/1.7				

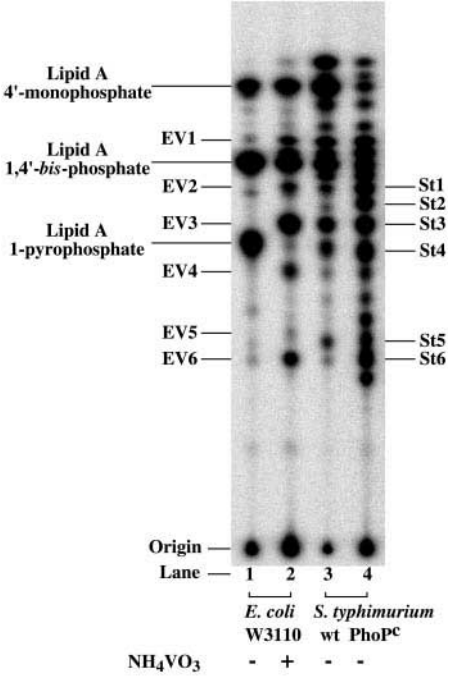

FIG. 2. **Rapid profiling of ^{32}P-labeled lipid A species of wild type and PhoP^C strains of *S. typhimurium* grown on LB broth.** Cells were uniformly labeled with $^{32}P_i$, and lipid A was released from lipopolysaccharide by hydrolysis at pH 4.5 in the presence of SDS, as described previously (7). Lipid A species were separated by TLC and detected with a PhosphorImager. Covalently modified lipid A variants that are induced by the presence of ammonium metavanadate in *E. coli* are designated *EV1–EV6* and represent chromatography standards, given that their structures have been determined (7). Selected lipid A species of *S. typhimurium* that were purified and characterized in the present study are designated *St1–St6*. Some lipid A 4′-monophosphate species arise by loss of the anomeric phosphate moiety during pH 4.5 hydrolysis at 100 °C.

CD_3OD was used for a field frequency lock. Both homo- and heteronuclear NMR experiments were performed as described previously (19, 24).

RESULTS

Rapid Profiling of Multiple Lipid A Species in S. typhimurium—Lipid A modifications in wild type *S. typhimurium* 14028 were initially surveyed by $^{32}P_i$ labeling of cells growing on LB broth, followed by hydrolysis of the cell residue at pH 4.5 to release all lipid A species, and analysis by TLC (Fig. 2) (7, 32). Previously characterized lipid A species of *E. coli* K-12 (including EV1 to EV6, which are induced by NH_4VO_3) were run in parallel as standards (Fig. 2, *lanes 1* and *2*) (7). Untreated *S. typhimurium* contained a more complex pattern of lipid A derivatives (Fig. 2, *lane 3*) than *E. coli* K-12 grown with or without 25 mM NH_4VO_3, but many common components were present (Fig. 2, *lanes 2* and *3*).

Lipid A modifications are controlled in part by the PhoP/PhoQ two-component signaling system (13). Lipid A profiling of a PhoP-constitutive (PhoP^C) *S. typhimurium* mutant using $^{32}P_i$ labeling (Fig. 2, *lane 4*) showed the expected increase in the extent and complexity of covalent lipid A modification.

Purification and Characterization of Selected S. typhimurium Lipid A Species—To evaluate the remarkable structural heterogeneity of *S. typhimurium* lipid A (Fig. 2), we purified six lipid A variants (designated St1 to St6) in milligram quantity from strain 14028. Each compound was analyzed by MALDI/TOF mass spectrometry in the negative ion mode (Fig. 3). The spectrum of St1 (Fig. 3) revealed two predominant peaks. The signal at m/z 2165.9 is consistent with the molecular ion $[M - H]^-$ of a lipid A 1,4′-bisphosphate bearing palmitate as a seventh acyl chain and a single L-Ara4N residue (Fig. 1*A* and Table II). St1 (Table II) therefore has the same composition as *E. coli* EV2 from NH_4VO_3-treated cells (7). The peak at m/z 2035.7 likely arises by loss of the L-Ara4N residue (132.1 atomic mass units) from the parent ion, formally $[M - H - \text{L-Ara4N} + H]^-$, during the MALDI process, a fragmentation known to occur with L-Ara4N-modified lipid A species under the mass spectrometry conditions employed (7).

The negative ion spectrum of St2 (Fig. 3) showed major peaks at m/z 2182.9 and 2052.1. The former is interpreted as the molecular ion $[M - H]^-$ of a lipid A derivative having the same composition as St1 but containing an additional oxygen atom (Table II), presumably because of the presence of a 2-hydroxymyristate chain in place of myristate, as indicated in Fig. 1*A*. The peak at m/z 2052.1 is attributed to the partial loss of the L-Ara4N residue (132.1 atomic mass units) from the parent ion $[M - H - \text{L-Ara4N} + H]^-$ because of fragmentation during mass spectrometry. A compound migrating like St2 is not seen in *E. coli* lipid A preparations, even when cells are exposed to NH_4VO_3 (7), because *E. coli* lacks the gene required for the formation of the 2-hydroxymyristoyl residue (9).

The spectrum of St3 (Fig. 3) is very similar to that reported previously (7) for EV3. The peak at m/z 1927.8 is consistent

TABLE IV
MALDI/TOF mass spectrometry of lipid A species purified from S. typhimurium pmrA^{c}pmrE$^-$ and pmrA^{c}pmrF$^-$ mutants

Lipid A species	Measured			Calculated			Type and number of covalent modifications		
	$[M-H]^-$	Y_1^-	B_1^+	M_r	Y_1^-	B_1^+	$C_{16:0}$	Oxygen atom	pEtN
S. typhimurium pmrA^C/pmrE$^-$ (JSG486)									
StE1	2158.1	1074.1	1088.2	2159.8	1072.3	1087.5	1	0	1
StE2	2174.4	1074.7	1104.7	2175.8	1072.3	1103.5	1	1	1
StE3	1920.7	835.2	1088.5	1921.4	833.9	1087.5	0	0	1
StE4	1936.9	836.5	1105.1	1937.4	833.9	1103.5	0	1	1
StE5	2282.3			2282.9			1	0	2
	2297.6			2298.9			1	1	2
	2043.2			2044.5			0	0	2
	2060.3			2060.5			0	1	2
S. typhimurium pmrA^c/pmrF$^-$ (JSG485)									
StF1	2157.4			2159.8			1	0	1
StF2	2175.0			2175.8			1	1	1
StF3	1920.5			1921.4			0	0	1
StF4	1935.6			1937.4			0	1	1
StF5	2282.1			2282.9			1	0	2
	2043.8			2044.5			0	0	2

The predicted M_r is calculated based on the number of proposed covalent modifications attached to the standard hexa-acylated lipid A 1,4′-bisphosphate (Fig. 1). $[M-H]^-$ is the observed molecular ion determined by MALDI/TOF mass spectrometry in the negative mode (Fig. 6). Positive mode spectra for determination of B_1^+ are not shown. B_1^+ arises from the distal unit and the Y_1^- from the proximal unit following cleavage of the glycosidic β,1′-6 linkage.

with the molecular ion $[M - H]^-$ of a hexa-acylated lipid A 1,4′-bisphosphate bearing a single L-Ara4N substituent (Fig. 1*A* and Table II). The peak at m/z 1797.2 is because of loss of the L-Ara4N moiety. The negative ion spectrum of St4 (Fig. 3) shows major peaks at m/z 1944.0 and 1813.1, which are interpreted as arising from a lipid A derivative having the same composition as St3 plus an extra oxygen atom because of the presence of a 2-hydroxymyristate moiety in place of the secondary myristate chain (Fig. 1*A* and Table II). The peak at m/z 1813.2 is explained by loss of the L-Ara4N group during the MALDI process.

Comparison of pure St1 through St4 (Fig. 3 and Table II) nicely illustrates the fact that for a given lipid A polar head group pattern (in this case a single L-Ara4N substituent) there are additional variants containing an extra oxygen atom and/or an additional palmitate chain. Previous workers (13, 16–18) were unable to separate these components from each other.

Lipid A Species Derivatized with two L-Ara4N Substituents in S. typhimurium 14028—The negative ion spectrum of St5 (Fig. 3) is more complex than those of St1 to St4 and reveals a novel pattern of polar head group substitution. The peak at m/z 2058.8 is interpreted as the molecular ion $[M - H]^-$ of a hexa-acylated lipid A 1,4′-bisphosphate bearing two L-Ara4N residues (Fig. 1*A* and Table II). The peaks at m/z 1928.0 and 1797.0 are therefore attributed to the loss of one or two of the labile L-Ara4N groups, respectively. The additional peaks at m/z 2080.8 and 1950.0 are interpreted as sodium adducts $[M - 2H + Na]^-$ and $[M - H -$ L-Ara4N $+ Na]^-$, respectively.

The spectrum of St6 (Fig. 3) contains three major peaks. The signal at m/z 2074.9 is attributed to the molecular ion $[M - H]^-$ of a hexa-acylated lipid A 1,4′-bisphosphate bearing two L-Ara4N residues plus an additional oxygen atom (Fig. 1*A* and Table II). The peaks at m/z 1944.0 and 1813.7 can therefore be explained by the loss of one and two L-Ara4N residues, respectively.

The observed molecular ions $[M - H]^-$ of St1 through St6 are consistent with different combinations of L-Ara4N, palmitate, and 2-hydroxymyristate substituents attached to the standard hexa-acylated lipid A 1,4′-bisphosphate (Table II and Fig. 1*A*). However, St5 and St6 represent the first examples of pure lipid A species that are derivatized with two L-Ara4N residues. Both are attached to lipid A via phosphodiester linkages (as shown in Fig. 1*A*), given the pattern of fragmentation (Fig. 3), which reflects the labile nature of such phosphodiester bonds.

Interestingly, pEtN-modified lipid A variants were not produced in large amounts by wild type *S. typhimurium* 14028 or by a *phoP* constitutive mutant grown in LB broth (pH 6.8), as evidenced by the small amount of ^{32}P-labeled material migrating with EV4 (Fig. 3), a hexa-acylated lipid A species known to contain a single pEtN appendage (7).

High Resolution NMR Spectroscopy of St3 and St4—As shown by mass spectrometry (Fig. 3 and Table II), St3 and St4 each contain a single L-Ara4N substituent. Identification of the attachment site of a single L-Ara4N group requires analysis by NMR spectroscopy. St4 contains a 2-hydroxymyristate unit in place of myristate as the secondary acyl chain appended at position 3′, given that St4 is ~16 atomic mass units larger than St3 (Fig. 3 and Table II). Pure lipid A preparations containing a 2-hydroxymyristate substituent were not previously available for study by NMR spectroscopy.

To validate the presence of L-Ara4N in both St3 and St4, each compound was dissolved in $CDCl_3/CD_3OD/D_2O$ (2:3:1, v/v), a solvent suitable for high resolution NMR spectroscopy of lipids (19, 24). Chemical shifts of key sugar and fatty acid protons in St3 and St4 were determined by two-dimensional COSY analysis (Fig. 4, *A* and *B*, and Table III). Both compounds contain three sugar spin systems, identical to those seen in the L-Ara4N containing substances EV3 and lipid II_A, characterized previously (19). When compared with the spectrum of St3 (Fig. 4*A*), the COSY analysis of St4 (Fig. 4*B*) revealed an important additional cross-peak between protons resonating at 4.26 and 1.7 ppm (Fig. 4*B, arrow*). This peak arises from the coupling between H-α′3′ and the two β′3′-protons of the 2-hydroxacyl chain of St4. It is very similar in appearance to the cross-peak between the H-α (4.19 ppm) and the two β-protons (1.7 ppm) of a 2-hydroxymyristate methyl ester standard (Table III). The analysis of St4 also provides direct NMR evidence that the 2-hydroxyacyl chain of St4 is attached to the 3′-(*R*)-3-hydroxymyristate moiety of the distal glucosamine (designated Gln_2 in Table III). The signals assigned to H-3′ of Gln_2 and to H-β3′ of the 3′-(*R*)-3-hydroxymyristate residue are shifted 0.05 and 0.12 ppm downfield, respectively, in comparison to the same protons in St3 (Fig. 4, *A versus B*, and Table III), consistent with the replacement of the secondary myristate chain by 2-hydroxymyristate in St4.

Selective inverse decoupling difference spectroscopy (19, 24) was used to determine the attachment site of the L-Ara4N group in St3 and St4. ^{31}P NMR spectra of St3 and St4 were

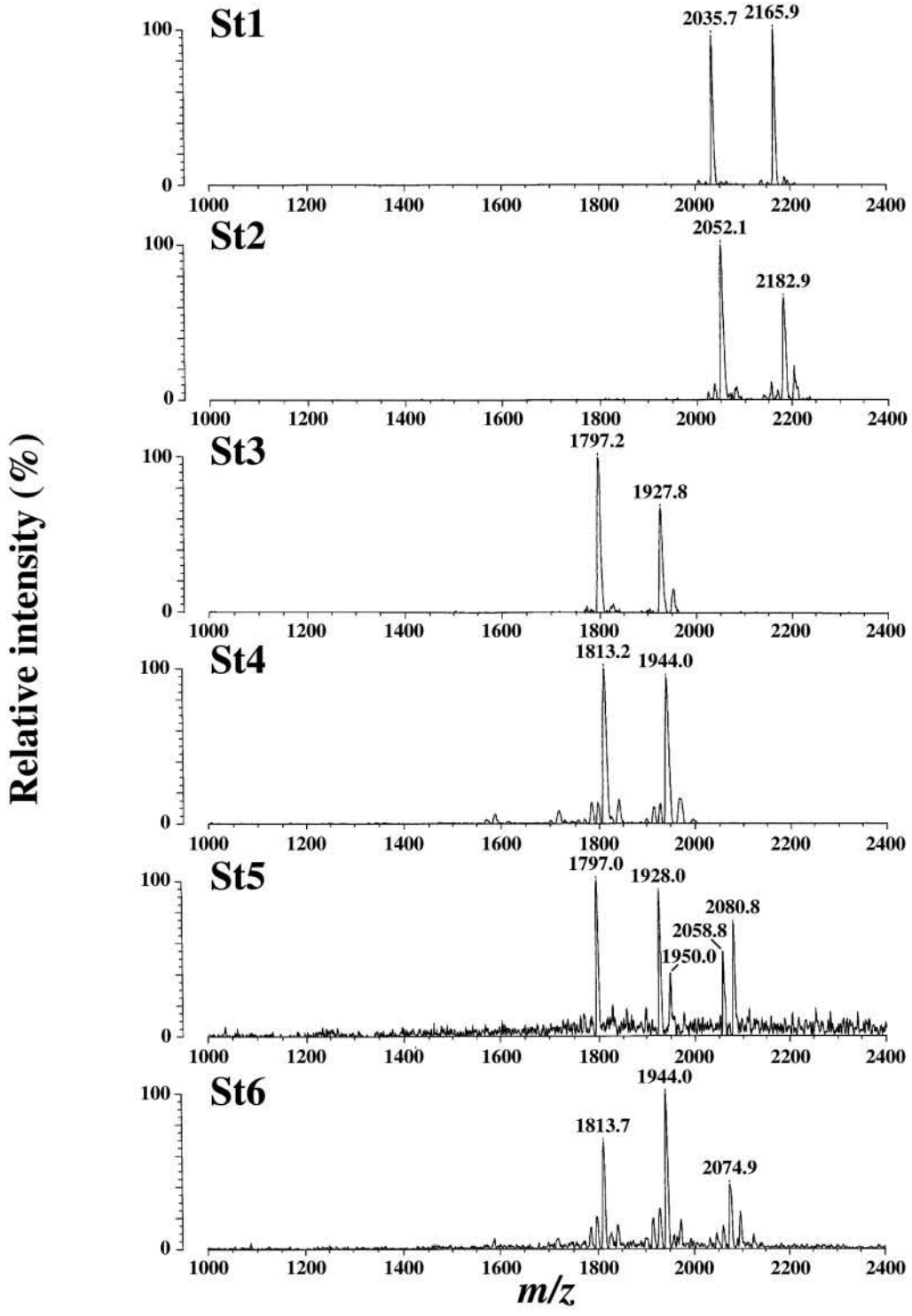

FIG. 3. **Negative-ion MALDI/TOF mass spectrometry of selected lipid A species purified from wild type *S. typhimurium*.** The proposed lipid A modifications that account for the observed mass ions of *St1–St6* are summarized in Table II, and structures are shown in Fig. 1.

recorded at 202 MHz in $CDCl_3/CD_3OD/D_2O$ (2:3:1, v/v) at 25 °C (data not shown). The ^{31}P NMR shifts were referenced to 85% phosphoric acid at 0 ppm. ^{31}P NMR spectra revealed that two phosphorus atoms were present in each compound, at −0.334 and −1.106 ppm for St3 and at −0.231 and −1.161 ppm for St4. The difference spectroscopy analysis, which involves the recording of the proton spectrum during selective on and off resonance irradiation of the individual phosphorus peaks, demonstrated unequivocally that the L-Ara4N residue is attached to the 4′-phosphate in both St3 and St4 (data not shown), as in EV3 and EV6 (19).

In summary, the NMR analysis of St3 and St4, taken together with the mass spectrometry of St5 and St6, shows that *S. typhimurium* 14028 has enzymes that can modify both the 1- and the 4′-phosphates of lipid A with L-Ara4N moieties. The 4′-phosphate group is normally the preferred site for L-Ara4N attachment. When Kdo biosynthesis is blocked, however, only the 1-phosphate residue is derivatized with the L-Ara4N substituent (6, 19), suggesting that addition of the L-Ara4N moiety to the 4′-position may be Kdo-dependent. Direct enzymatic evidence for this proposal is presented in the accompanying article (36).

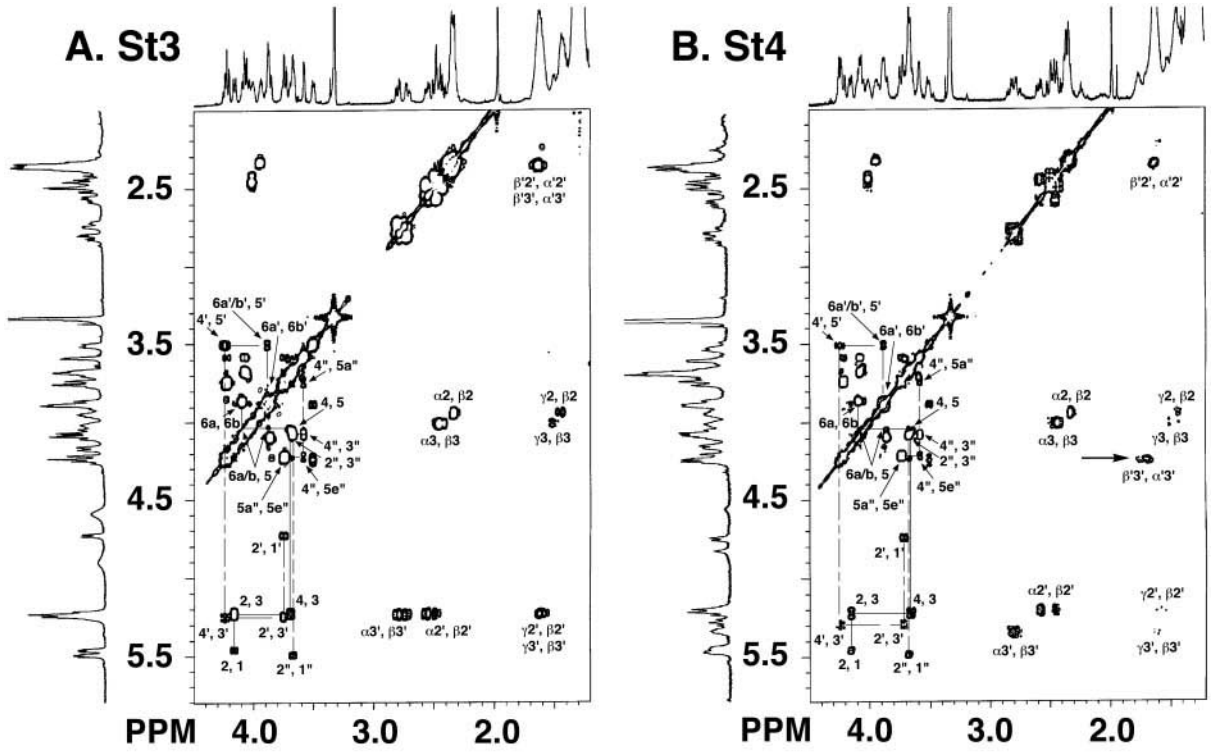

FIG. 4. **Partial two-dimensional ^{1}H-^{1}H COSY spectra at 500 MHz of St3 and St4 purified from wild type *S. typhimurium*.** *A,* St3 is a hexa-acylated lipid A species modified with a single L-Ara4N moiety attached to the 4′-phosphate group, as seen in EV3. *B,* St4 lipid A is the same as St3 but contains an additional oxygen atom, reflecting the presence of a 2-hydroxymyristate moiety as the secondary acyl chain at position 3′ (Table II and Fig. 1). The *arrow* highlights the distinct cross-peak in St4 that is consistent with the presence of a 2-hydroxyacyl substituent. Samples (about 4 mg each) were dissolved in 0.6 ml of $CDCl_3/CD_3OD/D_2O$ (2:3:1, v/v).

Lipid A Species Modified with pEtN Groups Accumulate in S. typhimurium pmrA^{C}pmrE$^-$ and pmrA^{C}pmrF$^-$ Mutants—PmrA-constitutive *S. typhimurium* mutants are polymyxin-resistant, and their lipid A is more extensively modified with L-Ara4N moieties than wild type (16, 22). By using a *pmrA^C* strain as the parent, Gunn *et al*. (18) isolated novel mutants with lesions in the *pmrE* and *pmrF* genes, resulting in the loss of polymyxin resistance and the L-Ara4N modification. The *pmrE* gene encodes UDP-glucose dehydrogenase (18), which generates UDP-glucuronic acid, the likely precursor of L-Ara4N (7). The function of the *pmrF* gene remains to be established (7). The presence of pEtN groups attached to lipid A was not reported in previous studies of such L-Ara4N-deficient *pmrA^C* mutants (18, 33).

^{32}P-Lipid A profiles were generated for strains JSG486 (*pmrA^C pmrE$^-$*) and JSG485 (*pmrA^C pmrF$^-$*) (Fig. 5). Previously characterized *E. coli* lipid A species, including those that accumulate in ammonium metavanadate-treated cells (7), were run as standards (Fig. 5, *lanes 1* and *2*). The parental organism JSG435 (*pmrA^C*) displayed a complicated pattern of lipid A modifications (Fig. 5, *lane 3*), distinctly different from *S. typhimurium* wild type or *phoP* constitutive strains (Fig. 2, *lanes 3* and *5*). JSG435 (*pmrA^C*) produced species migrating with EV2, EV3, and EV5, all of which are known to contain L-Ara4N (7). It also generated more of a species migrating with EV4, which contains a single pEtN substituent (7). As in wild type *S. typhimurium*, additional lipid A variants modified with 2-hydroxymyristoyl and/or palmitoyl groups were discernible. Neither of the double mutants, JSG486 (*pmrA^{C}pmrE$^-$*) (Fig. 5, *lane 4*) or JSG485 (*pmrA^{C}pmrF$^-$*) (Fig. 5, *lane 5*), produced significant amounts of lipid A species migrating with EV2, EV3, of EV5, consistent with the block in L-Ara4N biosynthesis. However, the species migrating with EV4 was produced in greater amounts when compared with JSG435 (PmrA^C)

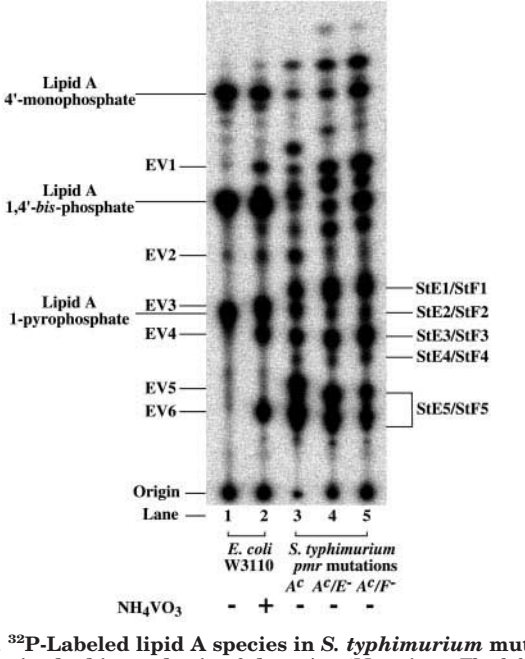

FIG. 5. **^{32}P-Labeled lipid A species in *S. typhimurium* mutants defective in the biosynthesis of the L-Ara4N moiety.** The labeling conditions are the same as in Fig. 2. *EV1–EV6* are standards that were characterized previously (7). In the present study, components *StE1–StE5* and *StF1–StF5* were isolated from strains JSG486 (*pmrA^{C}pmrE$^-$*) and JSG485 (*pmrA^{C}pmrF$^-$*), respectively. StE5 and StF5 were isolated as mixtures, as explained in Table IV, but the other components were purified as single chemical entities. Although StE5 and StF5 migrate very much like EV5 and EV6 (both of which contain the L-Ara4N moiety), none of the components present in StE5 and StF5 contain an L-Ara4N group; instead they are modified with two pEtN groups, as shown by mass spectrometry (Table IV), accounting for their slow mobility.

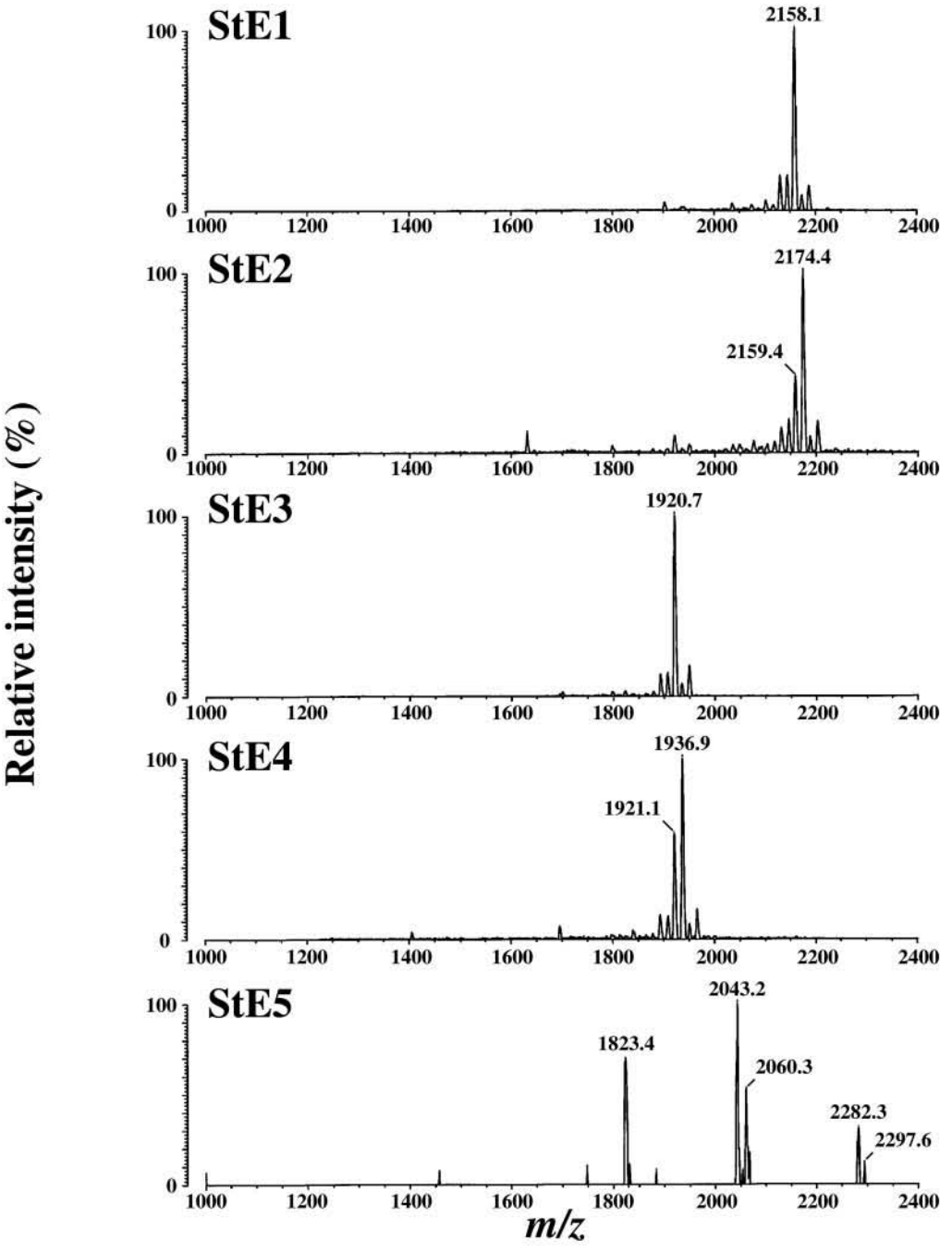

FIG. 6. **Negative ion MALDI/TOF mass spectrometry of lipid A species purified from *S. typhimurium pmrE*$^-$ mutants.** The proposed lipid A modifications that account for the observed mass ions of StE1-StE5 are summarized in Table IV. Negative ion mass spectra of StF1-StF5 are very similar (data not shown).

(Fig. 5). The results suggest that *pmrE* and *pmrF* are both required for modification of lipid A with L-Ara4N but not with pEtN.

Purification and Mass Spectrometry of Selected pEtN-modified Lipid A Species—To validate the structures of the polar lipid A modifications remaining in the absence of L-Ara4N biosynthesis, five major lipid A variants (designated *StE1* to *StE5* and *StF1* to *StF5* in Fig. 5) were purified from JSG486 (*pmrA*^{C} *pmrE*$^-$) and JSG485 (*pmrA*^{C} *pmrF*$^-$), respectively. These compounds were analyzed by MALDI/TOF mass spectrometry.

The negative ion spectrum of StE1 (Fig. 6 and Table IV) revealed a major peak at *m*/*z* 2158.1, consistent with the molecular ion $[M - H]^-$ of a lipid A 1,4′-bisphosphate bearing a palmitate group as a seventh acyl chain, and also modified with a single pEtN residue (Fig. 1*B* and Table IV). The positive ion spectrum of StE1 (not shown) demonstrated a B_1^+ ion at *m*/*z* 1088.2 (see Table IV for definition of ions), indicating that the distal glucosamine ($GlcN_2$) is not substituted with pEtN and that pEtN is attached to the 1-phosphate group. This was confirmed by fragmentation analysis in the negative mode (Table IV). StE2 appears to have the same structure of StE1, except that StE2 contains an additional oxygen atom (Fig. 6 and Table IV) localized to Gln_2. This oxygen atom is likely part of the secondary 2-hydroxymyristoyl group, as in St4 (Figs. 3 and 4*B*).

StE3 and StE4 were determined to have the same compositions as of StE1 and StE2, respectively, except for the absence

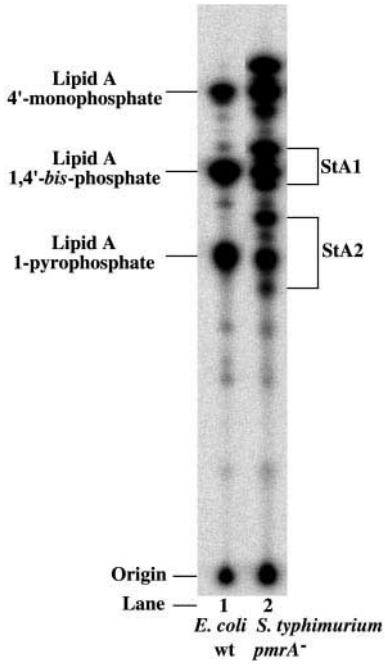

FIG. 7. **^{32}P-Labeled lipid A species in a *S. typhimurium pmrA* knockout mutant.** In the absence of *pmrA*, neither L-Ara4N nor pEtN-modified lipid A species are synthesized. The resulting lipid A profile therefore looks very similar to that of *E. coli* K-12 wild type (7), except that hepta-acylated and 2-hydroxyacyl variants are still present in the *S. typhimurium* lipid A. StA1 and StA2 were isolated and analyzed by mass spectrometry as mixtures. The proposed lipid A modifications that account for the observed mass ions seen in StA1 and StA2 are summarized in Table V.

of the palmitoyl group in StE3 and StE4. Thus, compounds StE1 through StE4 (Fig. 6 and Table IV) have the same relationship to each other as do St1 through St4 (Fig. 3 and Table II), except that the polar head group is a single pEtN unit, located at the 1-position.

StE5 was isolated as a mixture of closely migrating bands, as indicated in Fig. 5. The negative ion spectrum of the StE5 mixture (Fig. 6) shows that it consists of a set of related components. Major peaks at m/z 2282.3, 2297.6, 2043.2, and 2060.3 were attributed to lipid A derivatives having the same compositions as those of StE1 to StE4, respectively, but with the presence of an additional pEtN substituent (Table IV), presumably at position 4′. The peak at m/z 1823.1 could not be assigned.

The components, designated StF1 to StF5, were purified from JSG485 ($pmrA^C pmrF^-$) and analyzed in the same way as StE1 to StE5 (data not shown). Based on their mass spectrometry and migration on TLC plates (Fig. 5 and Table IV), StF1 to StF5 appear to be identical to StE1 to StE5.

In conclusion, the data presented in Fig. 5, Fig. 6, and Table IV reveal that $pmrA^C pmrE^-$ and $pmrA^C pmrF$ mutants, which are defective in L-Ara4N biosynthesis, make more pEtN-modified lipid A than the parental $pmrA^C$ strain or the wild type. As shown by the analysis of the StE5 mixture (Fig. 6), pEtN groups may be attached to both the 1- and 4′-positions. Because StE1 to StE4 contained a single pEtN residue, attached mainly to the 1-phosphate, it would appear that the 1-phosphate is the preferred site of modification with pEtN. Interestingly, however, in Kdo-deficient mutants only the 4′-position of lipid A can be modified with pEtN (6, 19), suggesting that the addition of pEtN to the 1-position may be Kdo-dependent.

A PmrA⁻ Mutant of S. typhimurium Lacks Both L-Ara4N and pEtN-modified Lipid A—To investigate the role of PmrA in the modification of lipid A with pEtN, the ^{32}P-lipid A profile of a *S. typhimurium pmrA*⁻ mutant was analyzed (Fig. 7). The *pmrA*⁻ mutant JSG421 displayed a much simpler lipid A modification pattern (Fig. 7, *lane 2*) than any other strain of *S. typhimurium* (Figs. 2 and 5). The complete absence of L-Ara4N- and pEtN-modified lipid A species was striking. However, lipid A species containing 2-hydroxymyristoyl and palmitoyl groups were observed. The *pmrA*⁻ mutant furthermore produced a lipid A species co-migrating with the *E. coli* hexa-acylated lipid A 1-pyrophosphate variant (Fig. 7, *lane 1*), which accounts for about one-third of the lipid A produced by wild type *E. coli* K-12 (7, 32).

To confirm the interpretation of the thin layer analysis (Fig. 7), lipid A species from the *pmrA*⁻ mutant were partially purified by means of a single DEAE-cellulose column. The lipid A 1,4′-bisphosphate fraction (StA1) and the lipid A 1-pyrophosphate fraction (StA2) were eluted from the DEAE column with chloroform, methanol, 240 mM ammonium acetate (2:3:1, v/v) but were not further resolved into their respective hexa-, hepta-, and/or hydroxylated subtypes by preparative TLC.

The negative ion spectrum of the StA1 mixture (Fig. 8) revealed peaks at m/z 2035.3, 2051.5, 1797.0, and 1813.1 attributed to $[M - H]^-$ of lipid A 1,4′-bisphosphate species partially modified with palmitate and/or an extra oxygen atom but lacking any additional polar head groups (Table V). The negative ion spectrum of the StA2 mixture (Fig. 8) consisted of peaks at m/z 2116.3, 2132.0, 1877.7, and 1893.7, which represent the same $[M - H]^-$ series as in StA1, but as the lipid A 1-pyrophosphate (Table V). In summary (Table V and Fig. 1*C*), the *pmrA*⁻ mutant JSG421 does not produce any L-Ara4N- or pEtN-modified lipid A species, demonstrating PmrA is absolutely essential for lipid A modification with either of these polar moieties. Acylation with palmitate and 2-hydroxylation of lipid A was not affected. The biosynthetic origin of the lipid A 1-pyrophosphate variant is unknown, but as in *E. coli* K12 (7), it is only formed in significant quantities when pEtN and L-Ara4N groups are not synthesized.

DISCUSSION

The structural characterization of lipid A species modified with different combinations of the two polar head groups, L-Ara4N and pEtN, is an important prerequisite to the elucidation of the molecular basis of polymyxin resistance. Our experiments demonstrate that *S. typhimurium* can add either one or two L-Ara4N and/or pEtN units to the 1 and 4′-phosphate moieties of lipid A (Figs. 1, 3, and 6). In wild type cells, the L-Ara4N residue is added predominantly to the 4′-phosphate group, whereas the pEtN residue is selectively incorporated at the 1-phosphate position, as judged by the distribution of the L-Ara4N and pEtN residues in the purified molecular species. However, some lipid A variants are synthesized in which either two L-Ara4N or two pEtN moieties are present (Tables II and IV), indicating that both the 1- and 4′-phosphates can also be substituted with the same polar head group.

A species of lipid A containing a stoichiometric 2-hydroxymyristate substituent as the secondary acyl chain at position 3′ was purified to homogeneity for the first time in quantities sufficient for analysis by high resolution NMR spectroscopy (Fig. 4*B*). This 2-hydroxymyristoyl-modified lipid A will be very helpful as a standard for identifying the enzyme responsible for the oxygen-dependent formation of the 2-hydroxymyristoyl moiety (9). The enzyme has not yet been identified by *in vitro* assay but is encoded by the *lpxO* gene of *S. typhimurium*, which is a member of the Fe^{2+}/ascorbate/α-ketoglutarate-dependent family of dioxygenases (9). The 2-hydroxymyristoyl modification of *S. typhimurium* lipid A is up-regulated by activation of the PhoP/PhoQ system (13) but is not eliminated by inactivation of *pmrA* (Fig. 2). Approximately 43% of the lipid A species

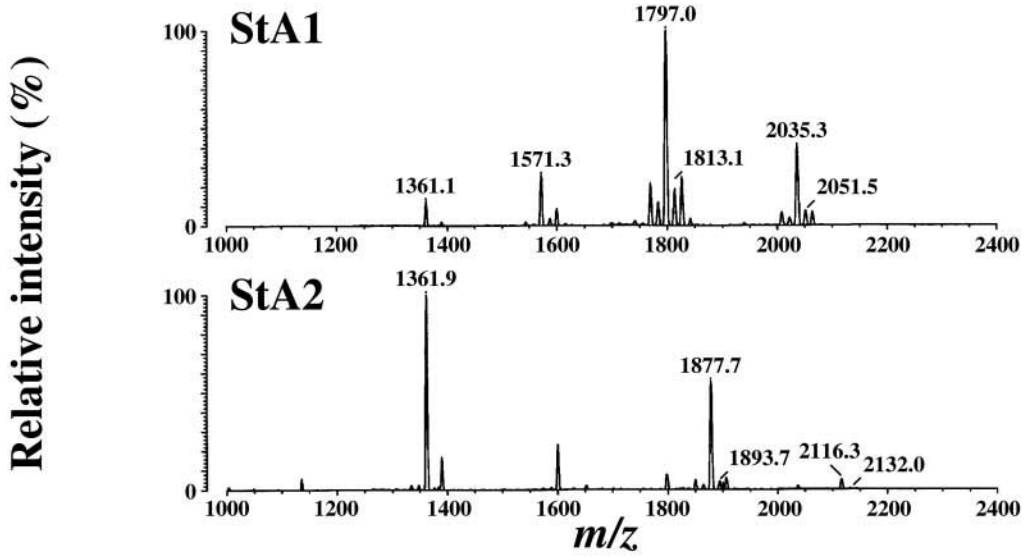

FIG. 8. **Negative ion MALDI/TOF mass spectrometry of lipid A species purified from a *S. typhimurium pmrA* knockout mutant.** The proposed lipid A modifications that account for the observed mass ions of StA1 and StA2 are summarized in Table V. The appearance of a 1-pyrophosphate lipid A variant shows that *S. typhimurium* has the enzymes to synthesize this species, which is not normally detected in this organism.

TABLE V
MALDI/TOF mass spectrometry of lipid A species purified from an S. typhimurium pmrA⁻ mutant grown on LB broth

Lipid A species	$[M - H]^-$	M_r	m_rTrype number of covalent modification		
			C16:0	Oxygen atom	Phosphate
StA1	2035.3	2036.8	1	0	0
	2051.5	2052.8	1	1	0
	1797.0	1798.4	0	0	0
	1813.1	1814.4	0	1	0
StA2	2116.3	2116.8	1	0	1
	2132.0	2132.8	1	1	1
	1877.7	1878.3	0	0	1
	1893.7	1894.3	0	1	1

The predicted M_r is calculated based on the number of proposed covalent modifications attached to the standard hexa-acylated lipid A, 1,4′-bis-phosphate (Fig. 1). $[M - H]^-$ is the observed molecular ion determined by MALDI/TOF mass spectrometry in the negative mode (Fig. 8).

contain a 2-hydroxymyristoyl group in PhoP-constitutive mutants of *S. typhimurium*, compared with about 13% in wild type cells grown aerobically in LB broth (Fig. 2). The biological significance of the 2-hydroxymyristoyl substituent of *S. typhimurium* lipid A is unclear, but its function may be revealed by the characterization of a *S. typhimurium lpxO* mutant recently isolated in our laboratory.

We suggest that L-Ara4N incorporation at the 4′-phosphate position may be Kdo-dependent, given the well established anomaly that *S. typhimurium* mutants defective in Kdo biosynthesis incorporate only a single L-Ara4N substituent exclusively at their 1-phosphate residue (6, 19). Furthermore, the *E. coli* Kdo transferase (KdtA) (34, 35) requires the presence of an unsubstituted 4′-phosphate moiety in its acceptor substrates,[2] consistent with the idea that incorporation of the L-Ara4N moiety at the 4′-phosphate position occurs later than Kdo addition during normal lipopolysaccharide biosynthesis. Conversely, given the structures of lipid A precursors in Kdo-deficient mutants of *S. typhimurium* in which the pEtN substituent occurs solely at the 4′-position (6, 19), it appears that pEtN addition to the 1-phosphate must also be Kdo-dependent, whereas pEtN transfer to the 4′-phosphate is not. The virtual exclusion of the pEtN moiety from the 4′-position under ordinary circumstances in wild type cells could be rationalized if the relative rate of L-Ara4N incorporation at the 4′-position following Kdo addition were much faster than pEtN transfer.

A priori, one might imagine that two L-Ara4N and two pEtN transferases with different substrate specificities are present, one for catalyzing 4′-phosphate substitution and the other for 1-phosphate modification. Development of assays for these transferases is under way in our laboratory. Evidence for a novel, membrane-associated L-Ara4N transferase is presented in the accompanying articles (36, 37). Expression cloning of the relevant L-AraN transferase structural gene, which is part of the *pmrF* operon, suggests unexpectedly that a single enzyme may be responsible for the incorporation of both the 4′ and 1 L-Ara4N substituents, but modification of the 4′-position appears to be Kdo-dependent.

The present study provides new insights into the roles of PmrA/PmrB signaling system in regulating lipid A modifications with L-Ara4N and pEtN moieties. Structural analyses of the lipid A species purified from various *pmr* mutant strains show that *pmrA* is required both for pEtN and for L-Ara4N addition, whereas *pmrE* and *pmrF* are required only for the L-Ara4N modification. Given that both *pmrE* and *pmrF* mutants produce greater than normal amounts of pEtN-modified lipid A species in a pmrA^C background, but are nevertheless polymyxin-sensitive, it is evident that the L-Ara4N moiety is important for maintenance of polymyxin resistance, whereas the pEtN substituent is not. Each L-Ara4N unit would be expected to neutralize two formal negative charges on lipid A, which would reduce the electrostatic interaction between lipid A and polymyxin.

It is intriguing that a *Salmonella pmrA⁻* mutant, lacking both types of polar modification, resembles *E. coli* K-12 in producing significant quantities of a lipid A species containing a 1-pyrophosphate moiety (7) (Figs. 1 and 8, and Table V). Conversely, *E. coli* K-12, which normally makes about one-third of its lipid A as the 1-pyrophosphate variant, is no longer able to produce this substance when induced to make lipid A that is modified with L-Ara4N and pEtN moieties by growth in the presence of NH_4VO_3 (7). These findings suggest that the incorporation of the L-Ara4N and/or the pEtN groups is incompatible with the biosynthesis of the lipid A 1-pyrophosphate,

[2] K. A. White, Z. Zhou, and C. R. H. Raetz, unpublished results.

possibly because of competition for a common donor substrate. As yet, however, the enzymatic mechanism for lipid A 1-pyrophosphate biosynthesis remains elusive.

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