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 sub-
stances are more polar than the predominant hexa-acy-
lated lipid A 1,4'-bisphosphate of *Escherichia coli* K-12. By using new isolation methods, we have purified six lipid A subtypes (S1 to S6) from wild type *S. typhi-
murium*. 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 deri-
vatized with a palmitoyl moiety and/or a 2-hydroxymyr-
ristoyl residue in place of the secondary myristoyl chain at position 3'. The unexpected finding is that S6 and S6 contain two L-Ara4N residues accounts for the anom-
alous structures of lipid A precursors seen in *S. typhi-
murium* mutants defective in 3-deoxy-α-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′pmrF− and pmrA′pmrF− mutants. Pu-
rification and analysis of selected compounds demon-
strate that one or two pEtN moieties may be present. Our findings show that *S. typhimurium* contains versa-
tile 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. How-
ever, PmrA is not needed for the incorporation of 2-hy-
droxymyristate or palmitate.

*Salamonella 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. 1A), such as 4-amino-4-deoxy-L-arabinose (L-Ara4N)3 (3–7), phosphoethanolamine (pEtN) (4–6), (S)-2-
hydroxy-myristate (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 signal-
ning systems (13–15). Addition of the L-Ara4N unit is required for resistance to polymyxin (16–18). Incorporation of the palmi-
toyl 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 constit-
tutive) mutants (17) or in wild type cells exposed to ammonium metavanadate (7, 19).

With the exception of PgpP, 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 L-phosphate (19). However, in *S. typhimurium* mutants defective in Kdo biosynthesis, lipid A precursors ac-
cumulate in which L-Ara4N is linked exclusively to the L-
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 resis-
tance and the biosynthesis of L-Ara4N-modified lipid A (18). The *pmrE* (upg) gene encodes UDP-glucose dehydrogenase (18), suggesting that L-Ara4N is derived from UDP-glucuronic acid. The *pmrF* gene encodes a homologue of yeast doliethyl phos-
phate-mannose synthase and is part of an operon (18) that
includes additional open reading frames hypothesized to en-
code 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. 1A), *S. typhimurium* lipid

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*The abbreviations used are: L-Ara4N, 4-amino-4-deoxy-L-arabinose; pEtN, phosphoethanolamine; Kdo, 3-deoxy-α-manno-octulosonic acid; MALDI/TOF mass spectrometry, matrix-assisted laser desorption-ionization/time of flight mass spectrometry.*
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/pmrB mutants (18), initially using 32P 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 components or two pEtN substituents were present in addition to the predominant, singly substituted species (Fig. 1). In the pmrA/pmrB mutants, 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. 1B). 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. 1C) 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**—NH4VO3, (RS)-2-hydroxyxymyristic acid methyl ester, and p-ansaldehyd (4-methoxybenzaldehyde) were obtained from Sigma. 32P was purchased from PerkinElmer Life Sciences. Pyridine, methanol, and 88% formic acid were obtained from Mallinckrodt, and chloroform was purchased from EM Science. CDCl3, CD3OD, and D2O were obtained 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 μg/ml NH4VO3 was made by mixing equal volumes of autoclaved (2-fold concentrated) LB medium and filter-sterilized aqueous 50 mM NH4VO3 (7). Rapid Profiling of Lipid A Species Released by Mild Acid Hydrolysis of 32P-Labeled Cells—Cells were grown in LB broth, 32P-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::pmrE), or JSG486 (pmrA:: pmrF) were inoculated into fresh LB broth (4.0 liters). Tetracycline was added to the cultures of all three mutants (18), initially using pmrA-constitutive (7). Lipid A species were then 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 St-1 to St-4 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::pmrE) or JSG485 (pmrA:: 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 bisphosphates) 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 N2 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
compounds with $M_r \sim 2000$. 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$_3$OD/D$_2$O (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 Spare 2 data system and a 5-mm Varian inverse probe. The $^2$H signal of
The atom numbering scheme is shown in Fig. 1A. The abbreviations used are: GlcN1, the proximal glucosamine; GlcN2, the distal glucosamine; L-Ara4N, 4-amino-4-deoxy-L-arabinose; 2-OH-C:14-Me, 2-hydroxymyristic acid methyl ester commercial standard. Lipids were dissolved in 0.6 ml of CDCl3/CD2OD/D2O (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 31P decoupled 1H spectra (data not shown).

Some lipid A species under the mass spectrometry conditions employed (7). The peak 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 of a lipid A derivative having the same composition as St1 but containing an additional oxygen atom of a lipid A derivative having the same composition as St1 but containing an additional oxygen atom of a lipid A derivative having the same composition as St1 but containing an additional oxygen atom of a lipid A derivative having the same composition as St1 but containing an additional oxygen atom of a lipid A derivative having the same composition as St1 but containing an additional oxygen atom. The signal at m/z 1897.5 was attributed to phosphatidylcholine 3,4-di-O-sulfo-L-α-tetradecanoylphosphatidylcholine. The negative ion spectrum of St2 (Fig. 3) showed major peaks. The signal at m/z 2165.9 is consistent with the molecular ion [M–H]– of a lipid A derivative having the same composition as St1 and PhoQ two-component signaling system (13). Lipid A profiling of a PhoP-constitutive (PhoPC) S. typhimurium mutant using 32P labeling (Fig. 2, lane 4) showed the expected increase in the extent and complexity of covalent lipid A modification.

**RESULTS**

**FIG. 2. Rapid profiling of 32P-labeled lipid A species of wild type and PhoP- strains of S. typhimurium grown on LB broth.** Cells were uniformly labeled with 32P, 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 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 derivative having the same composition as St1 and containing an additional oxygen atom. The signal at m/z 2165.9 is consistent with the molecular ion [M–H]– of a lipid A derivative having the same composition as St1 and containing an additional oxygen atom. The signal at m/z 2165.9 is consistent with the molecular ion [M–H]– of a lipid A derivative having the same composition as St1 and containing an additional oxygen atom. The signal at m/z 2165.9 is consistent with the molecular ion [M–H]– of a lipid A derivative having the same composition as St1 and containing an additional oxygen atom. The signal at m/z 2165.9 is consistent with the molecular ion [M–H]– of a lipid A derivative having the same composition as St1 and containing an additional oxygen atom. The signal at m/z 2165.9 is consistent with the molecular ion [M–H]– of a lipid A derivative having the same composition as St1 and containing an additional oxygen atom. The signal at m/z 2165.9 is consistent with the molecular ion [M–H]– of a lipid A derivative having the same composition as St1 and containing an additional oxygen atom. The signal at m/z 2165.9 is consistent with the molecular ion [M–H]– of a lipid A derivative having the same composition as St1 and containing an additional oxygen atom.
pmrA-dependent Lipid A Modifications in S. typhimurium

| Lipid A species | Measured | Calculated | Type and number of covalent modifications |
|-----------------|----------|------------|------------------------------------------|
|                  | [M – H]⁻ | Yᵢ⁻ | B₁⁺ | C₁₆₋₈ | Oxygen atom | pEtN |
| *S. typhimurium* pmrA⁺/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            | 2292.3   |         |        | 2292.9 |        |        | 1 | 0 | 1 |
|                 | 2297.6   |         |        | 2298.9 |        |        | 1 | 1 | 1 |
|                 | 2043.2   |         |        | 2044.5 |        |        | 0 | 0 | 2 |
|                 | 2060.3   |         |        | 2060.5 |        |        | 0 | 1 | 2 |

The predicted Mᵢ⁻ 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 B₁⁺ are not shown. B₁⁺ arises from the distal unit and the Yᵢ⁻ 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. 1A 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. 1A 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. 1A and Table II). The peaks at *m/z* 1928.0 and 1979.0 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. 1A 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 St5 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. 1A). 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. 1A), 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 32P-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 CDCl₃/CDₑₒₓ/D₂O (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 Π₄A, characterized previously (19). When compared with the spectrum of St3 (Fig. 4A), the COSY analysis of St4 (Fig. 4B) revealed an important additional cross-peak between protons resonating at 4.26 and 1.7 ppm (Fig. 4B, arrow). This peak arises from the coupling between H-α'3' and the two β'3'-protons of the 2-hydroxyl chain of St4. It is very similar in appearance to the 2-hydroxyacyl chain of St4. To validate the presence of l-Ara4N in both St3 and St4, each compound was dissolved in CDCl₃/CDₑₒₓ/D₂O (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 Π₄A, characterized previously (19). When compared with the spectrum of St3 (Fig. 4A), the COSY analysis of St4 (Fig. 4B) revealed an important additional cross-peak between protons resonating at 4.26 and 1.7 ppm (Fig. 4B, arrow). This peak arises from the coupling between H-α'3' and the two β'3'-protons of the 2-hydroxyl chain of St4. It is very similar in appearance to the 2-hydroxyacyl chain of St4.

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recorded at 202 MHz in CDCl₃/CD₃OD/D₂O (2:3:1, v/v) at 25 °C (data not shown). The ³¹P NMR shifts were referenced to 85% phosphoric acid at 0 ppm. ³¹P 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).
Lipid A Species Modified with pEtN Groups Accumulate in S. typhimurium pmrA CpmrE/H11002 and pmrA CpmrF/H11002 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 pmrAC 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 pmrAC mutants (18, 33).

32P-Lipid A profiles were generated for strains JSG486 (pmrAC pmrE/H11002) and JSG485 (pmrAC pmrF/H11002) (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 (pmrAC) 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 (pmrAC) 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 (pmrAC pmrE−) (Fig. 5, lane 4) or JSG485 (pmrAC pmrF−) (Fig. 5, lane 5), produced significant amounts of lipid A species migrating with EV2, EV3, or 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−)

![Image 1](image1.png)

**Lipid A Species Modified with pEtN Groups Accumulate in S. typhimurium pmrA CpmrE/H11002 and pmrA CpmrF/H11002 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 pmrAC 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 pmrAC mutants (18, 33).

32P-Lipid A profiles were generated for strains JSG486 (pmrAC pmrE/H11002) and JSG485 (pmrAC pmrF/H11002) (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 (pmrAC) 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 (pmrAC) 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 (pmrAC pmrE−) (Fig. 5, lane 4) or JSG485 (pmrAC pmrF−) (Fig. 5, lane 5), produced significant amounts of lipid A species migrating with EV2, EV3, or 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−)
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 (pmrAC pmrE/H11002) and JSG485 (pmrAC pmrF/H11002), 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. 1B and Table IV). The positive ion spectrum of StE2 (not shown) demonstrated a B3− ion at m/z 1088.2 (see Table IV for definition of ions), indicating that the distal glucosamine (GlcN2) 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). StE3 and StE4 were determined to have the same composition as StE1 and StE2, respectively, except for the absence of an additional oxygen atom (Fig. 6 and Table IV) localized to Gln2. This oxygen atom is likely part of the secondary 2-hydroxymyristoyl group, as in St4 (Figs. 3 and 4B).

StE5 and StE6 were determined to have the same compositions as of StE1 and StE2, respectively, except for the absence of a palmitate group as a seventh acyl chain, and also modified with a single pEtN residue (Fig. 1B and Table IV). The positive ion spectrum of StE5 (not shown) demonstrated a B3− ion at m/z 1088.2 (see Table IV for definition of ions), indicating that the distal glucosamine (GlcN2) 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 Gln2. This oxygen atom is likely part of the secondary 2-hydroxymyristoyl group, as in St4 (Figs. 3 and 4B).

StE3 and StE4 were determined to have the same compositions as of StE1 and StE2, respectively, except for the absence of a palmitate group as a seventh acyl chain, and also modified with a single pEtN residue (Fig. 1B and Table IV). The positive ion spectrum of StE1 (not shown) demonstrated a B3− ion at m/z 1088.2 (see Table IV for definition of ions), indicating that the distal glucosamine (GlcN2) 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 Gln2. This oxygen atom is likely part of the secondary 2-hydroxymyristoyl group, as in St4 (Figs. 3 and 4B).

StE3 and StE4 were determined to have the same compositions as of StE1 and StE2, respectively, except for the absence of a palmitate group as a seventh acyl chain, and also modified with a single pEtN residue (Fig. 1B and Table IV). The positive ion spectrum of StE5 (not shown) demonstrated a B3− ion at m/z 1088.2 (see Table IV for definition of ions), indicating that the distal glucosamine (GlcN2) 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 Gln2. This oxygen atom is likely part of the secondary 2-hydroxymyristoyl group, as in St4 (Figs. 3 and 4B).

StE3 and StE4 were determined to have the same compositions as of StE1 and StE2, respectively, except for the absence of a palmitate group as a seventh acyl chain, and also modified with a single pEtN residue (Fig. 1B and Table IV). The positive ion spectrum of StE5 (not shown) demonstrated a B3− ion at m/z 1088.2 (see Table IV for definition of ions), indicating that the distal glucosamine (GlcN2) 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 Gln2. This oxygen atom is likely part of the secondary 2-hydroxymyristoyl group, as in St4 (Figs. 3 and 4B).

StE3 and StE4 were determined to have the same compositions as of StE1 and StE2, respectively, except for the absence of a palmitate group as a seventh acyl chain, and also modified with a single pEtN residue (Fig. 1B and Table IV). The positive ion spectrum of StE5 (not shown) demonstrated a B3− ion at m/z 1088.2 (see Table IV for definition of ions), indicating that the distal glucosamine (GlcN2) 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 Gln2. This oxygen atom is likely part of the secondary 2-hydroxymyristoyl group, as in St4 (Figs. 3 and 4B).
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'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'pmrE' and pmrA'pmrF' mutants, which are defective in l-Ara4N biosynthesis, make more pEtN-modified lipid A than the parental pmrA' 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 32P-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. 1C), 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-hydroxylolation 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'-phospho 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. 4B). 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 Fe2+/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...
organism. 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<sup>-</sup> mutant grown on LB broth

| Lipid A species | [M - H]<sup>-</sup> | m<sub>r</sub> | m<sub>r</sub>-Type 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      |                                            |
|                 | 2130.0 2132.8       | 1 1 1      |                                            |
|                 | 1877.7 1878.3       | 0 0 1      |                                            |
|                 | 1893.7 1894.3       | 1 1 1      |                                            |

The predicted m<sub>r</sub> 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]<sup>-</sup> 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 in 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, consistent with the idea that incorporation of 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-Ara4N 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 pmrAc 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 pmr<sup>A</sup> 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 NH₄VO₃ (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,
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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