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Isolation and Chemical Characterization of Lipid A From the Outer Membrane of Gram-Negative Bacteria

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Abstract

SHORT ABSTRACT—Isolation and characterization of the lipid A domain of lipopolysaccharide (LPS) from Gram-negative bacteria provides insight into cell surface based mechanisms of antibiotic resistance, bacterial survival and fitness, and how chemically diverse lipid A molecular species differentially modulate host innate immune responses.

LONG ABSTRACT—Lipopolysaccharide (LPS) is the major cell surface molecule of Gram-negative bacteria, deposited on the outer leaflet of the outer membrane bilayer. LPS can be subdivided into three components: the distal O-polysaccharide, a core oligosaccharide, and the lipid A hydrophobic anchor, where lipid A is the only component essential for bacterial cell survival. Following its synthesis, lipid A is chemically modified in response to environmental stresses such as pH or temperature, to promote resistance to antibiotic compounds, and to evade recognition by mediators of the host innate immune response. The following protocol details the small- and large-scale isolation of lipid A from Gram-negative bacteria. Isolated material is then chemically characterized by thin layer chromatography (TLC) or mass-spectrometry (MS). In additional to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS we also describe tandem MS protocols for analyzing lipid A molecular species using electrospray ionization (ESI) coupled to collision induced dissociation (CID) and newly employed ultraviolet photodissociation (UVPD) methods. Our MS protocols allow for unequivocal determination of chemical structure, paramount to characterization of lipid A molecules that contain unique or novel chemical modifications. We also describe the radioisotopic labeling, and subsequent isolation, of lipid A from bacterial cells for analysis by TLC. Relative to MS-based protocols, TLC provides a more economical and rapid characterization method, but cannot be used to unambiguously assign lipid A chemical structures without the use of standards of known chemical structure. Over the last two decades isolation and characterization of lipid A has led to numerous

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DISCLOSURES

No conflicts of interest declared.

exciting discoveries that have improved our understanding of the physiology of Gram-negative bacteria, mechanisms of antibiotic resistance, the human innate immune response and have provided many new targets in the development of antibacterial compounds.

Keywords

lipid A; Bligh-Dyer; thin layer chromatography (TLC); lipopolysaccharide; mass spectrometry; Collision Induced Dissociation (CID); Photodissociation (PD)

INTRODUCTION

Lipopolysaccharide (LPS) is the major outer surface molecule of nearly all gram-negative organisms and consists of three molecular domains: a distal O-antigen polysaccharide, a core oligosaccharide, and the membrane associated lipid A deposited on the outer leaflet of the outer membrane bilayer[1,2]. The host innate immune response is specifically targeted against recognition of lipid A, formerly known as endotoxin[3]. Nine constitutively expressed genes, conserved throughout gram-negative bacteria, are responsible for the production of the standard hexa-acylated *bis*-phosphorylated lipid A[1,2]. Most bacteria contain an additional set of genes, which vary in degree of phylogenetic conservation, that participate in further chemical modification of lipid A[4]. Dephosphorylation, removal of acyl chains, and the addition of chemical moieties such as amino sugars (e.g. aminoarabinose) and/or phosphoethanolamine are the most commonly observed activities. Many of the enzymes responsible for lipid A modification are directly activated by environmental signals, such as divalent cations, or their expression is regulated by two component response-regulator systems[4].

Recognition of lipid A by the host innate immune system is mediated by the Toll-like receptor 4/myeloid differentiation factor 2 (TLR4/MD2) co-receptor[3]. Hydrophobic forces between MD2 and the lipid A acyl chains, as well as between TLR4 and the 1 and 4′phosphate groups of lipid A promote the strong association of lipid A with TLR4/MD2[3,5]. Modifications that alter acylation state or the negative charge of lipid A impact TLR4/MD2 based lipid A recognition and downstream stimulation of the innate immune response activators NF-κB and mediators of inflammation such as TNFα and IL1-β[6,7]. Modifications that mask the negative charge of lipid A also prevent bactericidal cationic antimicrobial peptides from binding to gram-negative cell surfaces[4,8]. Many lipid A modifications are hypothesized to increase bacterial fitness under specific environmental conditions, such as inside the human host or in an ecological niche. For this reason many modification enzymes are attractive targets in the rational development of antimicrobial compounds. The chemical diversity of lipid A structures, with respect to organism and/or environment, and the biological implications of these diverse structures make the structural characterization of lipid A an important endeavor in the study of Gram-negative bacteria.

Isolation of lipid A from whole bacteria involves the extraction of LPS from the bacterial cell surface, a hydrolytic step to liberate lipid A, followed by a final purification procedure[9–11]. The most frequently cited LPS extraction procedure is the hot-phenol water extraction procedure, first introduced by Westphal and Jann[10]. After extraction whole LPS is

subjected to mild-acid hydrolysis, which chemically separates the first sugar of the carbohydrate portion of LPS, 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), and the distal glucosamine sugar of lipid A (Fig. 1). Numerous pitfalls exist for the hot-phenol water procedure including the use of a high hazard reagent, the need to degrade co-extracted nucleic acids and proteins, and several days are needed to complete the protocol[10].

Our lab has further developed the extraction and isolation of lipid A as first developed by Caroff and Raetz[12,13]. Compared to hot-phenol water procedures, the method presented here is more rapid and efficient and accommodates a wide range of culture volumes is from 5 ml to multiple liters. Moreover, unlike hot-phenol water extractions, our method does not select for rough- or smooth-types of LPS, providing optimal recovery of lipid A species. In our protocol, chemical lysis of whole bacterial cells is performed using a mixture of chloroform, methanol and water, where LPS can be pelleted by centrifugation. A combination of mild-acid hydrolysis and solvent extractions (Bligh-Dyer) are used to liberate lipid A from covalently attached polysaccharide. The method of Bligh and Dyer was first applied to the extraction of lipid species from a variety of animal and plant tissues[14], modified here to separate hydrolyzed polysaccharide from lipid A. In this final separation step, chloroform soluble lipids selectively partition into the lower organic phase. To further purify lipid A, reverse-phase or anionic exchange column chromatography can be used[12].

After isolation of lipid A species from whole cells, a number of analytical methods can be used to characterize the chemical structure of the isolated material such as NMR, TLC, and MS-based analysis. NMR allows for non-destructive structural elucidation, and provides structural detail related to glycosidic linkages, unequivocal assignment of acyl chain positions, and assignment of attachment sites for lipid A modifications like aminoarabinose or phosphoethanolamine[15–17]. NMR analysis of lipid A is not discussed within our protocol, but has been described adequately elsewhere[15,16]. For rapid analysis TLC based methods are frequently used, but fail to provide direct information regarding fine chemical structure. MS based protocols are the most frequently employed method to characterize lipid A structures[18,19]. Matrix associated laser desorption ionization (MALDI)-MS is often used to initially survey intact lipid A species. Singly charged ions are generated from analyte prepared according to our extraction procedures. As more fine structural analysis is required, MS/MS based methods prove more informative than MALDI-MS. Coupled to electrospray ionization (ESI) singly or multiply charged lipid A precursor ions are further fragmented by collision induced dissociation (CID) or ultraviolet photodissociation (UVPD), to generate structurally informative product ions[18,20,21]. Neutral loss products from lipid A precursor ions are also frequently generated during ESI-MS providing an additional layer of structural information.

Tandem mass spectrometry (MS/MS) has proven to be an indispensable and versatile method for the elucidation of lipid A structures. During MS/MS, ions are activated to yield a diagnostic fragmentation pattern that can be used to elucidate the structure of the precursor ion. The most widely available MS/MS method is CID. This method produces fragments ions via collisions of the selected precursor ion with an inert target gas, resulting in energy deposition that leads to dissociation. CID has proven a critical tool in the assignment of lipid A structure for a wide range of bacterial species[22–33].

Although CID is the most universally implemented MS/MS method, it generates a limited array of product ions. 193 nm UVPD is an alternative and complementary MS/MS method. This method uses a laser to irradiate ions, and the absorption of photons results in energization of the ions and subsequent dissociation. This higher energy MS/MS technique produces a more diverse array of product ions than CID and thus provides more informative fragmentation patterns. In particular, UVPD affords information about subtle changes in lipid A species based on cleavages at glycosidic, amine, acyl and C-C linked bonds[18,21,34].

PROTOCOL

All solutions should be prepared with ultrapure water and HPLC grade methanol and chloroform. Prepared solutions that contain methanol or chloroform should be prepared and used under a chemical fume hood. All solutions can be stored at room temperature. Solvents should be measured in a graduated glass cylinder and stored in glass solvent bottles with Teflon lined caps. For long-term storage chloroform-containing solvents should be stored in tinted amber glass bottles to avoid the production of phosgene, a highly reactive acid chloride. Teflon centrifuge tubes and rotary evaporator flasks should be rinsed with methanol and chloroform before use. Follow necessary federal, state and/or institutional waste disposal regulations when disposing of solvents and/or radioactive waste.

1) Large Scale Lipid A Extraction (50 ml to 1.5 L)

1.1) Inoculate 5 ml of media (Luria broth or other) from a single bacterial colony. Grow overnight at 37 °C, or at required T °C for growth. A diagram of the extraction procedure is shown in Fig. 2.

1.2) Prepare a single-phase Bligh-Dyer mixture: chloroform-methanol-1X phosphate buffered saline (PBS) pH 7.4; 1:2:0.8 v/v). Combine 200 ml of chloroform, 400 ml of methanol and 160 ml of PBS in 1 L solvent bottle. Cap bottle and mix by inversion (>30 times). Loosen the cap periodically during mixing to vent and store sealed.

1.3) Prepare a pre-equilibrated two-phase Bligh-Dyer mixture: chloroform-methanol-water (2:2:1.8 v/v). Combine 400 ml of chloroform, 400 ml of methanol and 180 ml of water in 1 L solvent bottle. Cap bottle and mix by inversion, making sure to loosen cap periodically during mixing to vent. Let equilibrate overnight and store sealed.

1.4) Prepare the mild acid hydrolysis buffer (50 mM sodium acetate pH 4.5, 1% sodium dodecyl sulfate (SDS)). For 500 ml of mild acid hydrolysis buffer, weigh 2.05 g of sodium acetate and transfer to a 500 ml beaker. Add water to a volume of ~ 350 ml and stir, then add 50 ml of 10% SDS. Mix and adjust pH to 4.5, transfer to a graduated cylinder, and add water to 500 ml.

1.5) Prepare chloroform-methanol (4:1, v/v): Measure 100 ml of chloroform and transfer to solvent bottle. Measure 25 ml of methanol and mix with chloroform. Store in amber glass bottle.

1.6) The next day, measure the OD_{600} and use the 5 ml overnight culture to inoculate 200 ml of culture to a starting OD_{600} of 0.05. Grow cells until an OD_{600} of 0.8–1.0 is reached.

1.7) Harvest cells via centrifugation at 10,000 × *g* for 10 minutes. Longer spins may be required for strains that pellet poorly. Pour off media supernatant.

1.8) Wash cell pellet with 50 ml of 1X phosphate buffered saline (PBS). Repeat centrifugation to pellet cells. Pour off supernatant and store cell pellet at −20 °C, or proceed to step 1.5.

1.9) Resuspend cells in 40 ml of 1X PBS and divide between two 250 ml Teflon centrifuge tubes (yielding 20 ml of cell suspension per tube). Add 25 ml of chloroform and 50 ml of methanol to each tube, for a single phase Bligh-Dyer (chloroform, methanol, water; 1:2:0.8 v/v) (Fig. 2). Mix by inversion and incubate at room temperature for >20 minutes to ensure complete cell lysis.

1.10) Centrifuge the mixture at 2,000 × *g* for 20 minutes. LPS will pellet along with proteins and nucleic acids (Fig. 2); however, phospholipids, isoprenyl lipids, and small, hydrophobic peptides will remain in the supernatant. Discard the supernatant.

1.11) Wash the LPS pellet with ~100 ml single phase Bligh-Dyer mixture. Centrifuge at 2000 × *g* for 20 minutes. Discard supernatant. When isolating lipid A from *E. coli* or *Salmonella*, only one wash is required; however, additional wash steps may be needed to reduce phospholipid contamination when isolating lipid A from some organisms.

1.12) Add 27 ml of mild acid hydrolysis buffer (50 mM sodium acetate pH 4.5, 1% SDS; see step 1.3) to LPS pellet, and mix by pipetting up and down until only small particles remain. Sonicate to homogeneously resuspend LPS pellet in solution with probe tip sonicator (e.g. Branson Sonifier 250) at a constant duty cycle for 20 seconds at 50 % output. Repeat sonication of sample 2 times (20 seconds per burst, ~5 seconds between bursts).

1.13) Boil samples in a water bath for 30 minutes. Caution: make sure the caps are tight, but not fully sealed. Some organisms, like *Vibrio cholerae* require longer incubation times (1 h) to increase overall yield of Lipid A. Remove bottles from water bath and allow sample to cool to room temperature before proceeding.

1.14) To extract lipids after hydrolysis, convert the SDS solution into a two-phase Bligh-Dyer (Fig. 2) mixture by adding 30 ml of chloroform and 30 ml of methanol, for a chloroform-methanol-water (2:2:1.8, v/v) mixture. Mix by inversion and centrifuge the sample for 10 minutes at 2,000 × *g*. Extract the lower phase into a clean Teflon centrifuge tube using a glass pipet.

1.15) Perform a second extraction by adding 30 ml of lower phase from a pre-equilibrated Bligh-Dyer mixture (step 1.3), to the upper phase from step 1.13. Mix, then centrifuge at 2,000 × *g* for 10 minutes. Extract the lower phase, and pool with the lower phase extracted in step 1.13.

1.16) Wash the pooled lower phases (60 ml total) by adding 114 ml of pre-equilibrated Bligh-Dyer upper phase (step 1.3) to create a two-phase Bligh-Dyer mixture (chloroform-methanol-water; 2:2:1.8, v/v). Mix. Centrifuge at 2,000 × *g* for 10 minutes.

1.17) Remove the lower phase to a clean glass rotary evaporator flask and dry sample using rotary evaporation.

1.18) Add 5 ml of chloroform-methanol (4:1, v/v) to the rotary flask, and bath-sonicate (>30 seconds) to aid in suspension of lipid from sides of flask. Use a glass transfer pipet to transfer lipid to a clean glass tube (13×100mm or larger) capped with PTFE lined phenolic screw caps. Dry sample under a stream of nitrogen using a nitrogen dryer.

1.19) Resuspend dried lipid in 1 ml of chloroform-methanol (4:1, v/v). Transfer to small glass sample vial (12×32mm). Dry using a nitrogen dryer.

1.20) Dried sample can be stored at −20 °C until subsequent TLC or MS analysis.

2) Visualization of Lipid A Species via Thin-Layer Chromatography

2.1) Prepare the TLC mobile phase solvent system (chloroform-pyridine-88% formic acid-water; 50:50:16:5 v/v). Combine 200 ml of chloroform, 200 ml of pyridine, 64 ml of 88% formic acid, and 20 of ml water in a 1 L solvent bottle. Cap bottle, mix by inversion multiple times, and vent.

2.2) Use a TLC tank that will accommodate 20 × 20 cm plates. Line TLC tank with ~40 cm chromatography paper (Whatman 3MM Chr, 23.0 cm × 100 m).

2.3) To TLC tank add 200 ml of chloroform-pyridine-88% formic acid-water (50:50:16:5, v/v) mixture. Allow tank to pre-equilibrate for > 3 hours, often overnight is preferred and more convenient.

2.4) Remove dried lipid samples from freezer (step 1.19) and let warm to room temperature.

2.5) With a razor remove the silica from the top edge of a Silica gel 60 TLC plate. Using a dull pencil, draw a line parallel to the bottom of the plate, 2 cm from the bottom. This line is the origin for spotting samples. Mark increments along this reference line to spot samples 1 cm apart.

2.6) Dissolve dried lipid from step 2.4 in 200 μl of chloroform-methanol (4:1, v/v), vortex and sonicate (3 times ~15 seconds each), yields ~100–500 ng/μl lipid A if extracted from *E. coli*.

2.7) Using a microcapillary glass pipet, spot one-tenth of the volume (25 μl) onto the TLC plate as marked in step 2.5. Allow samples to air-dry for ~15 minutes.

2.8) Place TLC plate containing spotted samples into the pre-equilibrated tank. Once solvent front reaches the top of the TLC plate (~2.5–3h), remove the plate and air dry (>30 minutes).

2.9) While plate is drying, turn on hot plate at 250 °C for charring.

2.10) Prepare 10% sulfuric acid-ethanol mixture for charring lipids resolved on the silica TLC plate (concentrated sulfuric acid:100% ethanol; 1:9 v/v). Measure 10 ml of sulfuric acid and add slowly to 90 ml of 100% ethanol. Carefully mix and transfer to a glass chromatographic reagent atomizer.

2.11) In the fume hood, use a glass chromatographic reagent atomizer to spray the dried TLC plate with 10% sulfuric acid-ethanol mixture. Spray mixture evenly across the plate.

2.12) Place the TLC plate on the 250 °C hot plate until charred lipid samples appear as black/brown spots (<1 minute). Do not overexpose the plate, as this will cause the entire plate to turn brown and make visualization of lipid A species difficult.

3) Structural Characterization of Lipid A via MALDI-TOF Mass Spectrometry

3.1) From step 1.19 remove lipid A sample from freezer and let warm to room temperature. Resuspend dried lipid A sample in ~20 μl chloroform-methanol (4:1, v/v) and vortex to obtain a ~1–5 μg/μl lipid A solution if extracted from *E. coli*.

3.2) Prepare ATT matrix components saturated 6-aza-2-thiothymine in 50% acetonitrile and saturated ammonium citrate dibasic: add 500 μl of water and 500 μl of acetonitrile to a 1.5 ml microcentrifuge tube, and then add 6-aza-2-thiothymine so that the 50% acetonitrile is super-saturated. Prepare saturated ammonium citrate dibasic solution by adding >1 mg to 500 μl water, precipitate should be readily apparent. Vortex and centrifuge solutions before use; only saturated supernatant is used.

3.3) Prepare ATT matrix: saturated 6-aza-2-thiothymine in 50% acetonitrile, saturated ammonium citrate dibasic (20:1, v/v). Mix matrix components together by adding 20 μl of ATT to 1 μl ammonium citrate in 500 μl microcentrifuge tube, vortex to mix, and centrifuge before applying to MALDI plate.

3.4) Prepare MALDI plate by adding 0.5 μl calibrant mixture to the MALDI plate on a spot near where the samples will be deposited, to provide the most accurate mass/charge ratio determination. We use calibration mix 1 from sequazyme.

3.5) Deposit 0.5 μl of ATT matrix onto MALDI plate on each spot that a lipid A sample will be deposited.

3.6) Deposit 0.5 μl of sample onto the spot of ATT matrix, to mix on plate, and acquire spectra by scanning sample for optimal ion signals (Fig. 3). We use a MALDI-TOF/TOF ABI 4700 Proteomics Analyzer equipped with a Nd:YAG laser (355 nm) using a 200-Hz firing rate, and collect data averaged from >200 shots.

4) Electrospray Ionization Mass Spectrometry of Lipid A and Collision Induced Dissociation

4.1) Prepare a chloroform-methanol solvent mixture (1:1, v/v) by mixing a 200 µl stock of HPLC grade methanol with 200 ml HPLC grade chloroform in a glass solvent bottle.

4.2) Transfer 200 µl of the chloroform-methanol solvent mixture to the vial with lipid A and sonicate the lipid a solution for 5 minutes or until all material is dissolved.

4.3) Set up the mass spectrometer for negative mode electrospray ionization. We use an Orbitrap Elite mass spectrometer and a HESI-II electrospray ionization source, both from Thermo Fisher Scientific.

4.4) Using a 250 µl syringe and a syringe pump, directly infuse the diluted lipid A sample at a flow rate of 2.0 – 3.5 µl/min.

4.5) Optimize and enhance the lipid A ion signal by tuning the ion optics. A full mass spectrum can now be collected of the lipid A species (Fig. 4).

4.6) Isolate and activate the target lipid A by selecting CID as the MS/MS method.

4.7) Increase the CID voltage (or normalized collision energy, NCE) until the precursor lipid A species is about 10% relative abundance compared to the highest product ion.

4.8) Acquire and average spectra until sufficient signal-to-noise is achieved for the product ions. The number of scans needed is dependent on the signal intensity of the original precursor and can range from 3 – 300 scans (Figure 5A).

5) MS/MS on Lipid A by Ultraviolet Photodissociation

5.1) Prepare sample and mass spectrometer as in steps 4.2 to 4.5.

5.2) Turn on the laser interfaced to the Orbitrap Elite mass spectrometer. The Thermo Scientific Orbitrap Elite (Thermo Fisher Scientific, Bremen, Germany) was outfitted with a 193 nm excimer laser and modified to allow UV activation in the HCD cell of the instrument. Photodissociation was implemented in a manner similar to that described previously[35]. We use a 193 nm Coherent ExciStar XS excimer laser, and a modified vacuum manifold with a CaF_2 optical window to transmit photons into the higher-energy C-trap dissociation (HCD) cell. The laser is triggered during MS/MS by a transistor-transistor logic (TTL) signal from mass spectrometer to a pulse/delay generator (Model 505, Berkeley Nucleonics Corporation).

5.3) Set up the instrument software so that the laser will trigger the excimer laser when the ions enter the HCD cell. This requires a modest modification of the software.

5.4) Turn on the pulse generator such that the laser is pulsed every 2 ms (500 Hz).

5.5) Isolate the lipid A precursor ion by selecting HCD as MS/MS method and adjust the collisional energy to 1% NCE. This allows the isolation of precursor ions for

ultraviolet dissociation within the HCD cell. Although the HCD cell is used, the software is modified to perform UVPD during this interval.

5.6) Activate the isolated lipid A ion by increasing the laser energy and adjusting the number of laser pulses. A typical UVPD experiment will be performed using ten 6 ml pulses.

5.7) As in step 4.9, acquire and average spectra until sufficient signal-to-noise is achieved for the UVPD product ions. The number of scans needed is dependent on the signal intensity of the original precursor and can range from 3 – 300 scans (Fig. 5B).

6) ^{32}P-Labeling of Lipid A and Subsequent Isolation

6.1) Inoculate 5 ml of media (Luria broth or other media) from a single colony. Grow overnight at 37 °C or at required temperature.

6.2) The next day, measure the OD_{600} and use the overnight culture to inoculate 7 ml of culture to a starting OD_{600} of ~0.05. Add 2.5 μCi/ml of inorganic ^{32}P. Grow cells until an OD_{600} of 0.8–1.0 is reached.

6.3) Harvest cells in glass centrifuge tubes with Teflon lined cap using a fixed angle clinical centrifuge at 1,500 × *g* for 10 minutes. Remove supernatant into appropriate radioactive waste container.

6.4) Wash cell pellet with 5 ml of 1X phosphate buffered saline (PBS). Centrifuge for 10 minutes at 1,500 × *g*. Discard supernatant.

6.5) Resuspend cells in 5 ml of single phase Bligh-Dyer mixture (Fig. 2) consisting of chloroform-methanol-water (1:2:0.8, v/v). Vortex to mix, and incubate at room temperature for > 20 minutes to ensure complete cell lysis.

6.6) Centrifuge in clinical centrifuge at 1,500 × *g* for 20 minutes. Gently pour off supernatant, which contains phospholipids and isoprenyl lipids.

6.7) Resuspend the LPS pellet in 1.8 ml of 50 mM sodium acetate pH 4.5, 1% SDS buffer by vortexing. Sonicate sample using a bath sonicator until pellet is evenly dispersed (~ 30 seconds).

6.8) Incubate sample for 30 minutes in boiling water bath. Make sure caps are tight but not sealed.

6.9) Remove from water bath and allow sample to cool at room temperature for 5–10 minutes.

6.10) Convert the solution into a two-phase Bligh-Dyer mixture by adding 2 ml of chloroform and 2 ml of methanol, yielding a chloroform-methanol-aqueous (2:2:1.8 v/v) mixture. Vortex to mix, and centrifuge for 10 minutes in clinical centrifuge at 1,500 × *g* to separate phases.

6.11) Extract the lower phase into a clean glass centrifuge tube using a glass pipet (first extraction).

6.12) Perform a second extraction on the sample by adding 2 ml of pre-equilibrated Bligh-Dyer lower phase (prepared as in step 1.3) to the remaining upper phase from step 11. Vortex and centrifuge 10 minutes. Remove lower phase and combine with the lower phase from the first extraction.

6.13) To the pooled lower phase (~4 ml total volume), add 7.6 ml of pre-equilibrated upper phase (step 1.3), yielding a two-phase Bligh-Dyer solution (chloroform-methanol-water; 2:2:1.8, v/v). Vortex and centrifuge for 10 minutes.

6.14) Remove the lower phase to a clean glass tube and dry using a nitrogen dryer. Dried samples can be stored at −20 °C until further use.

7) Visualization of ^{32}P-labeled Lipid A Species via Thin-Layer Chromatography

7.1) Prepare ^{32}P-labeled lipid A sample, TLC tank, and TLC plates as described in steps 2.1–2.8.

7.2) Dissolve ^{32}P-labeled sample in 500 μl of 4:1 chloroform-methanol (v/v). Vortex and bath sonicate to completely dissolve lipid material. Add 5 μl of sample to scintillation vial containing 5 ml of scintillation cocktail. Count in scintillation counter and calculate total counts per minute of sample.

7.3) When visualizing radiolabeled lipid species, 10 × 20 cm or 20 × 20 cm TLC plates can be used. For 10 × 20 cm plates, samples should be spotted along the 10 cm edge of the plate to improve separation of lipid A species.

7.4) Using a microcapillary pipet, spot 10,000–20,000 cpm per sample on plate and allow spots to dry (> 15 min). Samples might need to be concentrated by drying under nitrogen and resuspended in an appropriate volume, to achieve 10,000–20,000 counts per minute per spot (2 μl or 2 μl at a time for <10 μl total).

7.5) Place TLC plate containing radiolabeled samples into the pre-equilibrated tank. Once solvent front reaches the top of the TLC plate (~3 hours), remove the plate and air dry (> 30 min).

7.6) Wrap the plate in plastic wrap and expose to PhosphorImager Screen overnight. The next morning, scan the screen to obtain the image (Fig. 6). Using image densitometry analysis software, this method allows for accurate, relative quantification of lipid A species.

REPRESENTATIVE RESULTS

Lipid A of *E. coli* and *Salmonella enterica* serovar Typhimurium is a hexa-acylated disaccharide of glucosamine with phosphate groups at the 1- and 4′-positions. During growth in rich media (e.g. Luria Broth) a portion of the lipid A contains a pyrophosphate group at the 1-position yielding a *tris*-phosphorylated species[36] (Figure 1). The first sugar of the core oligosaccharide, Kdo (3-deoxy-D-*manno*-octulosonic acid), is attached at the 6′-position and serves as a bridge to link lipid A to the remaining carbohydrate domains (i.e. core oligosaccharide and O-antigen domains) of LPS. Although Gram-negative bacteria share a conserved pathway for lipid A biosynthesis similar to that of *E. coli* K-12, there is a large amount of diversity in lipid A structures. This diversity arises from the action of latent

enzymes that modify the lipid A structure, which are activated in response to environmental stimuli. For example, in *S. enterica* the phosphate groups of lipid A can be modified with the cationic sugar L-4-aminoarabinose (Fig. 1; blue) or with a phosphoethanolamine residue (Fig. 1; magenta). In *Salmonella* these modifications are regulated by two-component response regulator systems, added in response to low [Mg^{2+}], mild-acid pH, and the presence of cationic antimicrobial peptides. Additionally in *Salmonella*, palmitate can be added to form a hepta-acylated lipid A, which promotes resistance to cationic antimicrobial peptides (Fig. 1; green). Other modifications include, but are not limited to, removal of phosphate groups and acyl chains, or dioxygenase catalyzed addition of a hydroxyl group to the 3′-linked secondary acyl chain observed in a number of organisms[1,4].

Isolation of intact lipid A from whole cells by our modification of the method of Caroff and Raetz is described in protocol 1 (Fig. 2). This isolation method has been used to estimate that 10^6 molecules of lipid A exist per bacterial cell of *E. coli*[37]. Following protocols 1 and 3, the negative ion MALDI-TOF mass spectrum of lipid A from *E. coli* K-12 (W3110) yields the singly deprotonated ion ($[M-H]^-$) at *m/z* 1795.2 as the major observed species (Fig. 3). MALDI-TOF MS was performed in negative-reflectron mode to improve spectral resolution. Alternatively, the same lipid sample subjected to negative mode nano-ESI (Protocol 4) yields predominately doubly deprotonated lipid A ions at *m/z* 898.1 denoted by $[M-2H]^{2-}$ (Fig. 4). Singly deprotonated lipid A ions $[M-H]^-$ at *m/z* 1796.20 are observable, but of lower relative abundance to $[M-2H]^{2-}$ ions (Fig. 4). Multiply charged species predominate when using ESI. Fragmentation by CID (Fig. 5A) or 193 nm UVPD (Fig. 5B) was performed on the $[M-H]^-$ lipid A ion *m/z* 1796.20. These techniques can be used to better assign chemical structures, particularly of lipid A species containing complex combinations of modifications, or previously uncharacterized chemical modifications. Fragmentation profiles are shown with dashed lines representing cleavage sites and are matched with the *m/z* values below each provided structure (Fig. 5). The *m/z* values and cleavage sites highlighted in red font represent unique product ions associated with UVPD.

As described in protocols 6 and 7, ^{32}P-labeled lipid A isolated from two different *E. coli* K-12 strains was analyzed by TLC (Fig. 6). W3110 contains mostly *bis*- and *tris*-phosphorylated lipid A (Fig. 6; left lane), whereas a more complex TLC pattern is observed with ^{32}P-lipid A isolated from *E. coli* strain WD101 (Fig. 6; right lane). WD101 produces lipid A heavily modified with aminoarabinose (L-Ara4N) and phosphoethanolamine (pEtN). Since both 1- and 4′-phosphates are available for modification, lipid A from WD101 can be described as singly modified, containing only one L-Ara4N or pEtN at either phosphate, or doubly modified where both phosphates are modified in a combinatorial manner. In addition to modification at the 1- and 4′- phosphates, palmitate addition is also observed (see Fig. 1) and increases the R_f value of lipid A species in this solvent system (Fig. 6). If desired, densitometry analysis using a phosphorimager, can be used to quantifiably estimate relative amounts of lipid A species within the same sample.

DISCUSSION

Culture volumes for lipid A extraction and isolation can be adjusted depending on the bacterial strain you are working with. For instance, *Vibrio cholerae* requires at least 200 ml

of culture in order to obtain high quality mass spectra; however, the culture volume for *Escherichia coli* can be scaled down to 5 ml. More starting material is required for some bacterial species because the hydrolysis step that releases lipid A from whole LPS is less efficient. For example organisms containing a functional Kdo dioxygenase or Kdo kinase exhibit decreased lipid A yields after mild-acid hydrolysis[38]. Often, mutants with altered LPS/lipid A structures or a particular bacterial species are often difficult to pellet during cell harvest. For these strains, the length of centrifugation can be extended to increase yield. Also of note, after cell lysis in a single-phase Bligh-Dyer mixture and LPS is pelleted (see step 1.9), additional wash steps may be required to reduce phospholipid contamination. When isolating lipid A from *E. coli* or *Salmonella*, only one wash is required. However, if isolating lipid A from other organisms (e.g., *V. cholerae* or *Helicobacter pylori*), additional wash steps are required.

After mild acid hydrolysis in SDS, the yield of lipid A species with reduced hydrophobicity (i.e. fewer phosphate groups < 2 or fewer acyl chains < 5) can be improved by using an acidic Bligh-Dyer extraction. For the volumes used in Protocol section 1, add 225 μl of concentrated HCl to the SDS solution containing hydrolyzed lipid A followed by 30 ml of chloroform and 30 ml of methanol for a two phase Bligh-Dyer mixture of chloroform, methanol, 0.1M HCl (2:2:1.8, v/v). For the volumes used in Protocol section 6 add 15 μl of concentrated HCl to the SDS solution containing hydrolyzed lipid A followed by 2 ml of chloroform and 2 ml of methanol yielding a chloroform, methanol, 0.1M HCl (2:2:1.8, v/v) mixture. After an acidic Bligh-Dyer extraction, pyridine can be added to the pooled lower phases to neutralize the acid (1 drop of pyridine per 2 ml of final sample volume). This additional step should be performed before drying the sample, in a chemical fume hood. Be careful not to use excess pyridine, which can lead to the removal of ester-linked fatty acids. Additionally, if the lipid sample is difficult to dry under nitrogen, add a few milliliters of chloroform-methanol (4:1, v/v) to the flask and continue to dry the sample to completion.

The complex chemical heterogeneity of lipid A species observed in some organisms (e.g. *Vibrio cholerae, Yersinia pseudotuberculosis*) can sometimes make TLC- or MS-based analysis difficult. Column chromatography can be employed upstream of these analytical techniques to pre-fractionate isolated lipid A species into more simple mixtures. Anion exchange with diethylaminoethyl (DEAE)-cellulose is most commonly used[39]. As a general guideline lipid A modified at either phosphate position, with non acidic groups such as aminoarabinose or phosphoetanolamine, elutes before unmodified lipid A species[39]. Similarly tris-phosphorylated lipid A elutes well after unmodified bisphosphorylated species[36,39]. Reverse phase chromatography can be used to fractionate lipid A species of varying degrees of hydrophobicity. Column chromatography is also useful in the removal of residual SDS after mild acid hydrolysis and subsequent Bligh-Dyer lipid A extraction steps. High levels of residual SDS can contribute to signal suppression in spectra obtained by our sensitive ESI-MS protocols. Tandem mass spectrometry is a powerful strategy for *de novo* structural characterization of biological compounds. CID and UVPD are two complementary activation methods that create different types of product ions that provide key fingerprints for lipid A molecules. MS/MS fragmentation using both CID and UVPD allows elucidation

of subtle differences of lipid A structures and supports the correlation of structure and function.

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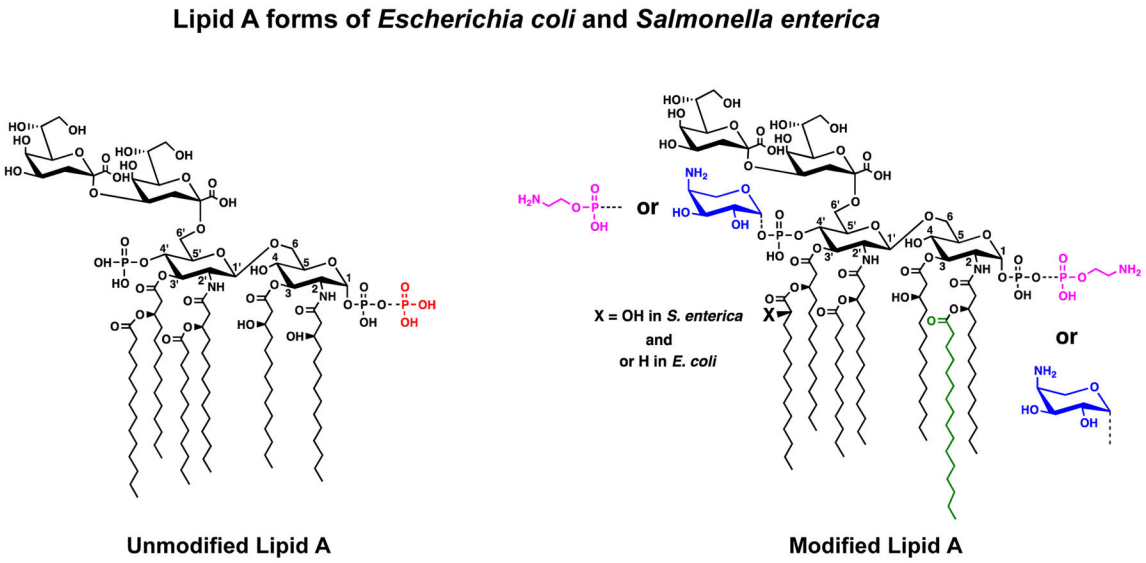

Figure 1.

Representative lipid A structures from *E. coli* K-12 and *S. enterica* serovar *typhimurium*. Modifications to the conserved lipid A structure are shown (right), as described in Representative Results.

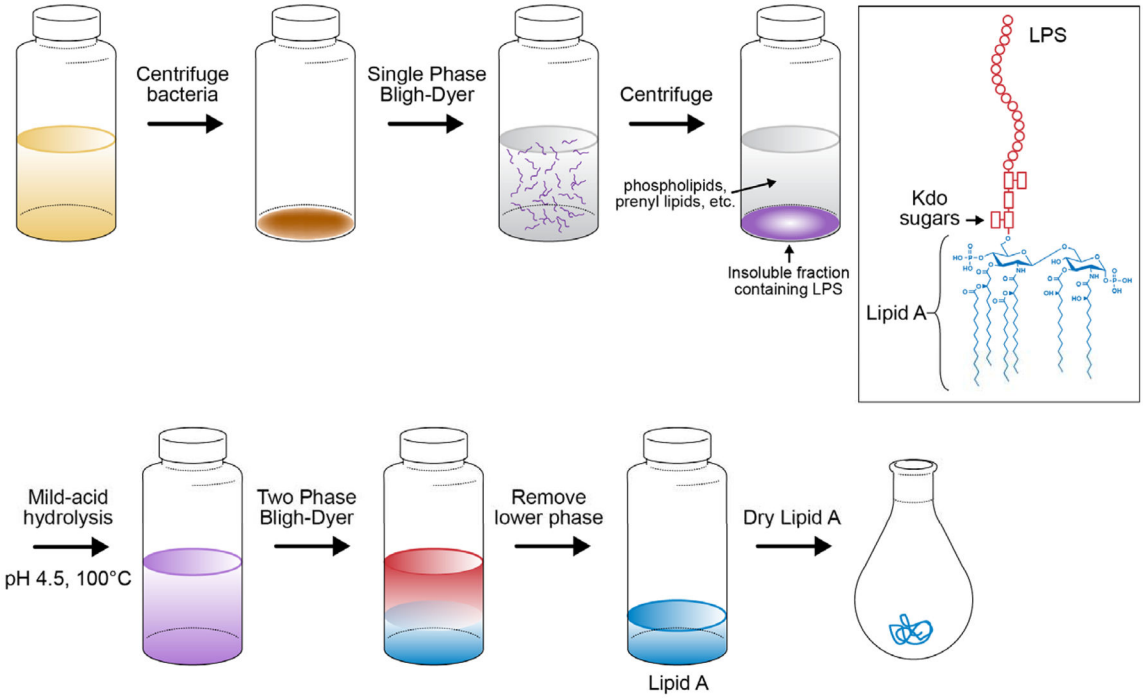

Figure 2.

Schematic of lipid A isolation procedure. Outline depicts chemical lysis of bacterial cell pellet using single phase Bligh-Dyer mixture, centrifugation of lysate to pellet LPS, mild-acid hydrolysis to liberate lipid A from attached polysaccharide, and final purification of lipid A using two phase Bligh-Dyer extraction.

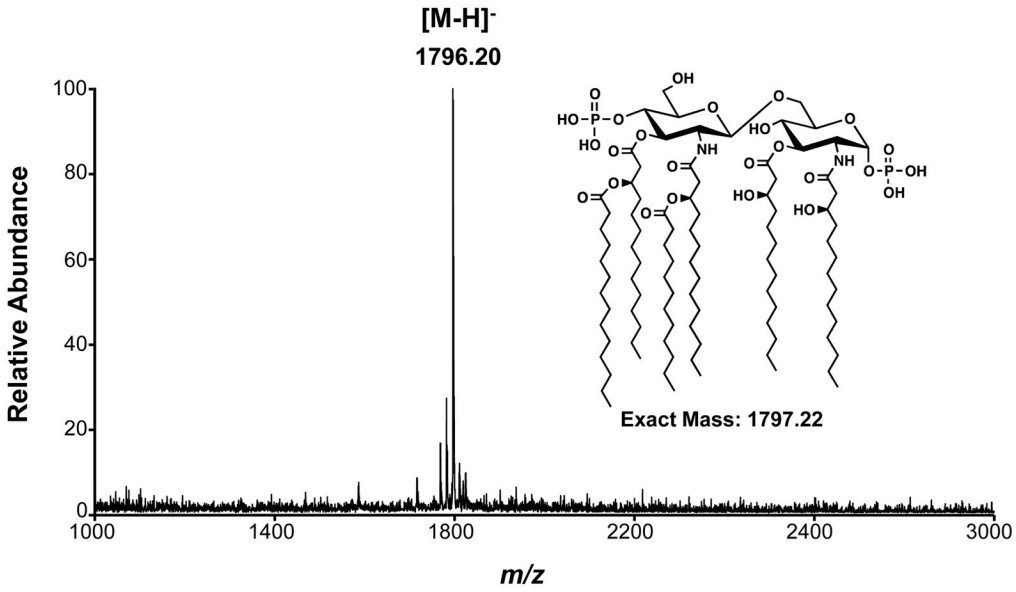

Figure 3.

MALDI-MS analysis of lipid A isolated from *E. coli* K-12 (W3110). Spectra obtained from the average of >300 shots. Singly charged $[M-H]^-$ lipid A is observed as the molecular ion at *m/z* 1796.2, which corresponds to a deprotonated species of the structure shown at right.

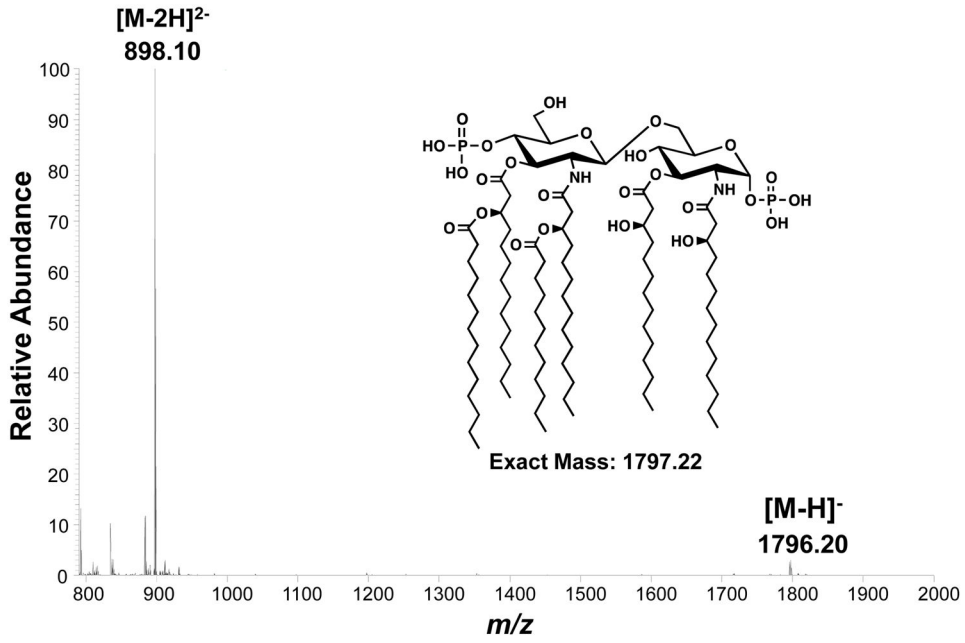

Figure 4.

ESI-MS analysis of lipid A isolated from *E. coli* K-12 (W3110). Singly $[M-H]^-$ and doubly-charged $[M-2H]^{2-}$ lipid A species are observed as molecular ions of *m/z* 1796.2 and *m/z* 898.1, respectively.

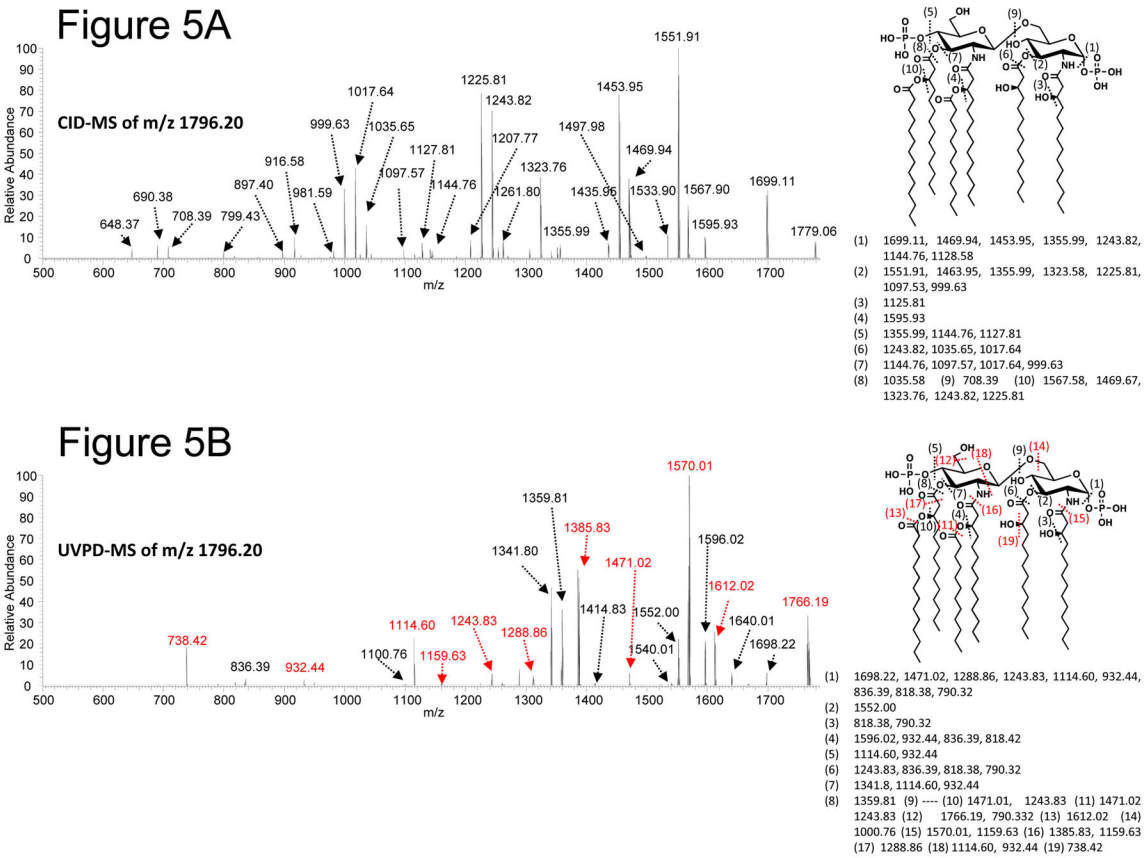

Figure 5.

MS/MS analysis of lipid A isolated from *E. coli* K-12 (W3110). Collision induced dissociation (A) or ultraviolet photodissociation (B) were used to fragment the precursor ion *m/z* 1796.2. Spectra are shown on the left with corresponding fragmentation maps on the right. Black dashed lines indicate fragmentation associated with both CID and UVPD, while red dashed lines (B) indicate UVPD specific product ions.

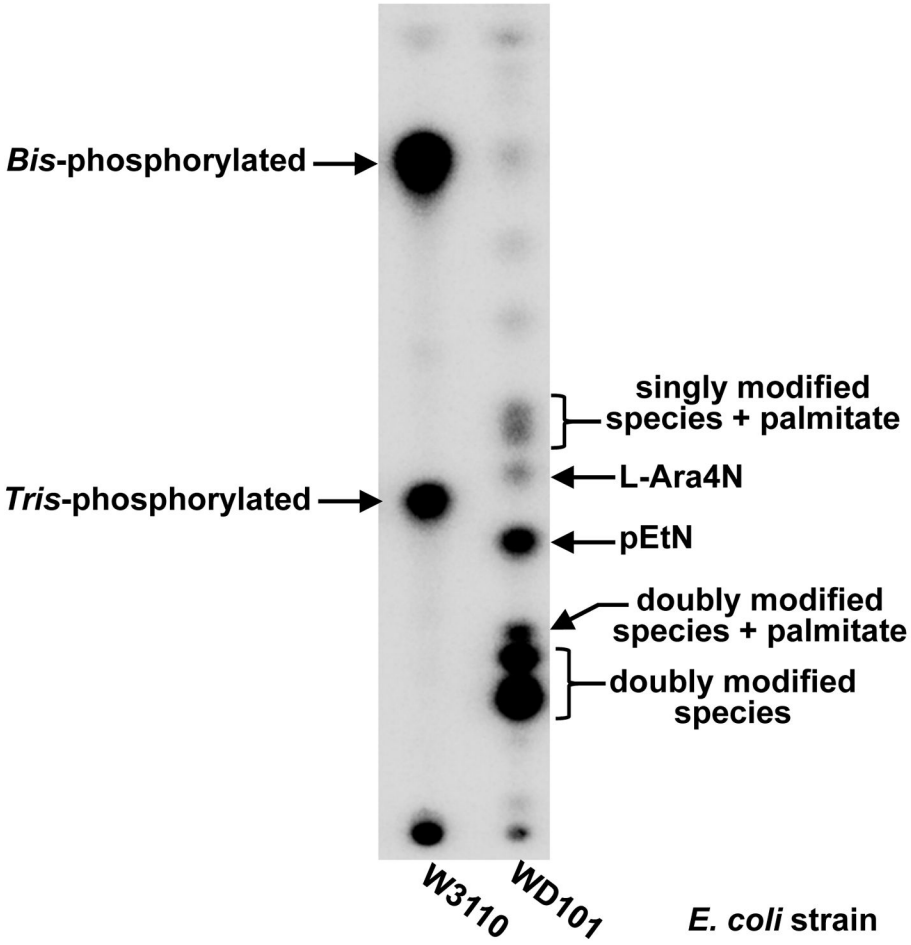

Figure 6.

TLC-based separation of lipid A species isolated from *E. coli* K-12. ^{32}P-labeled lipid A was isolated from W3110 (left lane) or WD101 (right lane) and separated in a TLC tank solvent system containing chloroform-pyridine-88% formic acid-water (50:50:16:5 v/v).