8-5-2005

Resistance to the Antimicrobial Peptide Polymyxin Requires Myristoylation of Escherichia Coli and Salmonella Typhimurium Lipid A

An X. Tran
Quillen-Dishner College of Medicine

Melissa E. Lester
Quillen-Dishner College of Medicine

Christopher M. Stead
Quillen-Dishner College of Medicine

Christian R.H. Raetz
Duke University Medical Center

Duncan J. Maskell
University of Cambridge

See next page for additional authors

Citation Information

Tran, An X.; Lester, Melissa E.; Stead, Christopher M.; Raetz, Christian R.H.; Maskell, Duncan J.; McGrath, Sara C.; Cotter, Robert J.; and Trent, M. Stephen. 2005. Resistance to the Antimicrobial Peptide Polymyxin Requires Myristoylation of Escherichia Coli and Salmonella Typhimurium Lipid A. *Journal of Biological Chemistry*. Vol.280(31). 28186-28194. https://doi.org/10.1074/jbc.M505020200 PMID: 15951433 ISSN: 0021-9258

Resistance to the Antimicrobial Peptide Polymyxin Requires Myristoylation of Escherichia Coli and Salmonella Typhimurium Lipid A

Creator(s)

An X. Tran, Melissa E. Lester, Christopher M. Stead, Christian R.H. Raetz, Duncan J. Maskell, Sara C. McGrath, Robert J. Cotter, and M. Stephen Trent

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 31, Issue of August 5, pp. 28186–28194, 2005

Printed in U.S.A.

Resistance to the Antimicrobial Peptide Polymyxin Requires Myristoylation of *Escherichia coli* and *Salmonella typhimurium* Lipid A*

Received for publication, May 5, 2005, and in revised form, June 8, 2005
Published, JBC Papers in Press, June 10, 2005, DOI 10.1074/jbc.M505020200

An X. Tran‡§, Melissa E. Lester‡§, Christopher M. Stead‡§, Christian R. H. Raetz¶, Duncan J. Maskell‖, Sara C. McGrath, Robert J. Cotter**, and M. Stephen Trent‡ ‡‡**

*From the ‡Department of Microbiology, J. H. Quillen College of Medicine, Johnson City, Tennessee 37614, the ¶Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710, the ‖Department of Veterinary Medicine, Centre for Veterinary Science, University of Cambridge, Cambridge CB3 OES, United Kingdom, and the **Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

Attachment of positively charged, amine-containing residues such as 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (pEtN) to *Escherichia coli* and *Salmonella typhimurium* lipid A is required for resistance to the cationic antimicrobial peptide, polymyxin. In an attempt to discover additional lipid A modifications important for polymyxin resistance, we generated polymyxin-sensitive mutants of an *E. coli pmrA^C* strain, WD101. A subset of polymyxin-sensitive mutants produced a lipid A that lacked both the 3′-acyloxyacyl-linked myristate (C_{14}) and L-Ara4N, even though the necessary enzymatic machinery required to synthesize L-Ara4N-modified lipid A was present. Inactivation of *lpxM* in both *E. coli* and *S. typhimurium* resulted in the loss of L-Ara4N addition, as well as, increased sensitivity to polymyxin. However, decoration of the lipid A phosphate groups with pEtN residues was not effected in *lpxM* mutants. In summary, we demonstrate that attachment of L-Ara4N to the phosphate groups of lipid A and the subsequent resistance to polymyxin is dependent upon the presence of the secondary linked myristoyl group.

Lipopolysaccharide (LPS)[1] is the major surface molecule of Gram-negative bacteria and is held in the outer membrane by a unique phospholipid domain known as lipid A. The typical lipid A backbone consists of a β-1′,6-linked disaccharide of glucosamine that is phosphorylated and multiply acylated. In *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (*Salmonella typhimurium*), the disaccharide backbone is acylated at the 2-, 3-, 2′-, and 3′-positions with (*R*)-3-hydroxymyristate and phosphorylated at the 1- and 4′-positions (1). A secondary lauroyl (C_{12}) and myristoyl (C_{14}) group is attached at the 2′- and 3′-positions, respectively, of the distal glucosamine in an acyloxyacyl linkage resulting in the hexa-acylated structure shown in Fig. 1*A* (1). The lipid A domain is attached to the polysaccharide portion of LPS via the Kdo (3-deoxy-D-*manno*-octulosonic acid) sugars (Fig. 1) (1).

Modification of the lipid A domain of *E. coli* and *S. typhimurium* with the cationic sugar 4-amino-4-dexoy-L-arabinose (L-Ara4N) and phosphoethanolamine (pEtN) promotes resistance to the cyclic antimicrobial lipopeptide, polymyxin (2–5). Although the mechanism of polymyxin killing is not completely understood, the peptide is thought to access the outer surface of the bacterium by interacting with the negatively charged phosphate groups of lipid A. A similar mechanism is employed by cationic antimicrobial peptides of the innate immune system (6). Masking of lipid A phosphate groups with positively charged amine-containing residues is predicted to decrease binding of polymyxin to the bacterial surface promoting survival. In *E. coli* and *S. typhimurium*, the polymyxin-resistant phenotype is primarily under the control of the PmrA/PmrB two-component regulatory system that is activated during growth under conditions of low pH, high Fe^{3+}, and in a PhoP/PhoQ-dependent manner during Mg^{2+} starvation (7–9).

Previously, Trent and co-workers (10) demonstrated that periplasmic addition of L-Ara4N to lipid A is catalyzed by L-4-aminoarabinose transferase (ArnT), a PmrA-regulated glycosyltransferase (10). Because the transferase utilizes an undecaprenyl-linked donor substrate, undecaprenyl-phosphate-α-L-Ara4N, its active site is predicted to lie in the periplasmic region of the cell (11). Furthermore, Doerrler *et al.* (12) have demonstrated that modification of lipid A with L-Ara4N and pEtN is dependent upon its transport across the inner membrane by MsbA. We now report that, in *E. coli* K12 and *S. typhimurium*, addition of L-Ara4N to the lipid A domain of LPS in living cells is dependent upon the presence of the acyloxyacyl-linked myristoyl group at the 3′-position. Loss of myristoylation of lipid A in both *E. coli* and *S. typhimurium* by inactivation of *lpxM* resulted in loss of L-Ara4N modification and in a significant decrease in polymyxin resistance. However, the pEtN modification of the lipid A phosphate groups was not effected by loss of myristoylation.

EXPERIMENTAL PROCEDURES

Chemicals and Other Materials—[γ-^{32}P]ATP and $^{32}P_i$ were obtained from Amersham International. Silica Gel 60 (0.25-mm) thin layer plates were purchased from EM Separation Technology (Merck). Yeast extract and Tryptone were from Difco. Triton X-100 and bicinchoninic acid were from Pierce. Polymyxin B sulfate was purchased from Sigma. All other chemicals were reagent grade and were purchased from either Sigma or Mallinckrodt.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.

‡‡ To whom correspondence should be addressed: J. H. Quillen College of Medicine, Box 70579, Johnson City, TN 37614. Tel.: 423-439-6293; Fax: 423-439-8044; E-mail: trentms@mail.etsu.edu.

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

TABLE I
Strains and plasmids

Strain or plasmid	Description	Source or Ref.
E. coli		
W3110	Wild type, F^-, λ^-	*E. coli* Genetic Stock Center, Yale University
WD101	W3110, *pmrA*^{c}, polymyxin^r	11
MLK1067	W3110, *lpxM*::Ωcam	13, 14
WD103	WD101, *lpxM*::Ωcam	This work
F2–1	WD101 polymyxin^s mutant	This work
C1–1	WD101 polymyxin^s mutant	This work
A3–1	WD101 polymyxin^s mutant	This work
S. typhimurium		
C5	Wild type mouse virulent strain	28
C5 *lpxM*::kan	Kan^r	28
C5 *lpxM*::kan/pR1A	Kan^r,Amp^r	This work
C5 *lpxM*::kan/pRM17A	Kan^r,Amp^r	This work
Plasmids		
pR1A	Derivative of pACYC177, Amp^r	This work
pRM17A	pR1A containing *S. typhimurium lpxM*	This work
pWSK29	Low-copy expression vector, Amp^r	15
pWSLpxM	pWSK29 containing *E. coli lpxM*	This work

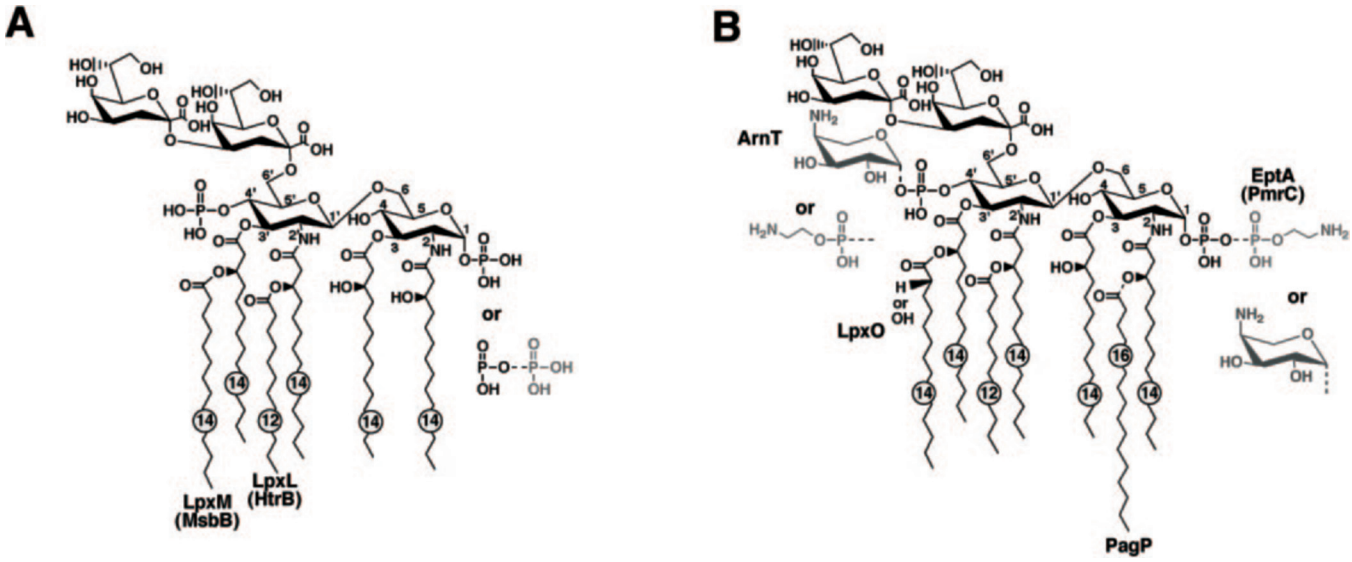

FIG. 1. **Structure of the modified Kdo$_2$-lipid A of *E. coli* and *S. typhimurium*.** The covalent modifications of lipid A are indicated with *dashed bonds*, and lengths of the acyl chains are designated with *enclosed circles*. *Panel A*, the lipid A backbone of *E. coli* and *S. typhimurium* is a hexa-acylated disaccharide of glucosamine that is substituted at the 1- and 4′-positions with phosphate and glycosylated at the 6′-position with two Kdo (3-deoxy-D-*manno*-octulosonic acid) moieties (1, 43). In wild type *E. coli* K12 strains, a portion of the lipid A contains a pyrophosphate group at the 1-position (36). Acyl chains catalyzed by the so-called late acyltransferases, LpxL and LpxM, are indicated. *Panel B*, the modification of phosphate moieties of lipid A is regulated by PmrA (5) and may be substituted with L-Ara4N or pEtN groups. In addition, the protein product of the PhoP-activated gene, *pagP*, further modifies lipid A by the addition of a palmitoyl chain to the hydroxyl group of the *N*-linked-*R*-3-hydroxymyristate chain on the proximal glucosamine unit of lipid A. In *Salmonella*, 2-hydroxymyristate can be found in place of myristate. The reported enzymes required for their respective modifications are indicated in the figure.

Bacterial Strains and Growth Conditions—Bacterial strains are described in Table I. Typically bacteria were grown at 37 °C in LB broth containing 10 g of NaCl, 10 g of Tryptone, and 5 g of yeast extract per liter. When required for plasmid selection, cells were grown in the presence of 100 μg/ml ampicillin, 12 μg/ml tetracycline, 30 μg/ml chloramphenicol, or 30 μg/ml kanamycin.

Recombinant DNA Techniques—Plasmids were isolated using the Qiagen Spin Prep Kit. Custom primers were obtained from Integrated DNA Technologies. PCR reagents were purchased from Stratagene. PCR clean up was performed using the Qiaquick PCR Purification Kit (Qiagen). DNA fragments were isolated from gels using the Qiaquick Gel Extraction Kit (Qiagen). Restriction endonucleases, T4 DNA ligase, and shrimp alkaline phosphatase were purchased from New England Biolabs. All modifying enzymes were used according to the manufacturer's instructions.

Isolation of Polymyxin-sensitive Mutants—Polymyxin-sensitive mutants were generated by random mutagenesis of the *E. coli* strain, WD101. WD101, a polymyxin-resistant *E. coli* K12 strain, contains a mutation in the *pmrA* (*basR*) gene resulting in a *pmrA*^{C} phenotype promoting polymyxin resistance (11). *E. coli* WD101 was treated with 40 μg/ml of the mutagen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine for 10 min at 37 °C. To obtain clones that were sensitive to polymyxin, mutagenized cells were first grown on LB agar plates (~100/plate). Sensitive mutants were identified by replica plating onto LB agar plates containing 2 μg/ml polymyxin B sulfate at 37 °C. Sensitive clones were re-purified and their sensitivity to polymyxin verified. Approximately 10,000 colonies were screened by the above method, and ~100 polymyxin-sensitive mutants were identified including strains F2-1 and C1-1.

Generation of E. coli Strain WD103—WD103 was generated by P1*vir* transduction of the polymyxin-resistant genotype (*pmrA*^{C}) of strain WD101 into the *E. coli lpxM* mutant MLK1067 (W3110 *lpxM*::Ωcam) (13, 14).

Construction of LpxM Covering Plasmids, pWSLpxM and pRM17A—*E. coli lpxM* was cloned into the multiple cloning site of the low-copy expression vector pWSK29 (15) resulting in plasmid pWSLpxM. The latter was used to complement the *pmrA*^{C} *E. coli lpxM* mutant, WD103. The *S. typhimurium lpxM* covering plasmid was constructed by subcloning the *Salmonella lpxM* gene into a pACYC derivative, pR1A. The resulting plasmid was named pRM17A and was used to complement the *S. typhimurium lpxM* mutant.

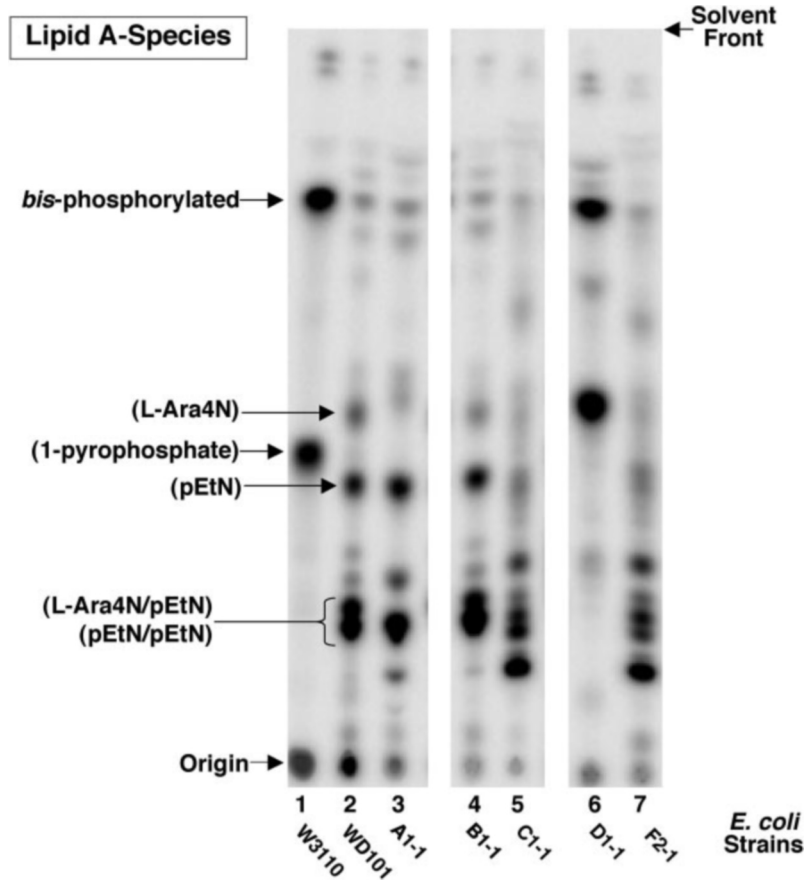

FIG. 2. **Comparison of ^{32}P-labeled lipid A of polymyxin-sensitive derivatives of *E. coli* WD101.** ^{32}P-Labeled lipid A species from polymyxin-sensitive mutants generated by random chemical mutagenesis of *E. coli* strain WD101 were isolated as described under "Experimental Procedures." The labeled lipid A species were separated by TLC using the solvent chloroform, pyridine, 88% formic acid, water (50:50:16:5, v/v), and visualized by phosphorimaging. The proposed lipid A species are indicated based upon previous work by Zhou *et al.* (16, 36). Lipid A from the polymyxin-sensitive strain W3110 and the polymyxin-resistant strain WD101 were analyzed as controls.

Isolation and Analysis of ^{32}P-Labeled Lipid A Species—$^{32}P_i$-Labeled lipid A was isolated from cells uniformly labeled with 2.5 μCi/ml of $^{32}P_i$ in 5 ml of LB broth as previously described (16). The ^{32}P-labeled lipid A domain was released from LPS by hydrolysis of the Kdo dissacharide and processed by the method of Zhou and co-workers (16). Lipids were spotted onto a Silica Gel 60 TLC plate (10,000 cpm/lane) and developed in the solvent chloroform, pyridine, 88% formic acid, water (50:50:16:5, v/v). Labeled lipids were visualized by phosphorimaging.

Isolation and Analysis of ^{32}P-Labeled Undecaprenyl-Phosphate-α-L-Ara4N—Cells were uniformly labeled with $^{32}P_i$ as described above. The glycerolphospholipid fraction containing the undecaprenyl-phosphate-α-L-Ara4N was isolated and analyzed by TLC as described by Trent and co-workers (11).

Preparation of Cell-free Extracts, Double-Spun Cytosol, and Washed Membranes—Typically, 200-ml cultures of *E. coli* or *S. typhimurium* were grown at 37 °C to an A_{600} of 1.0 and harvested by centrifugation at 6,000 × *g* for 30 min. All samples were prepared at 4 °C. Cell-free extract, double-spun cytosol, and washed membranes were prepared as previously described (17) and were stored in aliquots at −20 °C. Protein concentration was determined by the bicinchoninic acid method (18), using bovine serum albumin as the standard.

Preparation of [4′-^{32}P]Lipid IV$_A$—The substrate [4′-^{32}P]lipid IV$_A$ was generated from 100 μCi of [γ-^{32}P]ATP and the tetraacyldisaccharide 1-phosphate lipid acceptor, using the overexpressed 4′-kinase present in membranes of *E. coli* BLR(DE3)/pLysS/pJK2 (17, 19, 20), as previously described (17, 20).

Assay of E. coli ArnT—The ArnT was assayed as previously described by Trent and co-workers using [4′-^{32}P]lipid IV$_A$ as the substrate (10). Addition of the L-Ara4N was visualized as a shift to a slower migrating lipid species previously termed Lipid II$_A$ (21).

Polymyxin Sensitivity Assays—E. coli and *S. typhimurium* strains were assayed for polymyxin resistance as described by Gunn and Miller (8). After growth to an $A_{600\ nm}$ of ~0.5, bacteria were diluted to ~2,500 colony forming units/ml in LB broth. Cells (200 μl) were treated for 1 h at 37 °C with increasing concentrations of polymyxin in a microtiter plate. Following exposure to polymyxin, 100 μl of treated bacteria were plated on LB agar plates and incubated overnight at 37 °C. Colony counts were determined for each concentration of polymyxin tested, and the results were expressed as the percentage of colonies resulting from untreated cells.

Large Scale Isolation of Lipid A—E. coli or *S. typhimurium* were cultured in 200 ml of LB medium at 37 °C or N-minimal media containing 10 μM Mg^{2+}, respectively. The cultures were allowed to reach an $A_{600\ nm}$ ~ 0.8, harvested by centrifugation at 6000 × *g* for 15 min, and washed once with phosphate-buffered saline. The final cell pellets were resuspended in 20 ml of phosphate-buffered saline. Lipid A was released from cells and purified as previously described (16, 22), and stored frozen at −20 °C. Prior to mass spectrometry various lipid A species were separated by anion-exchange chromatography as previously described (22, 37).

Mass Spectrometry of Lipid A Species—Mass spectra of the purified lipids were acquired in the negative ion mode using a matrix-assisted laser desorption-ionization-time of flight (MALDI-TOF) mass spectrometer (AXIMA-CFR, Kratos Analytical, Manchester, UK), equipped with a nitrogen laser (337 nm). The instrument was operated using 20-kV extraction voltage and time-delayed extraction, providing a mass resolution of about ±1 atomic mass units for compounds with M_r ~ 2000. Each spectrum represented the average of 100 laser shots. Saturated 6-aza-2-thiothymine in 50% acetonitrile and 10% tribasic ammonium citrate (9:1, v/v) served as the matrix. The samples were dissolved in chloroform/methanol (4:1, v/v), and deposited on the sample plate followed by an equal portion of matrix solution (0.3 μl). The sample was dried at 25 °C prior to mass analysis.

RESULTS

Isolation and Characterization of Polymyxin-sensitive Mutants of E. coli WD101—Addition of the amine-containing residues, L-Ara4N and pEtN, to the phosphate groups of lipid A (Fig. 1) is associated with increased resistance to cationic antimicrobial peptides, including the peptide polymyxin (2–5). *E. coli* strain WD101 contains a mutation in the *pmrA* gene resulting in a *pmrA^C* phenotype and modification of the lipid A structure with L-Ara4N and pEtN, helping to provide resistance to polymyxin at concentrations of up to 20 μg/ml on solid

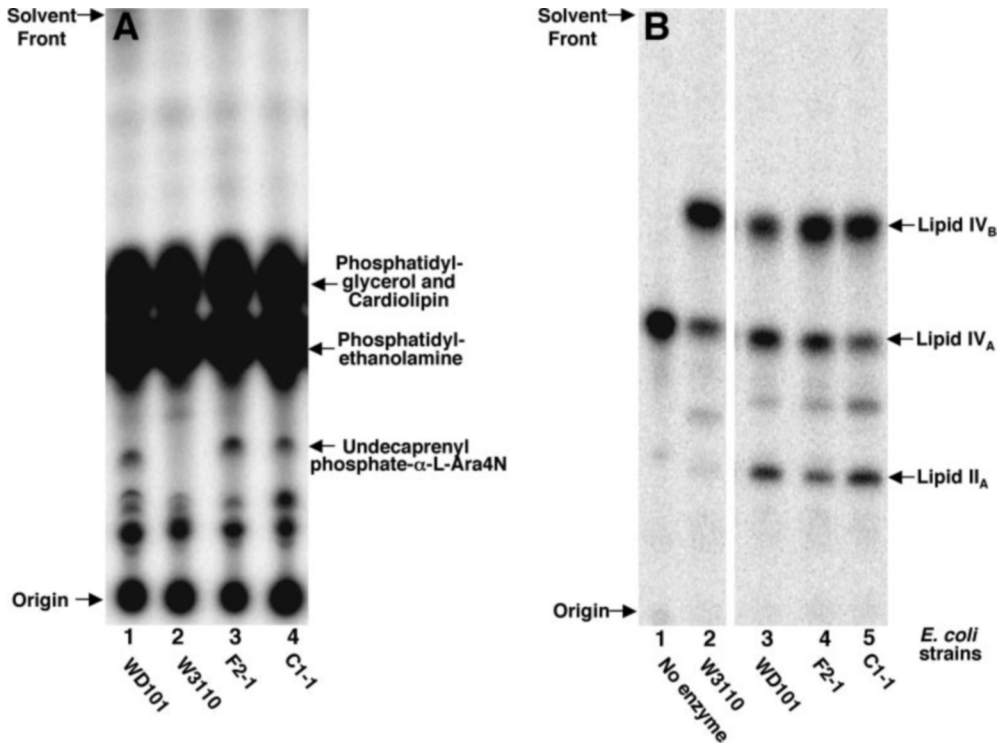

FIG. 3. **Detection of the L-Ara4N donor lipid and L-Ara4N transferase activity in polymyxin-sensitive mutants C1-1 and F2-1.** *Panel A*, ^{32}P-labeled phospholipids were isolated from the indicated strains as described under "Experimental Procedures" and separated by TLC. The migration of undecaprenyl-phosphate-α-L-Ara4N is indicated, and based on work by Trent and co-workers (11). *Panel B*, membranes from the indicated strains were assayed for the presence of the L-Ara4N-lipid A transferase (ArnT) activity as previously described (10). Formation of the slower migrating species, lipid II$_A$, was indicative of ArnT activity.

media (10). Originally, we sought to determine additional modifications to the lipid A structure that were important for polymyxin resistance. Using WD101 as the parent strain, a library of mutants displaying sensitivity to polymyxin at 2 μg/ml was generated via random chemical mutagenesis. Approximately 10,000 colonies were replica plated on LB agar containing 2 μg/ml of polymyxin, and ~100 colonies showing sensitivity to the peptide were purified for further analysis. Each mutant was uniformly labeled in the presence of $^{32}P_i$, and the lipid A fraction analyzed by TLC. Fig. 2 shows the modification patterns found in the lipid A fractions isolated from selected polymyxin-sensitive mutants.

Overall, four different lipid A phenotypes were found among the polymyxin-sensitive mutants. For example, some mutants produced lipid A identical to that of wild type *E. coli* strain W3110. W3110 synthesizes a *bis*-phosphorylated hexa-acylated lipid A species lacking modified phosphate groups (Fig. 2, *lane* 1) and shows sensitivity to polymyxin at concentrations <0.1 μg/ml on solid media (data not shown). Mutant B1-1 produced lipid A species (Fig. 2, *lane* 4) identical to those of the WD101 parent strain (Fig. 2, *lane* 2) modified with L-Ara4N, and pEtN yet showed sensitivity to the peptide at 2 μg/ml. These results suggest that other mechanisms involved in conferring polymyxin resistance to *E. coli* may remain as yet unidentified. As expected, in some cases sensitivity arose from the inability to synthesize L-Ara4N- or pEtN-modified species. For example, strain A1-1 (Fig. 2, *lane* 3) produced lipid A modified with one or two pEtN residues, whereas mutant D1-1 produced species modified with only L-Ara4N (*lane* 6). The changes in the lipid A domain of A1-1 and D1-1 most likely arose from lack of L-Ara4N or pEtN transferase activities, respectively (data not shown). Finally, strains C1-1 and F2-1 produced very hydrophilic lipid A species that had not been previously identified, and for this reason these strains were chosen for further analysis.

Strains C1-1 and F2-1 Have the Enzymatic Machinery to Synthesize and Transfer L-Ara4N to Lipid A—Transfer of L-Ara4N to lipid A occurs in the periplasmic region of the cell catalyzed by the inner membrane glycosyltransferase, ArnT. The enzyme utilizes an undecaprenyl carrier lipid, undecaprenyl-phosphate-α-L-Ara4N, as the donor substrate (10, 11). Loss of ArnT function or the inability to synthesize the undecaprenyl-linked substrate results in loss of polymyxin resistance (5, 23). Because mutants C1-1 and F2-1 showed sensitivity to polymyxin, we determined if these mutants contained the necessary enzymatic machinery to synthesize and transfer L-Ara4N to the lipid A domain of their LPS. The phospholipid fraction from $^{32}P_i$-labeled cells was analyzed by TLC for the presence of the undecaprenyl-phosphate-α-L-Ara4N donor lipid. As previously shown by Trent *et al.* (11), the L-Ara4N donor lipid was produced by the polymyxin-resistant strain WD101 (Fig. 3*A*, *lane* 1), but absent in the polymyxin-sensitive wild type strain, W3110 (*lane* 2). However, examination of the phospholipid fraction from mutants C1-1 and F2-1 (Fig. 3*A*, *lanes* 3 and *4*) showed the presence of the L-Ara4N donor lipid. Furthermore, mutants C1-1 and F2-1 contained functional ArnT. Membranes isolated from either C1-1 or F2-1 catalyzed the transfer of L-Ara4N from the donor substrate to [4′-^{32}P]lipid IV$_A$ (Fig. 3*B*, *lanes 4* and *5*), a tetra-acylated *bis*-phosphorylated precursor of lipid A. From this data we were able to conclude that a decrease in polymyxin resistance in strains C1-1 and F2-1 did not arise from the inability to produce or transfer L-Ara4N to lipid A.

Analysis of Lipid A Produced by Polymyxin-sensitive Mutants C1-1 and F2-1 by MALDI-TOF Mass Spectrometry—The lipid A species of mutants C1-1 or F2-1 were isolated as previously described and separated based upon charge using anion-exchange chromatography (16, 22). Mass spectrometry of the lipid A species containing unmodified phosphate groups re-

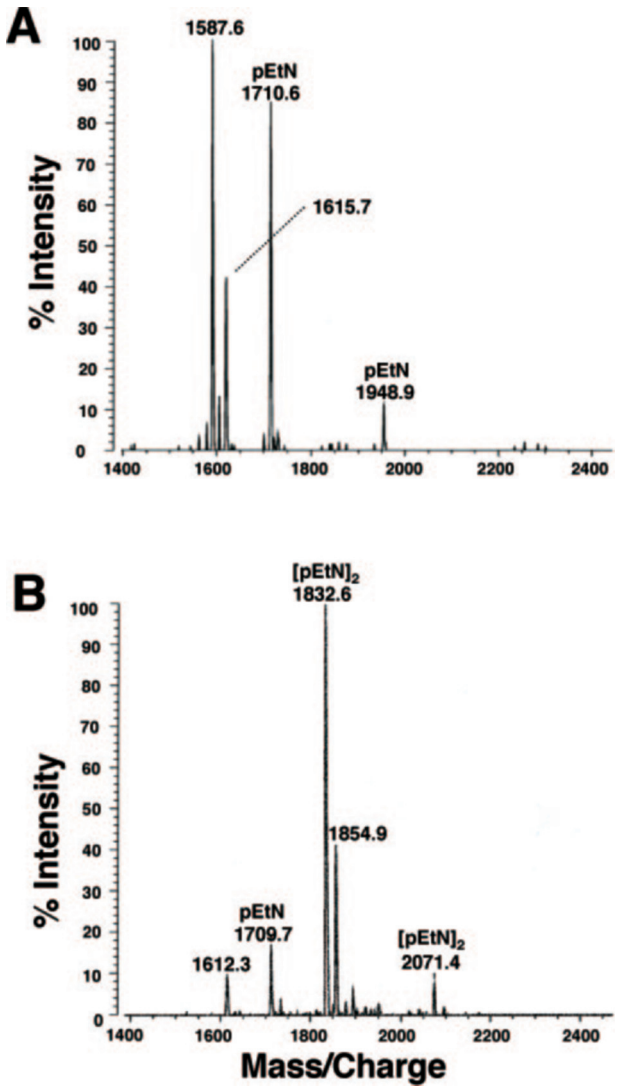

FIG. 4. **Negative ion MALDI-TOF mass spectrometry of lipid A from *E. coli* polmyxin-sensitive derivative C1-1.** Lipid A of *E. coli* strain C1-1 was isolated and fractionated by DEAE-cellulose chromatography using published protocols (16). Mass spectrometry data were acquired in the negative ion mode for all lipids eluting from the DEAE column. However, only the most representative data are shown. Data shown in *panels A* and *B* are from lipids eluting in the 120 and 60 mM salt fractions, respectively. Identical data were seen for the mutant F2-1.

vealed the presence of a penta-acylated *bis*-phosphorylated species as indicated by $[M\text{-}H]^-$ at *m/z* 1587.6 in the negative mode. The major ion peak at 1587.6 (Fig. 4*A*) corresponds to the loss of myristate (C_{14}) from *E. coli* lipid A that is found in *E. coli* mutants lacking a functional myristoyltransferase, LpxM (MsbB) (24, 25). The majority of the penta-acylated lipid was modified with either one or two pEtN groups as indicated by major ion peaks at *m/z* 1710.6 (Fig. 4*A*) and 1832.6 (Fig. 4*B*), respectively. Interestingly, mutants C1-1 or F2-1 were unable to produce lipid A modified with L-Ara4N even though both mutants contain the necessary enzymatic machinery required for transfer of L-Ara4N to lipid A (see Fig. 3). The only additional modification present was addition of palmitate (C_{16}) to species containing either one or two pEtN groups producing minor ion peaks at *m/z* 1948.9 and 2071.4, respectively (Fig. 4). Addition of the palmitoyl group is catalyzed by the outer membrane enzyme, PagP (26, 27). This data were suggestive that the increased polymyxin sensitivity seen displayed by mutants C1-1 or F2-1 resulted from a loss of L-Ara4N addition. Second, these results suggest that myristoylation of *E. coli* lipid A *in vivo* is a requirement for L-Ara4N addition.

Generation and Characterization of a pmrA^C, lpxM::Ωcam E. coli Mutant—To determine whether loss of L-Ara4N modification in mutants C1-1 and F2-1 arose from a loss of addition of myristate to lipid A, we constructed the *E. coli* strain WD103. A $P1_{vir}$ bacteriophage lysate of polymyxin-resistant WD101 was used to transduce the *pmrA^C* mutation into an existing *E. coli lpxM* (*msbB*) mutant, MLK1067 (13, 14). The resulting strain was referred to as WD103. Cultures of the various mutants and their corresponding parent strains were labeled with $^{32}P_i$, and the lipid A fraction isolated to determine possible modifications *in vivo*. As expected, the polymyxin-sensitive parent strains MLK1067 (Fig. 5, *lane 3*) and W3110 (Fig. 5, *lane 1*) produced unmodified penta- or hexa-acylated lipid A, respectively. The lipid A species shown in *lane* 5 isolated from WD103 (*pmrA^C*, *lpxM*::Ωcam) were found to migrate with identical R_F values of those isolated from mutants C1-1 (Fig. 5, *lane* 4) or F2-1 (data not shown).

Complementation of strain WD103 with a low-copy vector expressing *E. coli lpxM*, pWSLpxM, resulted in production of lipid A identical to that found in the polymyxin-resistant parent strain WD101 (Fig, 5, *lane* 7). This was not the case when WD103 was complemented with the empty vector control, pWSK29 (*lane* 6). Analysis of the lipid A fraction of *E. coli* WD103/pWSK29 by MALDI-TOF mass spectrometry produced results identical to those shown in Fig. 4 for the polymyxin-sensitive mutant C1-1 (data not shown). WD103/pWSK29 produced pEtN-modified lipid A lacking both myristate and L-Ara4N. However, the lipid A of WD103/pWSLpxM produced major ions at *m/z* 1929.7, 2052.7, and 2290.7, corresponding to hexa-acylated lipid A modified with L-Ara4N (Fig. 5*B*). Table II provides a summary of the lipid A species of strains C1-1 and WD103/pWSLpxM identified by mass spectrometry.

Myristoylation of S. typhimurium Lipid A Enhances Addition of L-Ara4N—Because myristoylation of LPS appears to determine the extent of L-Ara4N addition to the lipid A domain of *E. coli* K-12, we investigated whether or not the same was true for *S. typhimurium* LPS. Using an existing *lpxM* mutant of *S. typhimurium* strain C5 (28), we determined the extent of aminoarabinose addition in bacteria containing a vector control (pR1A) or an *lpxM* covering plasmid (pRM17A). *Salmonella* were cultured in N-minimal media under Mg^{2+}-limiting conditions to activate the PhoP/PhoQ two-component regulatory system. Under these conditions, *Salmonella* PhoP has been shown to induce activation of PmrA (5, 8, 29), leading to increased substitution of lipid A phosphate groups with L-Ara4N and pEtN (5, 23).

Previously, Khan and co-workers (28) demonstrated that the *S. typhimurium* C5 *lpxM* mutant produced an LPS showing an approximate 10-fold decrease in the amount of myristate (C_{14}). However, the extent to which phosphate groups were modified with L-Ara4N was not examined. As expected, analysis of intact lipid A from strain C5 *lpxM* (data not shown), or C5 *lpxM* containing the empty vector pR1A by MALDI-TOF mass spectrometry showed a major peak at *m/z* 1588.3 corresponding to penta-acylated lipid A (Fig. 6*B*). The *Salmonella lpxM* mutant does produce a hexa-acylated lipid A with a major ion peak at *m/z* 1826.5 (or 1826.1) atomic mass units (Fig. 6, *A* and *B*). However, the signal at 1826.5 (or 1826.1) corresponds to a lipid A species containing palmitate (C_{16}) rather than myristate (C_{14}). The *lpxM* mutant also produced pEtN-modified lipid A with mass spectrometry showing two ion peaks at *m/z* 1949.8 and 1977.9 (Fig. 6*B*) (see Table II). Addition of L-Ara4N to the lipid A of *E. coli*

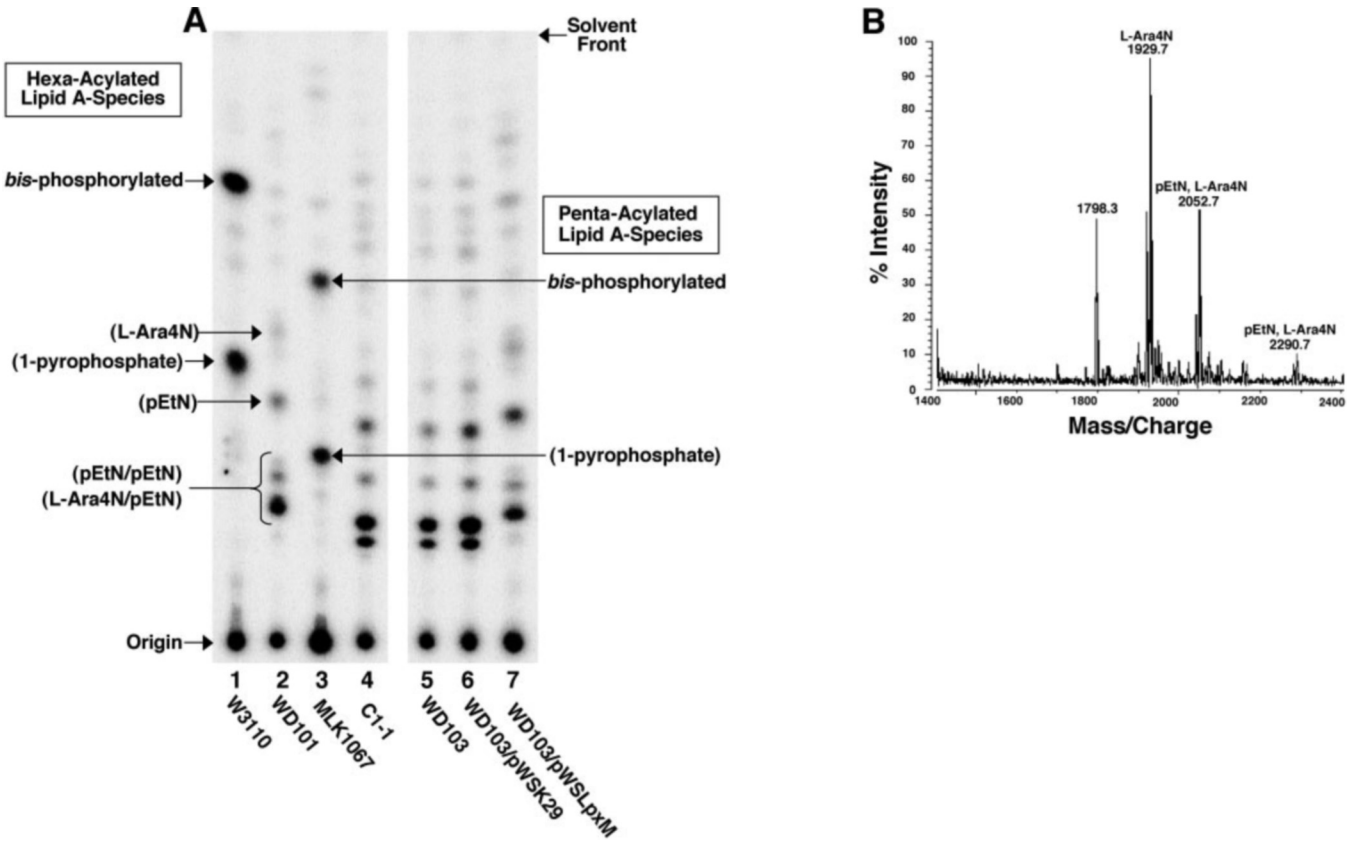

FIG. 5. **Analysis of lipid A species of *E. coli* WD103 (*pmrA*^{C}, *lpxM*::Ω*cam*) and WD103/pWSLpxM.** *Panel A*, ^{32}P-labeled lipid A species from the indicated strains were analyzed by TLC. The proposed modifications are indicated *in parentheses* for either hexa- or penta-acylated lipid A. Strains W3110 and MLK1067 are both polymyxin-sensitive producing either the hexa- or penta-acylated lipid A species, respectively (24). *Panel B*, lipid A of *E. coli* strain WD103 containing an *lpxM* covering plasmid (pWS*lpxM*) was processed as described in the legend of Fig. 4 and subjected to mass spectrometry. The data shown are from lipids eluting in the 120 mM fraction. Lipid A isolated from *E. coli* WD103 containing the empty vector control (pWSK29) produced mass spectrometry results identical to that of mutant C1-1 shown in Fig. 4 (data not shown).

TABLE II
Structural interpretation of lipid A species detected by MALDI/TOF mass spectrometry

Strain	*Lipid A modifications*					*Expected [M-H]*$^-$	*Observed [M-H]*$^-$
	L-Ara4N	pEtN	C16–0	C14–0	2-OH		
E. coli C1-1[a]	0	0	0	0	0	1588.0	1587.6
	0	1	0	0	0	1711.1	1709.7/1710.6
	0	2	0	0	0	1834.1	1832.6
	0	1	1	0	0	1949.5	1948.9
	0	2	1	0	0	2072.5	2071.4
E. coli WD103/pLpxM	0	0	0	1	0	1798.4	1798.3
	1	0	0	1	0	1929.5	1929.7
	1	1	0	1	0	2052.5	2052.7
	1	1	1	1	0	2290.9	2290.7
S. typhimurium lpxM/pR1A	0	0	0	0	0	1588.0	1588.3
	1	0	0	0	0	1719.1	1718.5
	0	0	0	1	0	1826.4	1826.5
	0	1	0	1	0	1949.5	1949.8
S. typhimurium lpxM/pRM17A	0	0	1	0	0	1798.4	1797.7
	0	0	1	0	1	1814.4	1813.8
	1	0	1	0	0	1929.5	1928.2
	1	0	1	0	1	1945.5	1944.5/1945.5
	1	1	1	0	0	2052.5	2052.3/2051.6
	1	1	1	0	1	2068.5	2068.2
	2	0	1	0	1	2076.6	2074.3
	0	1	1	1	0	2159.8	2159.4
	1	0	1	1	0	2167.9	2167.8
	0	1	1	1	1	2175.8	2176.2
	1	0	1	1	1	2183.9	2183.7/2182.0
	1	1	1	1	1	2306.9	2305.8/2306.3

[a] Similar data were obtained for *E. coli* strains F2-1 and WD103.

strain WD103 (*pmrA*^{C}, *lpxM*::Ω*cam*) was not detectable by mass spectrometry. However, a very minor peak at *m/z* 1718.5 (Fig. 6*A*) corresponding to the addition of a single L-Ara4N sugar to penta-acylated lipid A of the *Salmonella* mutant was present.

Complementation of the *Salmonella* mutant with the *lpxM*

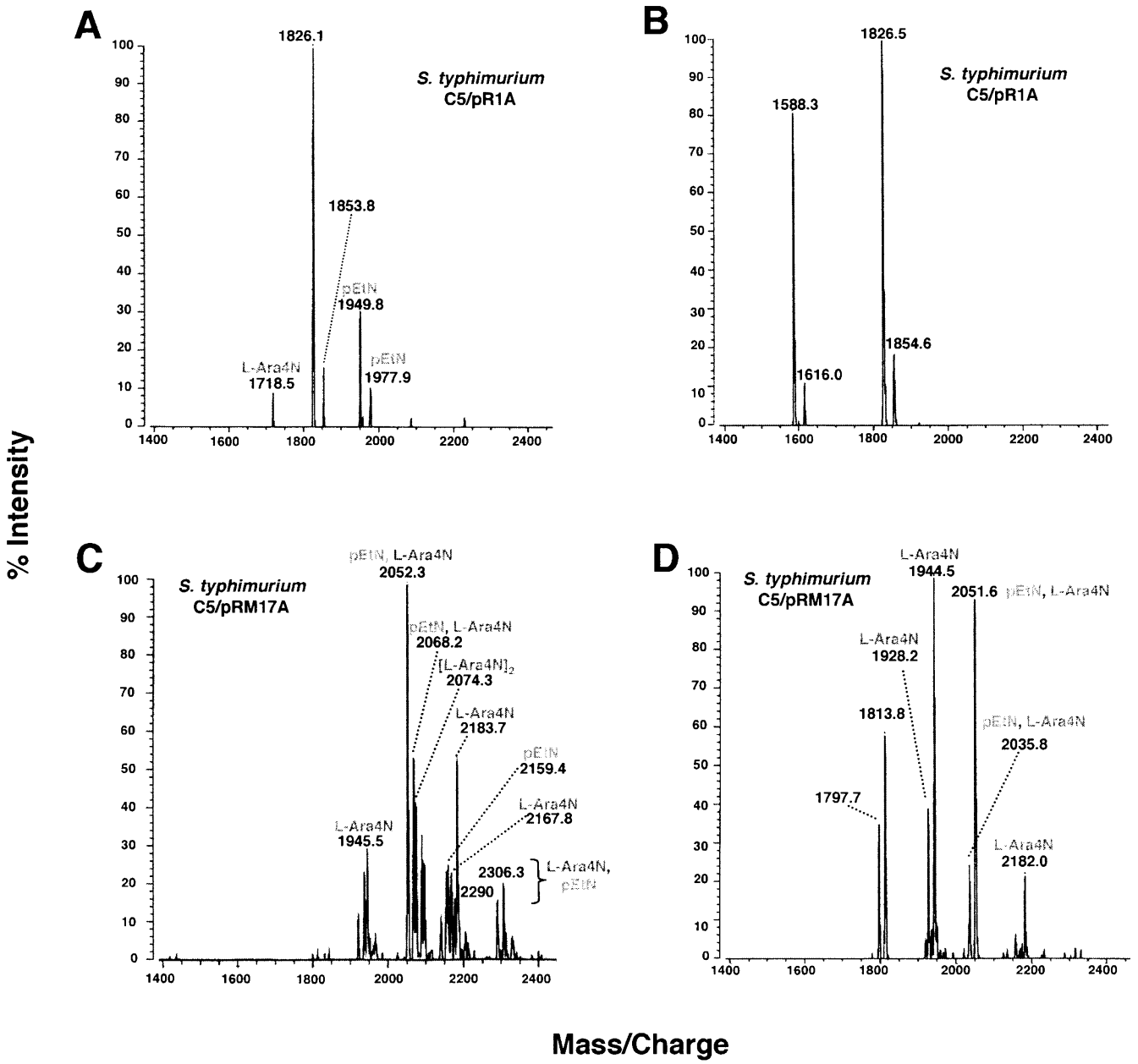

FIG. 6. **Negative ion MALDI-TOF mass spectrometry of lipid A from *S. typhimurium* C5/pR1A (*panels A* and *B*) or C5/pRM17A (*panels C* and *D*).** Lipid A of *S. typhimurium* strain C5 grown under Mg^{2+}-limiting (10 μM) conditions was isolated and fractionated by DEAE-cellulose chromatography as previously described (16). *Panels A* and *B* show spectra acquired for the lipid A isolated from *S. typhimurium C5* containing the low-copy vector, pR1A. *Panels C* and *D* show spectra acquired for the lipid A from *S. typhimurium* C5 containing the *lpxM* covering plasmid, pRM17A. Peaks containing modifications to the phosphate head groups of lipid A are indicated. Mass spectrometry data were acquired in the negative ion mode for all lipids eluting from the DEAE column, but only the most representative data are shown. *Panel A*, 120 mM fraction; *panel B*, 240 mM fraction; *panel C*, 60 mM fraction; *panel D*, 120 mM fraction.

covering plasmid resulted in the production of lipid A species typically seen in *Salmonella* grown under Mg^{2+}-limiting conditions (27, 30). The amount of lipid A containing L-Ara4N-modified species was drastically increased as compared with the *Salmonella lpxM* mutant with major peaks at *m/z* 1945.4 (1944.5) and 2052.3 (2051.6) (Fig. 6, *C* and *D*). Additionally, a species modified with two L-Ara4N sugars was present as indicated by the peak at 2074.3 *m/z* (see Table II). The mass spectrometry data of lipid A isolated from the recovered mutant was further complicated by the appearance of the hydroxylated lipid A species characterized by the addition of 16 mass units. This arises from the action of LpxO, a lipid A hydroxylase required for the addition of 2-OH myristate to *S. typhimurium* lipid A (see Table II).

Effect of Lipid Myristoylation on the Polymyxin Resistance of E. coli and S. typhimurium Strains—E. coli WD101 harbors a mutation in *pmrA* giving rise to a $pmrA^C$ phenotype resulting in increased resistance to polymyxin. As judged by disc diffusion assays, the polymyxin-resistant phenotype is significantly reduced upon loss of a functional copy of *lpxM* (see Table III). A similar result was found when comparing the survivability of *E. coli* after treatment with increasing concentrations of polymyxin in Luria broth. As shown in Fig. 7, *E. coli* strain WD103 showed a marked increase in polymyxin sensitivity compared with its $pmrA^C$ parent strain, WD101. Complementation of WD103 with the vector pWSLpxM resulted in restoration of polymyxin resistance to the levels displayed by WD101 (Table III and Fig. 7). *Salmonella lpxM* mutants grown under Mg^{2+}-

TABLE III
Increased polymyxin sensitivity of E. coli harboring an lpxM mutation

Strain[a]	Polymyxin (zone of clearing diameter (mm)[b]					
	0.2 μg	0.5 μg	1.0 μg	2.0 μg	10.0 μg	20.0 μg
W3110	9	10	12	13.5	16	19
WD101	≤ 6	≤ 6	≤ 6	≤ 6	9.5	12
MLK1067	8	9	11	14	16.5	20
WD103	≤ 6	7	8	8.5	12	15.0
WD103/pWSK29	≤ 6	≤ 6	8	9	13	15.0
WD103/pWSlpxM	≤ 6	≤ 6	≤ 6	≤ 6	10	12

[a] Strains are described in Table I.
[b] A zone of inhibition of ≤6 mm corresponds to complete resistance and is equal to the diameter of the disc.

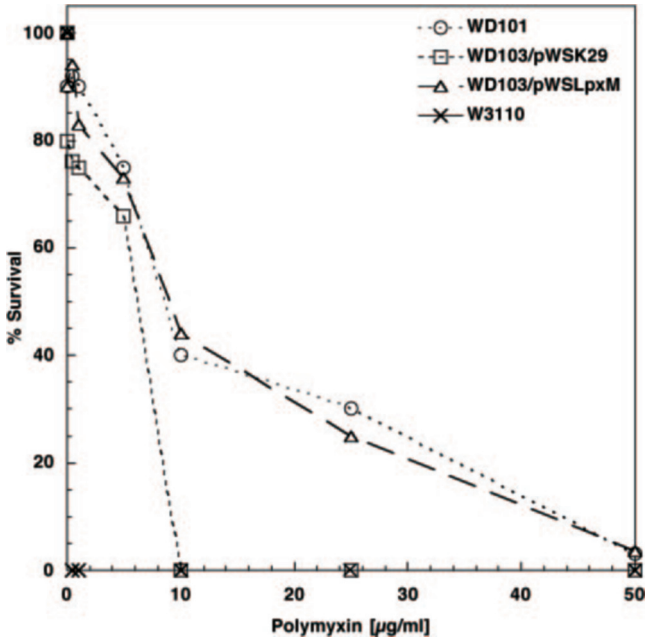

FIG. 7. **Effect of *lpxM* mutation on the polymyxin resistance of *E. coli*.** Strains of *E. coli* were treated with increasing concentrations of polymyxin and the survival percentages determined as described under "Experimental Procedures." Strains WD101 and W3110 (or MLK1067) served as polymyxin-resistant and -sensitive controls, respectively. Plasmid pWSlpxM containing *E. coli lpxM* was used to complement strain WD103. Symbols: ○, WD101; □, WD103 containing pWSK29 (vector control); Δ, WD103 containing pWSlpxM; ×, W3110.

limiting (10 μM) conditions also displayed increased sensitivity to polymyxin that could be restored by introduction of an *lpxM* covering plasmid (data not shown).

DISCUSSION

In *E. coli* and *S. typhimurium*, the synthesis of the lipid A domain of LPS is completed by the addition of myristate catalyzed by the so-called "late" acyltransferase, LpxM (1). Acyl-ACP serves as the acyl-donor for the acyltransferase (13), therefore restricting the reaction to the cytoplasmic side of the inner membrane. Lipid A attached to the core oligosaccharide is then transported across the inner membrane by the ABC transporter, MsbA (31, 32). Following transport to the periplasmic face, lipid A can be modified with various substituents including the cationic sugar L-Ara4N (12). The latter is transferred from undecaprenyl-phosphate-α-L-Ara4N (11) by the inner membrane enzyme ArnT to the phosphate groups of lipid A (10). Addition of L-Ara4N to the lipid A domain of Gram-negative bacterial LPS helps to promote resistance to cationic antimicrobial peptides, such as polymyxin (2, 23, 33, 34).

In an attempt to identify lipid A modifications necessary for polymyxin resistance, we isolated polymyxin-sensitive mutants of an *E. coli* K12 *pmrA*^{C} strain. A subset of mutants unable to decorate their lipid A domain with L-Ara4N also failed to incorporate myristate at the 3′-position. These results were verified by constructing an *E. coli* strain (WD103) lacking a functional copy of *lpxM*, but retaining the *pmrA*^{C} phenotype. This led to the possibility that *in vivo* modification of LPS with L-Ara4N requires LpxM-dependent myristoylation of the lipid A domain. However, we felt it necessary to determine whether the same were true for *S. typhimurium*.

Both *E. coli* and *S. typhimurium* synthesize a *bis*-phosphorylated hexa-acylated lipid A species with LpxM catalyzing the final step before transport across the cytoplasmic membrane. We isolated the lipid A fraction from wild type *S. typhimurium*, strain C5, containing an inactivated *lpxM* gene grown under conditions known to increase modification to the lipid A domain. When the *Salmonella lpxM* mutant was cultured under Mg^{2+}-limiting conditions, lipid A species modified with pEtN were detected by mass spectrometry. Only a very small fraction of the lipid A was modified with L-Ara4N when isolated from the *S. typhimurium lpxM* mutant (Fig. 6, *A* and *B*). However, upon introduction of the *Salmonella lpxM* gene using the covering plasmid, pRM17A, an impressive increase in L-Ara4N-modified lipid A species were detected by mass spectrometric analysis. Second, the bacteria produced lipid A species containing L-Ara4N attached to both phosphate groups of its lipid A (Fig. 6, *C* and *D*).

Typically, L-Ara4N is found attached via a phosphodiester linkage to the 4′-phosphate of *E. coli* and *S. typhimurium* lipid A (16, 35, 36). However, in wild type *S. typhimurium* a portion of the lipid A does contain two L-Ara4N moieties (Fig. 1) (35). We found that inactivation of *lpxM* in *E. coli* results in a total loss of L-Ara4N addition, whereas the *Salmonella lpxM* mutant produced a very minor amount of L-Ara4N-modified lipid A. Previously, Trent and co-workers (10) demonstrated that *in vitro* the L-Ara4N transferase of *Salmonella* transfers L-Ara4N to the 1-phosphate group when using the tetra-acylated lipid A precursor, lipid IV_A, as the substrate. However, if Kdo_2-lipid IV_A is used as the substrate in the assay system two sugars can be added to the molecule (10). Because Kdo-deficient *S. typhimurium* mutants accumulate species modified with L-Ara4N only at the 1-position (21, 37), it was proposed that modification of the 4′-phosphate group is dependent upon the presence of the Kdo dissacharide (10). Therefore, it is likely that the small amount of L-Ara4N present in the *Salmonella lpxM* mutant is linked to the 1-phosphate group.

Based upon our present findings, perhaps efficient transfer of L-Ara4N to the 4′-phosphate group of lipid A *in vivo* requires both the presence of the Kdo moiety and myristate. Because ArnT activity is partitioned in the periplasmic region of the cell, it is reasonable that the enzyme prefers a hexa-acylated lipid A domain for activity. A detailed study of the substrate preference of ArnT would be required to determine the validity of this argument. Other lipid A modifying enzymes have been shown to require specific structural changes to their substrate for activity. For example, Stead *et al.* (38) demonstrated that an *Helicobacter pylori* Kdo hydrolase requires dephosphorylation

at the 1-position by an *H. pylori* lipid A 1-phosphatase for activity. Also, the outer membrane 3-*O*-deacylase of *Salmonella*, PagL, appears to be activated in the absence of L-Ara4N modification (39). Therefore, modification of LPS within the Gram-negative membrane can also be regulated at the enzymatic level.

On the whole, we have presented an additional phenotype of *lpxM* mutants. *Salmonella* and *E. coli* lacking functional copies of *lpxM* are attenuated in their ability to activate human macrophages producing an LPS with reduced endotoxicity (28, 40). Also, *lpxM* mutants of either a clinical isolate of *E. coli* (41) or of *S. typhimurium* (28, 40) show a reduction in virulence in murine infection models. However, it should be noted that extragenic suppressors of *lpxM* mutants (42) might also influence the virulence phenotype. Given our current data, it is possible that the reduction in lethality shown by the *lpxM* mutants of these organisms results, in part, from their inability to modify their lipid A with L-Ara4N, because loss of the modification would result in increased sensitivity to cationic antimicrobial peptides.

REFERENCES

1. Raetz, C. R., and Whitfield, C. (2002) *Annu. Rev. Biochem.* **71,** 635–700
2. Helander, I. M., Kilpelainen, I., and Vaara, M. (1994) *Mol. Microbiol.* **11,** 481–487
3. Nummila, K., Kilpelainen, I., Zahringer, U., Vaara, M., and Helander, I. M. (1995) *Mol. Microbiol.* **16,** 271–278
4. Lee, H., Hsu, F. F., Turk, J., and Groisman, E. A. (2004) *J. Bacteriol.* **186,** 4124–4133
5. Gunn, J. S., Lim, K. B., Krueger, J., Kim, K., Guo, L., Hackett, M., and Miller, S. I. (1998) *Mol. Microbiol.* **27,** 1171–1182
6. Vaara, M. (1992) *Microbiol. Rev.* **56,** 395–411
7. Wosten, M. M., Kox, L. F., Chamnongpol, S., Soncini, F. C., and Groisman, E. A. (2000) *Cell* **103,** 113–125
8. Gunn, J. S., and Miller, S. I. (1996) *J. Bacteriol.* **178,** 6857–6864
9. Groisman, E. A. (2001) *J. Bacteriol.* **183,** 1835–1842
10. Trent, M. S., Ribeiro, A. A., Lin, S., Cotter, R. J., and Raetz, C. R. (2001) *J. Biol. Chem.* **276,** 43122–43131
11. Trent, M. S., Ribeiro, A. A., Doerrler, W. T., Lin, S., Cotter, R. J., and Raetz, C. R. (2001) *J. Biol. Chem.* **276,** 43132–43144
12. Doerrler, W. T., Gibbons, H. S., and Raetz, C. R. (2004) *J. Biol. Chem.*, 45102–45109
13. Clementz, T., Zhou, Z., and Raetz, C. R. (1997) *J. Biol. Chem.* **272,** 10353–10360
14. Karow, M., and Georgopoulos, C. (1992) *J. Bacteriol.* **174,** 702–710
15. Wang, R. F., and Kushner, S. R. (1991) *Gene* (*Amst.*) **100,** 195–199
16. Zhou, Z., Lin, S., Cotter, R. J., and Raetz, C. R. (1999) *J. Biol. Chem.* **274,** 18503–18514
17. Trent, M. S., Pabich, W., Raetz, C. R., and Miller, S. I. (2001) *J. Biol. Chem.* **276,** 9083–9092
18. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85
19. Garrett, T. A., Kadrmas, J. L., and Raetz, C. R. (1997) *J. Biol. Chem.* **272,** 21855–21864
20. Basu, S. S., York, J. D., and Raetz, C. R. (1999) *J. Biol. Chem.* **274,** 11139–11149
21. Raetz, C. R., Purcell, S., Meyer, M. V., Qureshi, N., and Takayama, K. (1985) *J. Biol. Chem.* **260,** 16080–16088
22. Odegaard, T. J., Kaltashov, I. A., Cotter, R. J., Steeghs, L., van der Ley, P., Khan, S., Maskell, D. J., and Raetz, C. R. (1997) *J. Biol. Chem.* **272,** 19688–19696
23. Gunn, J. S., Ryan, S. S., Van Velkinburgh, J. C., Ernst, R. K., and Miller, S. I. (2000) *Infect. Immun.* **68,** 6139–6146
24. Vorachek-Warren, M. K., Ramirez, S., Cotter, R. J., and Raetz, C. R. (2002) *J. Biol. Chem.* **277,** 14194–14205
25. Somerville, J. E., Jr., Cassiano, L., Bainbridge, B., Cunningham, M. D., and Darveau, R. P. (1996) *J. Clin. Investig.* **97,** 359–365
26. Bishop, R. E., Gibbons, H. S., Guina, T., Trent, M. S., Miller, S. I., and Raetz, C. R. (2000) *EMBO J.* **19,** 5071–5080
27. Guo, L., Lim, K. B., Poduje, C. M., Daniel, M., Gunn, J. S., Hackett, M., and Miller, S. I. (1998) *Cell* **95,** 189–198
28. Khan, S. A., Everest, P., Servos, S., Foxwell, N., Zahringer, U., Brade, H., Rietschel, E. T., Dougan, G., Charles, I. G., and Maskell, D. J. (1998) *Mol. Microbiol.* **29,** 571–579
29. Kox, L. F., Wosten, M. M., and Groisman, E. A. (2000) *EMBO J.* **19,** 1861–1872
30. Guo, L., Lim, K. B., Gunn, J. S., Bainbridge, B., Darveau, R. P., Hackett, M., and Miller, S. I. (1997) *Science* **276,** 250–253
31. Chang, G., and Roth, C. B. (2001) *Science* **293,** 1793–1800
32. Doerrler, W. T., Reedy, M. C., and Raetz, C. R. (2001) *J. Biol. Chem.* **276,** 11461–11464
33. Vaara, M., Vaara, T., Jensen, M., Helander, I., Nurminen, M., Rietschel, E. T., and Makela, P. H. (1981) *FEBS Lett.* **129,** 145–149
34. Baker, S. J., Gunn, J. S., and Morona, R. (1999) *Microbiology* **145,** 367–378
35. Zhou, Z., Ribeiro, A. A., Lin, S., Cotter, R. J., Miller, S. I., and Raetz, C. R. (2001) *J. Biol. Chem.* **276,** 43111–43121
36. Zhou, Z., Ribeiro, A. A., and Raetz, C. R. (2000) *J. Biol. Chem.* **275,** 13542–13551
37. Strain, S. M., Armitage, I. M., Anderson, L., Takayama, K., Qureshi, N., and Raetz, C. R. (1985) *J. Biol. Chem.* **260,** 16089–16098
38. Stead, C., Tran, A., Ferguson, D., Jr., McGrath, S., Cotter, R., and Trent, S. (2005) *J. Bacteriol.* **187,** 3374–3383
39. Kawasaki, K., Ernst, R. K., and Miller, S. I. (2005) *J. Bacteriol.* **187,** 2448–2457
40. Low, K. B., Ittensohn, M., Le, T., Platt, J., Sodi, S., Amoss, M., Ash, O., Carmichael, E., Chakraborty, A., Fischer, J., Lin, S. L., Luo, X., Miller, S. I., Zheng, L., King, I., Pawelek, J. M., and Bermudes, D. (1999) *Nat. Biotechnol.* **17,** 37–41
41. Somerville, J. E., Jr., Cassiano, L., and Darveau, R. P. (1999) *Infect. Immun.* **67,** 6583–6590
42. Murray, S. R., Bermudes, D., de Felipe, K. S., and Low, K. B. (2001) *J. Bacteriol.* **183,** 5554–5561
43. Raetz, C. R. H. (1996) in *Escherichia coli and Salmonella, Cellular and Molecular Biology* (Niedhardt, F., ed) pp. 1035–1063, American Society of Microbiology, Washington, D. C.