In Vitro Assembly of the Outer Core of the Lipopolysaccharide from Escherichia coli K-12 and Salmonella typhimurium

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Supporting Information

ABSTRACT: There are five distinct core structures in the lipopolysaccharides of Escherichia coli and at least two in Salmonella isolates, which vary principally in the outer core oligosaccharide. Six outer core glycosyltransferases, E. coli K-12 WaaG, WaaB, and WaaO and Salmonella typhimurium WaaI, WaaJ, and WaaK, were cloned, overexpressed, and purified. A novel substrate for WaaG was isolated from ΔwaaG E. coli overexpressing the lipid A phosphatase lpxE and the lipid A late acyltransferase lpxM. The action of lpxE and lpxM in the ΔwaaG background yielded heptose-1,6-dephospho Kdo-1-lipid A, a 1-dephosphorylated hexa-acylated lipid A with the inner core sugars that is easily isolated by organic extraction. Using this structurally defined acceptor and commercially available sugar nucleotides, each outer core glycosyltransferases was assayed in vitro. We show that WaaG and WaaB add a glucose and galactose sequentially to heptose-1,6-dephospho Kdo-1-lipid A. E. coli K-12 WaaO and S. typhimurium WaaI add a galactose to the WaaG/WaaB product but can also add a galactose to the WaaG product directly without the branched core sugar added by WaaB. Both WaaG and WaaO require divalent metal ions for optimal activity; however, WaaO, unlike WaaL, can add several glucose residues to its lipid acceptor. Using the product of WaaG, WaaB, and WaaL, we show that S. typhimurium WaaJ and WaaK transfer a glucose and N-acetylgalcosamine, respectively, to yield the full outer core. This is the first demonstration of the in vitro assembly of the outer core of the lipopolysaccharide using defined lipid A-oligosaccharide acceptors and sugar donors.

Lipopolysaccharide (LPS) is the major constituent of the outer leaflet of the outer membrane of Gram-negative bacteria and essential for the integrity of the outer membrane, making it a potential target for the development of novel therapeutics. The general features of many LPS structures have been characterized; it is composed of lipid A (the hydrophobic membrane anchor), the core region (a nonrepeating oligosaccharide), and O-antigen (a distal, repeating oligosaccharide).1–3

The core oligosaccharide (OS) can be further divided into two structurally distinct regions: the inner (lipid A proximal) and outer core OS. The inner core typically contains residues of 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and L-glycero-D-manno-heptose and is less variable among Gram-negative bacteria. The outer core OS, in contrast, varies considerably and helps define five distinct core structures in Escherichia coli, termed K-12 and R1=R4, and at least two core structures in Salmonella isolates.5,6 Nevertheless, the overall outer core OS structure shows conserved structural themes as shown in the simplified outer core structures of E. coli K-12 and Salmonella typhimurium in Figure 1. Both have three connected hexose residues on the main chain, and the first and third hexose residues are branched with other sugars. In all of the E. coli and Salmonella outer core OS, HexI, the first hexose on the main chain, is a glucose residue.7 WaaG has been identified as the UDP-glucose:(heptosyl) lipopolysaccharide α-1,3-glucosyltransferase in E. coli K-12 and S. typhimurium.7,8 In E. coli K-12, HexII and HexIII are both glucose residues, and in S. typhimurium, HexII is a galactose residue and HexIII is a glucose residue (Figure 1). WaaO [UDP-glucose:(galactosyl) lipopolysaccharide α-1,3-galactosyltransferase] from E. coli K-12, WaaI [UDP-galactose:(glucosyl) lipopolysaccharide α-1,3-galactosyltransferase] from S. typhimurium, and WaaJ [UDP-glucose:(galactosyl) lipopolysaccharide α-1,2-galactosyltransferase] are responsible for the transfer of these hexose residues.1,2,9,10

In both E. coli K-12 and S. typhimurium, branched sugars are added to the outer core OS. In both organisms, galactose is added to HexI in an α-1→6 linkage by WaaB, the UDP-galactose:(glucosyl) lipopolysaccharide α-1,6-galactosyltransferase.9 HexIII is appended with an α-1→2‐linked N-acetylglucosamine residue in S. typhimurium by WaaK, the UDP-N-acetyl glucosamine:(glucosyl) lipopolysaccharide α-1,6-N-acetyl glucosamine transferase. E. coli K-12 differs at this position; it is partially substituted at the C-6 position with a β-d-GlcNAc-(1→7)-l-α-d-Hep disaccharide, both of which are proposed to be added by WaaU.1,2

The presence of an intact inner and outer core OS is critical for cell function in both E. coli and S. typhimurium. The outer membranes of E. coli cells possessing a mutated waaG have a
E. coli K-12  

\[
\begin{array}{ccc}
\text{GlcNAc} & \text{O-antigen} \\
\text{Hep IV} & \text{WaaU} \\
\text{Glc III} & \text{WaaU} \\
\text{WaaR} & \\
\text{WaaO} & \\
\text{Gal I} & \text{WaaB} \\
\text{WaaG} & \\
\text{Hep II} & \\
\text{S. typhimurium} \\
\text{GlcNAc} & \text{O-antigen} \\
\text{Glc II} & \text{WaaK} \\
\text{WaaR} & \\
\text{WaaJ} & \\
\text{WaaI} & \\
\text{Gal II} & \text{WaaB} \\
\text{WaaG} & \\
\text{Hep II} & \\
\end{array}
\]

Figure 1. E. coli K-12 and S. typhimurium LPS outer core oligosaccharides (OS’s). The enzymes proposed to catalyze each glycosylation are shown. The linkages among the sugars are indicated; all are \(\alpha\) linkages except sugars added by WaaU from E. coli K12, which are \(\beta\) linkages. The dashed line attached to HepII indicates where the rest of the inner core and lipid A is attached. The location at which the O-antigen is attached to the outer core OS is indicated.

decreased level of phosphorylation of the inner core heptose, leading to decreased membrane stability; this phosphorylation increases membrane stability by mediating cross-linking with divalent cations. In Salmonella enterica serovar Typhi, the outer terminal glucose residue (Glc II) is required for the entry of the bacteria into epithelial cells. In addition, the inner and outer core OS links lipid A and the O-antigen, both of which are potent modulators of innate immunity.

Detailed analysis of the outer core OS glycosyltransferases of E. coli and S. typhimurium has been limited by the lack of biochemical data derived from purified enzymes and structurally defined acceptors and structural characterization of in vitro reaction products. Previously, only WaaJ from E. coli R3 has been characterized in vitro using pure enzyme and substrates. Previous work on the biochemical characterization of the other outer core OS glycosyltransferases has relied on in vivo complementation of chromosomal insertion mutations. In these studies, the LPS was characterized using polyacrylamide gel electrophoresis, not by directly assessing the required lipid acceptors and sugar donors.

Cloning, Overexpression, and Purification of E. coli K-12 WaaG, WaaB, and WaaO and S. typhimurium WaaL, WaaJ, and WaaK. E. coli K-12 waaG, waaB, and waaO were amplified via PCR from genomic DNA of W3110 using the primers described in Table S1 of the Supporting Information. For each gene, an Ndel site was incorporated at the N-terminus and a BamHI or Xhol site was incorporated at the C-terminus. Each PCR product was digested with Ndel and BamHI or Xhol and ligated into a similarly digested pET-21b or pET-28b expression vector as indicated in Table 1. S. typhimurium waaL, waaJ, and waaK were similarly cloned from genomic DNA of S. typhimurium into the pET-28b expression vector.

Each plasmid was transformed into BL21(DE3) for overexpression of the respective protein. LB (750 mL) containing the appropriate antibiotics was inoculated from a 50 mL overnight culture to a final A600 of 0.02 and grown to an A600 of 0.7 at 37 °C. Protein expression was induced by the addition of IPTG (final concentration of 1 mM). For JQ2, arabinose (0.2%) was also added for the induction. The cultures were grown for an additional 4 h and then harvested at 4 °C by centrifugation at 4000 g for 20 min. The cell pellet was washed with phosphate-buffered saline (PBS) [137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄ (pH 7.5)], and the cells were harvested by centrifugation. This and all subsequent steps were conducted at 4 °C. Cell debris and unbroken cells were removed by centrifugation at 27000 g for 30 min. The final cell pellet was resuspended in 30 mL of buffer.

MATERIALS AND METHODS

Materials. Reagent grade chloroform, methanol, and silica gel 60 thin-layer chromatography (TLC) plates (layer thickness of 150–200 μm) were obtained from EMD Chemicals Inc. (Gibbstown, NJ). [32P]P₃, UDP-[U-14C]glucose, UDP-[U-14C]-galactose, and GDP-[U-14C]mannose were from PerkinElmer Life and Analytical Sciences, Inc. (Waltham, MA). Yeast extract, agar, and tryptone were from BD Biosciences. Sodium chloride and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were from VWR International (West Chester, PA). Triton X-100 was from Thermo Fisher Scientific (Waltham, MA). Isopropyl 1-thio-β-D-galactopyranoside (IPTG) was from Invitrogen Corp. (Carlsbad, CA). UDP-glucose, UDP-galactose, UDP-glucuronic acid, UDP-galacuronic acid, ADP-glucose, GDP-glucose, UDP-N-acetylgalcosamine, and ADP-mannose were purchased from Sigma-Aldrich. The P-10 desalting column was from GE. All other chemicals were reagent grade and were purchased from either Sigma-Aldrich (St. Louis, MO) or Mallinckrodt Baker, Inc. (Phillipsburg, NJ).

Bacterial Strains and Molecular Biology Applications. All bacteria were grown in Luria-Bertani broth (LB, 10 g of NaCl, 10 g of bacto-tryptone, and 5 g of yeast extract per liter at 37 °C). When required for the selection of plasmids, cells were grown in the presence of 100 μg/mL ampicillin, 30 μg/mL chloramphenicol, or 30 μg/mL kanamycin. Plasmids were prepared using the Qiagen mini-prep kit (Qiagen). Restriction endonucleases (New England Biolabs) and T4 ligase (Invitrogen) were used according to the manufacturers’ instructions. Genomic DNA was isolated using the protocol for bacterial cultures in the Easy-DNA kit (Invitrogen). Transformation-competent E. coli cells were prepared by the method of Inoue et al. Double-stranded DNA sequencing was performed with an ABI Prism 377 instrument at the Duke University DNA Analysis Facility. Primers were purchased from Integrated DNA Technologies, Inc.

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Table 1. Bacterial Strains and Plasmids

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<td>Novagen</td>
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<td>JQ2</td>
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<tr>
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<td>pET21b harboring <em>E. coli</em> K-12 waaO</td>
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<tr>
<td>pSTI28</td>
<td>pET28b harboring <em>S. typhimurium</em> waaI</td>
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<tr>
<td>pSTJ28</td>
<td>pET28b harboring <em>S. typhimurium</em> waaJ</td>
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<tr>
<td>pSTK28</td>
<td>pET28b harboring <em>S. typhimurium</em> waaK</td>
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“lpxM was subcloned out of the pET vector and into the pBAD vector using the SacI and XbaI sites, yielding a fragment that included the pET-21a(+) ribosome binding site.

A [50 mM Hepes (pH 7.5) containing 300 mM KCl] with 0.5% Triton X-100 and disrupted by being passed through a French pressure cell at 18000 psi.

Each of the overexpressed proteins was purified using Ni-NTA chromatography. The membrane-free cytosol (derived from a 750 mL culture of *E. coli* overexpressing an outer core glycosyltransferase) was loaded onto 1 mL of Ni-NTA resin at 4 °C and washed with 30 column volumes of buffer A with 30 mM imidazole and 1 column volume of buffer A with 120 mM imidazole. The enzyme was eluted with buffer A with 500 mM imidazole. The imidazole was removed from the enzyme preparation using a P10 desalting column. Purified, desalted enzymes were stored at −80 °C until they were needed.

Preparation of Radiolabeled Substrates. A 10 mL culture of G56 medium containing 100 μg/mL ampicillin, 30 μg/mL chloramphenicol, 1 mM IPTG, and 0.02% arabinose was inoculated with 100 μL of an overnight culture of JQ2 (Table 1). The culture was incubated at 37 °C while being shaken at 225 rpm until A600 reached 0.5. Cells were collected by centrifugation at 4 °C and 2000g for 20 min and then resuspended in 10 mL of G56 medium without phosphate. The cell suspension was centrifuged again, the supernatant discarded, and the cell pellet resuspended in 10 mL of fresh G56 medium without phosphate. [32P]P_i (1 mCi) was added, and the cells were grown at 37 °C for 4 h. The radiolabeled cells were pelleted by centrifugation in a clinical centrifuge for 15 min. The cell pellet was resuspended in 3.8 mL of a neutral single-phase Bligh–Dyer extraction mixture [1:2:0.8 (v/v/v) CHCl_{3}/CH_{2}OH/PBS] and incubated at room temperature for 30 min. The cell debris was pelleted by centrifugation as described above. The supernatant was transferred to a fresh tube, and 1 mL of CHCl_{3} and 1 mL of PBS were added to generate a two-phase neutral Bligh–Dyer extraction mixture [2:2:1.8 (v/v/v) CHCl_{3}/CH_{2}OH/PBS]. After the mixture had been vortexed, the phases were resolved by centrifugation for 15 min as described above. The lower phase was transferred to a fresh glass tube. The remaining upper phase was extracted a second time by the addition of the fresh, pre-equilibrated neutral lower phase. The two lower phases were pooled and dried under a stream of N_{2}. The dried lipid films were redissolved in a CHCl_{3}/CH_{2}OH mixture [4:1 (v/v)] and spotted onto a 10 cm × 20 cm TLC plate. The TLC plate was developed in the CHCl_{3}/CH_{2}OH/CH_{3}COOH/H_{2}O solvent system [25:15:4:4 (v/v/v/v)]. The plate was dried under a cold air stream and exposed to X-ray film for 30 s to locate the [32P]heptose1,1-dephosphorylated-KDO2-lipid A (Hep2,1-deP-KLA). The region of the silica plate containing the product was dampened with H_{2}O, removed by scraping, transferred to a thick-walled glass tube, and resuspended in 4 mL of a neutral single-phase Bligh–Dyer mixture. The suspension was vigorously mixed and subjected to sonic irradiation in a sonicator bath for 30 s. The silica particles were pelleted by centrifugation as in a clinical centrifuge for 10 min. The supernatant containing the [32P]-labeled Hep2,1-deP-KLA was removed and passed through a 4 mL glass-wool column to remove the remaining silica particles. The solution was converted to a neutral two-phase Bligh–Dyer mixture by the addition of CHCl_{3} and PBS. The phases were separated in a clinical centrifuge as described above. The lower phase was transferred to new glass tubes. The upper phases was extracted a second time by the addition of 1.8 mL of fresh, pre-equilibrated neutral lower phase. The lower phases were pooled and dried under a stream of N_{2}. The [32P]Hep2,1-deP-KLA was resuspended in buffer B [25 mM Tris-HCl (pH 7.8) containing 1 mM EDTA, 1 mM EGTA, and 0.1% Triton X-100] and then stored at −20 °C. Typically, the yield of [32P]Hep2,1-deP-KLA recovered was ~0.6% of the total input [32P]P_i.

Radiolabeled Hep2,1-deP-KLA was used to prepare [32P]Gal-Glc-Hep2,1-deP-KLA using WaaG and WaaB. WaaG (0.2 mg/mL) was incubated with 3 μCi of [32P]Hep2,1-deP-KLA and 5 mM UDP-Glc in the presence of 50 mM Hepes (pH 7.5) and 0.1% Triton X-100 for 2 h at 25 °C in a total volume of 100 μL. The reaction mixture was converted to a one-phase neutral Bligh–Dyer mixture by the addition of CHCl_{3} and CH_{2}OH. After a brief centrifugation, the single-phase mixture was transferred to a fresh tube and converted to a two-phase neutral Bligh–Dyer mixture by the addition of CHCl_{3} and CH_{2}OH. The reaction was continued for an additional 2 h at 25 °C.
mixture by the addition of CHCl₃ and PBS. After centrifugation to resolve the phases, the upper phase was washed as described above and the two lower phases were combined. After being dried under a stream of N₂, the lipid films were dissolved in 50 µL of buffer B. WaαB (0.2 mg/mL) and 5 mM UDP-Gal were added to the extracted WaαG product, producing a final volume of 100 µL, and incubated at 25 °C for 3 h. The reaction mixture was spotted onto a 10 cm × 20 cm TLC plate and developed in a CHCl₃/CH₃OH/CH₃COOH/H₂O system [25:15:4:4 (v/v/v/v)].

**Large-Scale Preparation of Hep₂-1-deP-KLA.** JQ₂ cells were grown under conditions similar to those described above for the expression of the outer core enzymes. A 3 L culture of JQ₂ was grown to an A₆₀₀ of 1.8 and harvested by centrifugation at 4000 g for 10 min at 4 °C. The cell pellet was washed once with 40 mL of PBS and the centrifugation repeated. The final cell pellet was resuspended in 40 mL of PBS, and 50 mL of CHCl₃ and 100 mL of CH₃OH were added to generate a neutral single-phase Bligh–Dyer mixture. The extraction mixture was incubated for 1 h at room temperature. The cell debris was removed by centrifugation at 4000g for 15 min at room temperature. The supernatant was transferred to a clean 250 mL Teflon bottle and converted to a two-phase neutral Bligh–Dyer system by the addition of 50 mL of CHCl₃ and 50 mL of PBS. The phases were resolved by centrifugation as described above. The upper phase was removed and re-extracted with 90 mL of the fresh, pre-equilibrated neutral lower phase. The lower phases were pooled and dried by rotary evaporation. Next, the dried lipid film was redissolved in 25 mL of a CHCl₃/CH₃OH/H₂O mixture [2:3:1 (v/v/v)] and applied to a 5 mL diethylaminoethyl (DEAE) cellulose (acetate form) column²⁴ equilibrated with the same solvent mixture. The column was washed with 10 column volumes of the CHCl₃/CH₃OH/H₂O mixture [2:3:1 (v/v/v)]. The bound phospholipids and LPS were eluted using 25 mL steps of the CHCl₃/CH₃OH/CH₃COONH₄ mixture [2:3:1 (v/v/v)], with successive CH₃COONH₄ concentrations of 60, 120, 240, and 480 mM in the aqueous component. For each elution step, 5 mL fractions were collected. The fractions containing Hep₂-1-deP-KLA were identified by spotting 10 µL portions of each fraction onto a TLC plate. The plate was developed in a CHCl₃/CH₃OH/CH₃COOH/H₂O system [25:15:4:4 (v/v/v/v)]. Lipids were visualized by spraying the plate with 10% sulfuric acid in ethanol and charring on a hot plate. All fractions containing Hep₂-1-deP-KLA were pooled and the appropriate amounts of CHCl₃ and H₂O added to generate a 2:2:1.8 (v/v/v) CHCl₃/CH₃OH/aqueous mixture. The lower phase was dried under a stream of N₂. The collected lipid was redissolved in a CHCl₃/CH₃OH mixture [4:1 (v/v)] and spotted along the bottom of a 10 cm × 20 cm TLC plate. A 10 µL sample was separately spotted onto the same TLC plate as a control to identify the location of the Hep₂-1-deP-KLA. After the plate had been developed in the CHCl₃/CH₃OH/CH₃COOH/H₂O solvent system [25:15:4:4 (v/v/v/v)], the plate was dried under cold air, and the portion of the plate containing the control was charred.

**Figure 2.** Structure of Hep₂-1-deP-KLA. The structure of the lipid A OS purified from JQ₂ is shown.

**Figure 3.** Glycosylations catalyzed by *E. coli* WaαG and WaαB. [³²P]Hep₂-1-deP-KLA (marked A) was incubated with WaαG and/or WaαB (0.01 mg/mL each) for the times indicated as described in Materials and Methods. Both UDP-Glc (1 mM) and UDP-Gal (1 mM) were included in the reaction mixture. B marks the location of the WaαG product, Glc-Hep₂-1-deP-KLA. C marks the location of the WaαB product, Gal-Glc-Hep₂-1-deP-KLA.
The region of the TLC plate containing the Hep2-1-deP-KLA was scraped, extracted, and purified as described above. Typically, 3 mg of Hep2-1-deP-KLA was isolated from 3 L of JQ2 grown to an A$_{600}$ of 1.8.

The concentration of Hep2-1-deP-KLA was determined as follows. UDP-$^{14}$C-Glc (1 mM) and different concentrations of Hep2-1-deP-KLA (from 0.01 to 0.05 mM) were reacted with 0.2 mg/mL WaaG for 30 min as described above until all of the Hep2-1-deP-KLA had been completely consumed. Assays with $[^32P]$Hep2-1-deP-KLA showed that WaaG can convert most of the Hep2-1-deP-KLA to Glc-Hep2-1-deP-KLA at high UDP-Glc concentrations (Figure 3). By following the incorporation of $[^{14}C]$Glc into $[^{14}C]$Glc-Hep2-1-deP-KLA, we could determine the concentration of the unlabeled Hep2-1-deP-KLA.

**In Vitro Assay Conditions.** The standard reaction mixtures contained purified glycosyltransferase(s) (0.1 mg/mL), 50 mM Hepes (pH 7.5), 0.1% Triton X-100, 10 μM $[^{32}P]$Hep2-1-deP-KLA, and 1 mM UDP-hexose donor(s). Reactions were initiated by the addition of enzyme and mixtures incubated at 25 °C for the times indicated. For coupling reactions of WaaG, WaaB, and WaaO from *E. coli* K-12, WaaG (0.01 mg/mL) was reacted with UDP-Glc for 15 min using the standard assay conditions described above (final volume of 50 μL). Next, WaaB (0.1 mg/mL) and 1 mM UDP-Gal were added to the reaction mixture (final volume of 52 μL) and incubated at 25 °C for 30 min. WaaO (0.1 mg/mL) and 1 mM MgCl$_2$ were added to the reaction solution after WaaB reacted at 25 °C for 30 min (final volume of 54 μL).

For reactions of *E. coli* K-12 WaaO and *S. typhimurium* WaaI with Gal-Glc-Hep2-1-deP-KLA, the product of the reaction of WaaG and WaaB, only the $[^{32}P]$Gal-Glc-Hep2-1-deP-KLA product (final concentration in the nanomolar range) was included as the LPS acceptor along with the appropriate UDP-hexoses and 1 mM MgCl$_2$. The preparation of $[^{32}P]$Gal-Glc-Hep2-1-deP-KLA is described above.

Reactions were stopped by spotting 3−5 μL portions of the reaction mixtures onto a TLC plate. After being dried in a stream of cold air, plates were developed in the CHCl$_3$/CH$_3$OH/CH$_3$COOH/H$_2$O solvent [25:15:4:4 (v/v/v/v)]. The amount of product formed was calculated from the percent conversion of radioactive substrate to product, quantified using a Molecular Dynamics PhosphorImager.

**Data Fitting.** To determine the kinetic parameters for outer core enzymes, one substrate, either a sugar donor or an acceptor, was varied from approximately 5-fold below to 5-fold above the predicted K$_m$ while the other substrate was held constant 5-fold.

![Figure 4](https://example.com)
above the predicted $K_m$. The data were fit to the Michaelis–Menten equation using Kaelidagraph (Synergy Software, Reading, PA).

Electrospray Ionization Mass Spectrometry. Mass spectra were acquired on an ABI QSTAR XL tandem quadrupole time-of-flight mass spectrometer (ABI/MDS-Sciex, Toronto, ON) equipped with an electrospray ionization (ESI) source. Spectra were acquired in the negative ion mode and typically were the accumulation of 60 scans collected from $m/z$ 200 to 2000. Typically, lipids were dissolved in 200 µL of a CHCl₃/CH₂OH mixture [2:1 (v/v)], supplemented with 1% piperidine, and immediately infused into the ion source at a rate of 5–10 µL/min. The negative ion ESI was conducted at −4200 V. The acquisition and analysis of data were performed using Analyst QS (ABI/MDS-Sciex).

RESULTS

Preparation of the *E. coli* WaaG Substrate, Hep₂₋₁-deP-KLA. An LPS lacking an outer core OS accumulates in CW303, an *E. coli* strain with the gentamycin gene inserted into the waaG gene. The lipid A 1-phosphatase *lpxE* and the lipid A late acyl transferase *lpzM* were introduced into CW303 to generate strain JQ2 and promote the accumulation of a modified LPS that contains a fully hexa-acylated lipid A portion that is dephosphorylated at the 1-position and the inner core sugars, two Kdo and two heptose residues (Hep₂₋₁-deP-KLA). The accumulating Hep₂₋₁-deP-KLA (Figure 2) was purified as described in Materials and Methods, and the structure was confirmed using negative ion electrospray ionization mass spectrometry (ESI-MS) (Figure 4A). The major ions at $m/z$ 1269.73 and 846.15 correspond to the [M − 3H]⁻ and [M − 3H]⁻ ions of Hep₂₋₁-deP-KLA, respectively (expected values of $m/z$ 1269.7409 and 846.1582, respectively).

WaaG Transfers the Outer Core Hexl. When purified *E. coli* WaaG was incubated in an *in vitro* reaction mixture with UDP-Glc as the sugar donor and [³²P]Hep₂₋₁-deP-KLA (A, Figure 3) as the lipid acceptor, a more slowly migrating product was formed (B, Figure 3), consistent with the addition of a glucose to the Hep₂₋₁-deP-KLA to form Glc-Hep₂₋₁-deP-KLA. This product was purified and analyzed by negative ion ESI-MS (Figure 4B). The two predominant ions at $m/z$ 1351.34 and 900.23 correspond to the [M − 2H]²⁻ and [M − 3H]⁻ ions expected for Glc-Hep₂₋₁-deP-KLA, respectively (expected values of $m/z$ 1351.2690 and 900.1758, respectively).

Using this *in vitro* reaction, WaaG was further characterized. The Glc-Hep₂₋₁-deP-KLA product was formed in a time-dependent manner (Figure 3). No Glc-Hep₂₋₁-deP-KLA was formed when other sugar donors, such as UDP-galactose, UDP-glucuronic acid, UDP-galacuronic acid, GDP-mannose, ADP-glucose, and GDP-glucose, were used in place of UDP-Glc (Figure S1A,B of the Supporting Information). The apparent $K_m$ values for UDP-Glc and Hep₂₋₁-deP-KLA are 0.162 ± 0.028 mM and 3.85 ± 1.80 µM, respectively, and the $k_{cat}$ is 0.5 s⁻¹, similar to the values reported previously when WaaG was characterized in crude extracts.

The Triton X-100 dependence of the WaaG-catalyzed formation of Glc-Hep₂₋₁-deP-KLA was investigated (Figure S2 of the Supporting Information). The activity of WaaG increased as the Triton X-100 concentration was increased from 0 to 0.2%; higher concentrations of detergent were inhibitory. On the basis of these results, 0.1% Triton X-100 was used in subsequent *in vitro* assay reactions of WaaG.

The WaaG activity is dramatically decreased by inclusion of Mg²⁺ in the *in vitro* assay reaction mixture (Figure S3 of the Supporting Information). Less than 20% of WaaG activity is left when 0.8 mM Mg²⁺ is added to the assay. WaaG was similarly inhibited by other divalent metal ions such as Mn²⁺, Ca²⁺, Zn²⁺, Co²⁺, Ni²⁺, and Cu²⁺ (Figure S4 of the Supporting Information).

WaaG can hydrolyze the sugar donor, UDP-Glc, in the absence of a lipid acceptor as shown in Figure 5. When ¹⁴C-labeled UDP-Glc is incubated with WaaG, glucose is released in a WaaG-dependent reaction (in Figure 5, compare lanes 1 and 2). The $K_m$ and $k_{cat}$ values for this hydrolysis reaction are 3.6 ± 0.8 mM and 0.014 s⁻¹, respectively. WaaG was not able to catalyze the release of the 4-epimer of glucose, galactose, from UDP-Gal (Figure S5 of the Supporting Information).

### Figure 5.

WaaG hydrolyzes UDP-Glc in the absence of a lipid acceptor. UDP-[U-¹⁴C]Glc was incubated in the presence (lane 1) or absence (lane 2) of WaaG (0.25 mg/mL) and then displayed using TLC as described in Materials and Methods. [U-¹⁴C]Glc was spotted as a control in lane 3 to indicate the migration of free glucose.

WaaB Catalyzes the Transfer of Galactose to the *E. coli* K₁₂ Outer Core. Using the product of WaaG, Glc-Hep₂₋₁-deP-KLA, the ability of *E. coli* WaaB to catalyze the transfer of galactose from UDP-Gal to the outer core glucose was assessed. When purified WaaB was combined with WaaG, a more slowly migrating product was formed (product C, Figure 3), consistent with the addition of galactose to Glc-Hep₂₋₁-deP-KLA. This product, Gal-Glc-Hep₂₋₁-deP-KLA, was isolated and analyzed using negative ion ESI-MS (Figure 4C). The ion at $m/z$ 1431.78 is consistent with the [M − 2H]²⁻ ion of Gal-Glc-Hep₂₋₁-deP-KLA (expected value of $m/z$ 1431.7937). No product was formed when Hep₂₋₁-deP-KLA was used as the sugar acceptor. This strongly indicates that the galactose added by WaaB depends only on the glucose added by WaaG. In addition, no activity was observed for other sugar nucleotides, such as UDP-glucose, UDP-glucuronic acid, UDP-galacturonic acid.
UDP-galacuronic acid, GDP-mannose, ADP-glucose, and GDP-glucose (Figure S5A,B of the Supporting Information). As with WaaG, in the absence of a lipid acceptor, WaaB can hydrolyze its sugar donor, UDP-galactose, to UDP and galactose but was not able to hydrolyze UDP-Glc (Figure S5A,B of the Supporting Information).

**E. coli K-12 LPS Outer Core HexII Is Transferred by WaaO.** In *E. coli* K-12 LPS, the outer core HexII is a glucose residue, which is transferred by WaaO.1 When purified *E. coli* WaaO is coupled to the reaction of WaaG and WaaB, several more slowly migrating products were detected (Figure 6). When 0.1 mM Mg2+ was included in the assay, low levels of only one product, presumably Glc-[Gal]-Glc-Hep2-1-deP-KLA ([Gal] indicates the branched galactose added to the 6-position of the glucose as shown in Figure 1), was detected (D, Figure 6). As the concentration of Mg2+ is increased, multiple products were observed (D*, Figure 6). These glucosylated products (Glcn-[Gal]-Glc-Hep2-1-deP-KLA) were detected only in the presence of Mg2+ (data not shown); multiple glucosylated products were observed only in the presence of Mg2+. These results confirm the divalent cation dependence of WaaO and indicate that WaaO does not require the branched galactose added by WaaB. These data also strongly suggest that UDP-Glc is the sugar donor for the multiple sugar additions catalyzed by WaaO (Figure 8) because there is no UDP-galactose present in this reaction mixture.

To exclude the possibility that the products observed in the presence of WaaO might be due to the coupling enzyme WaaG or WaaB, Gal-Glc-Hep2-1-deP-KLA, the product of WaaG and WaaB, was isolated (C, Figure 9) and used directly as the substrate for WaaO. As shown in Figure 9, multiple products consistent with the addition of a single glucose (product D, Figure 9) but also multiple glucoses (D*, Figure 9) were observed, indicating that WaaO alone is responsible for the formation of those lipid A OS’s.

Like WaaB and WaaG, WaaO can hydrolyze its sugar donor, UDP-Glc, but not UDP-Gal (Figure S5A,B of the Supporting Information). The *Km* for UDP-Glc in this hydrolysis reaction is 1.95 ± 0.4 mM, and the *kcat* is 0.013 s⁻¹.

**S. typhimurium LPS Outer Core HexII Is Transferred by WaaI.** The LPS outer core structure of *S. typhimurium* is similar to that of *E. coli* K-12; both HexI and the branched substitution for HexI are the same (Figure 1). HexII, added by WaaO in *E. coli* and WaaI in *S. typhimurium*, differs between the two
organisms; in *S. typhimurium*, a galactose is added instead of glucose. This suggests the *S. typhimurium* WaaI will preferentially use a galactose from Gal-Glc-Hep₂₁-deP-KLA, the product of the coupled reaction of *E. coli* K-12 WaaG and WaaB. To test this, *S. typhimurium* WaaI was purified and reacted with Gal-Glc-Hep₂₁-deP-KLA in the presence of UDP-galactose or UDP-glucose. Similar to *E. coli* WaaO, *S. typhimurium* WaaI catalyzed the formation of a product consistent with the addition of a sugar to Gal-Glc-Hep₂₁-deP-KLA (Dₙ, Figure 10). The glycosylation occurred only in the presence of UDP-galactose, not UDP-glucose, consistent with the predicted specificity for the HexII glycosyltransferase based on the outer core OS structure.

Like *E. coli* WaaO, *S. typhimurium* WaaI also requires magnesium ions for optimal activity. When the *S. typhimurium* WaaI was reacted with Glc-Hep₂₁-deP-KLA through coupling only to the WaaG reaction, a faint product consistent with Gal-Glc-Hep₂₁-deP-KLA (D₀, Figure 10) was observed, suggesting that WaaI does not require the branched α₁→6 galactose added by WaaB (Figure S6 of the Supporting Information).

The *S. typhimurium* LPS Outer Core HexIII Substitution with UDP-GlcNAc Is Catalyzed by WaaK. The ability of *S. typhimurium* WaaK to add a GlcNAc residue to Glc-Gal-[Gal]-Glc-Hep₂₁-deP-KLA was tested. Gal-Glc-Hep₂₁-deP-KLA, the product of *E. coli* WaaG and WaaB, was isolated using preparative TLC. *S. typhimurium* WaaI and WaaJ were reacted with Gal-Glc-Hep₂₁-deP-KLA in the presence of UDP-Glc and UDP-Gal to form Glc-Gal-[Gal]-Glc-Hep₂₁-deP-KLA (Figure 12, product Dₙ) and then Glc-Gal-[Gal]-Glc-Hep₂₁-deP-KLA (Figure 12, product Eₙ). When *S. typhimurium* WaaK was added to the reaction mixture in the absence of UDP-GlcNAc, a faint band was observed, suggesting WaaK has some activity using UDP-Glc or UDP-Gal as a sugar donor. However, when UDP-GlcNAc is added, WaaK activity is dramatically enhanced, strongly suggesting that UDP-GlcNAc is a preferred sugar donor. The formation of product Fₙ, GlcNAc-Glc-Gal-[Gal]-Glc-Hep₂₁-deP-KLA, is dependent on WaaK. The formation of this product demonstrates, for the first time, the *in vitro* assembly of the entire *S. typhimurium* outer core OS using purified proteins and defined substrates.

**DISCUSSION**

This work relates several leaps forward for the biochemical characterization of the enzymes involved in outer core OS.
synthesis in Gram-negative bacteria. First, we have developed a method for the production of a lipid A OS to use as an acceptor for the enzymes involved in outer core OS assembly. The LPS isolated from CWG303, an E. coli strain deficient in waaG, possesses only the inner core sugars and decreased the level of phosphorylation of the inner core heptoses. However, the major lipid A OS in CWG303 is still too hydrophilic to be easily purified using standard lipid extraction protocols. The incorporation of lpxE, the Francisella novicida lipid A 1-phosphatase, into a strain lacking waaG decreases the hydrophilicity of the major LPS species. Furthermore, the incorporation of lpxM, the lipid A late acyltransferase, increases the proportion of hexa-acylated lipid A. All of these modifications result in a high yield of a lipid A OS, Hep2-1-deP-KLA (Figure 2), that is structurally defined, can be isolated via organic extraction, and can be radiolabeled efficiently with $^{32}$P.

Detailed analysis of outer core glycosyltransferases has been limited by the purity of the LPS acceptor and the assay method. Usually, assays depended on following the incorporation of $[^{14}C]$glucose or $[^{14}C]$galactose with scintillation counting into the LPS acceptor. The availability of Hep$_2$-1-deP-KLA allows us to follow the addition of the outer core sugars in vitro using TLC analysis. These reactions can be followed either by labeling the lipid A OS acceptor with $^{32}$P or by using $^{14}$C-labeled sugar donors. The increased sensitivity afforded by the use of radiolabeled substrates allows us to fully define the biochemical properties of the outer core glycosyltransferases by detecting expected and unexpected products.

We purified three E. coli and three S. typhimurium outer core glycosyltransferases previously shown through numerous genetic and in vivo experiments to be involved in outer core biosynthesis. Each protein was efficiently overexpressed in E. coli and purified to homogeneity using Ni affinity chromatography.

Combining the purified enzymes with a defined lipid acceptor and sugar donors, we characterized the biochemical activity of each of these enzymes from E. coli and S. typhimurium. We have demonstrated by TLC and ESI-MS that WaaG can add a glucose residue to its LPS acceptor, Hep$_2$-1-deP-KLA (Figures 3 and 4). While E. coli WaaG is inhibited by Mg$^{2+}$ (Figure S2 of the Supporting Information), E. coli WaaO can add multiple sugars under similar conditions (Figure 6). Because the physiological concentration of Mg$^{2+}$ in E. coli could be in the millimolar range, these in vitro activities may be physiologically relevant.

WaaB has not been characterized previously in pure enzyme form in vitro. The obvious activity of WaaB is observed only when UDP-Gal is used as the sugar donor for addition to the WaaG product, Glc-Hep$_2$-1-deP-KLA (Figure S5 of the Supporting Information). ESI-MS (Figure 4) of the WaaB in vitro product is consistent with the addition of a galactose residue to Glc-Hep$_2$-1-deP-KLA. The role of WaaB as the second enzyme to act in the LPS outer core assembly in E. coli K-12 is supported by these in vitro results. Furthermore, both HexII glycosyltransferases, E. coli K-12 WaaO and S. typhimurium WaaI, have much lower activity toward Glc-Hep$_2$-1-deP-KLA than toward Gal-Glc-Hep$_2$-1-deP-KLA, the product of WaaG and WaaB, suggesting that the addition of HexII occurs after Hex substitution by WaaB in vivo (compare Figures 8 and 9 for E. coli WaaO and Figure S6 of the Supporting Information and Figure 10 for S. typhimurium WaaI).
As expected, WaaI from *S. typhimurium* can add a galactose residue to the *E. coli* K-12 WaaG, WaaB product, Gal-Glc-Hep2-1-deP-KLA (Figure 10). It can also add a second galactose residue to its first product, though the activity is low; after reaction for 30 min, a faint second product band can be observed (Figure 10). Building off of the major WaaI product, the *S. typhimurium* WaaJ adds a glucose residue (Figure 11), and *S. typhimurium* WaaK adds a GlcNAc residue to yield a fully assembled *S. typhimurium* LPS outer core (Figure 12).

Previously, it has been reported that only one hexose residue was observed in the outer core of the majority of LPS isolated from *E. coli* K-12 waaB and Δgal mutants, but both mutants still produce minor LPS bands that migrate as though they have additional sugars added to the core. The authors proposed that *E. coli* K-12 WaaO cannot work unless the branched galactose added by WaaB is present and that the HexIII glycosyltransferase (WaaR in *E. coli* K-12) might mistake the GlcI added by WaaG for GlcII added by WaaO and inefficiently add a terminal glucose. Our *in vitro* data for *S. typhimurium* WaaO do not support this proposal. However, WaaI can add, with a low efficiency, glucose to Glc-Hep2-1-deP-KLA (Figure 10, slight spot that appears after 30 min). This suggests that the more glycosylated LPS species detected in the *waaB* and Δ*gal* mutants are due to WaaI adding glucose to Glc-Hep2-1-deP-KLA. This is also consistent with previous observations with an *S. typhimurium* waaB mutant. Despite lacking the branched galactose, additional core sugars were detected in a fraction of the isolated LPS molecules. Our work clearly shows that the branched galactose added by WaaB is not absolutely necessary for the action of other enzymes involved in outer core biosynthesis, consistent with the LPS structures observed in *waaB* mutants.

WaaB and WaaG are members of glycosyltransferase carbohydrate-active enzyme (CAZy) family 4, characterized by a GT-B fold as well as retention of configuration. WaaO and WaaR from *E. coli* K12 and WaaJ and WaaR from *S. typhimurium*, however, belong to CAZy family 8, typified by LgtC being a retaining glycosyltransferase of CAZy family 8 that has been well characterized enzymatically and structurally. These glycosyltransferases are characterized by the GT-A fold, a DXD motif that is implicated in coordination of catalytically important divalent metal ions involved in sugar binding, and by retention of configuration at the anomeric carbon of the donor sugar. One LgtC mechanism invokes an aspartate fairly distant (~9 Å) from the sugar binding site and...
structural dynamics. Like LgtC, *E. coli* K-12 WaaO is capable of specifically hydrolyzing their uridine diphosphate sugar donors in the absence of a lipid acceptor, perhaps suggesting a similar reliance on dynamic structural changes for catalysis. No obvious hydrolysis activity was observed for *S. typhimurium* outer core enzymes, indicating that sugar nucleotide hydrolysis is not necessarily a shared characteristic of GT-A fold glycosyltransferases. The comparison of these similar, yet catalytically distinct, glycosyltransferases may shed further light on the mechanism of this class of enzymes.

WaaO from *E. coli* K-12 was originally proposed to be a nonprocessive enzyme. However, we have shown that, in vitro, WaaO can add several sugars (at least three) to Gal-Glc-Hep$_2$-1-deP-KLA and Glc-Hep$_2$-1-deP-KLA, especially in the presence of MgCl$_2$. Similar results have been observed previously. Shibayama et al. reported that overexpression of *E. coli* K-12 waaO in the waaO-inactivated *E. coli* K-12 C600 strain revealed a minor slow-migrating band besides the prominent band of LPS in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), both of which could be the WaaO product, and the minor band may be due to the overexpression of waaO adding an additional glucose residue due to overexpression of waaI. In addition, Schnaitman et al. reported a doublet band on the SDS–PAGE gel for LPS of *E. coli* K-12 lacking waaR. Given our observation that WaaO can add multiple glucose, the additional sugars observed in vivo may be due to the processive activity of WaaO and deserve further investigation. *S. typhimurium* WaaI, which, like *E. coli* WaaO, also transfers a HexII to Gal-Glc-Hep$_2$-1-deP-KLA, does not add multiple sugars (Figure 10). As *E. coli* WaaO and *S. typhimurium* WaaI are quite similar (~56% identical and ~75% similar), further comparisons will yield insights into the mechanism by which *E. coli* WaaO adds multiple sugars to Gal-Glc-Hep$_2$-1-deP-KLA. The synthesis of these defined lipid A OS's opens the possibility for careful characterization of the glycosyltransferase mechanisms of these enzymes as well as glycosyltransferases from other Gram-negative bacteria. The synthesis of the entire *S. typhimurium* outer core OS on a defined lipid acceptor opens the possibility for careful biochemical characterization of enzymes involved in O-antigen ligation.

Further experiments might focus on creating novel lipid A OS's by using outer core glycosyltransferases from other Gram-negative bacteria that have different sugar nucleotide specificities. Both Hep$_2$-Kdo$_2$-lipid A and the various in vitro-synthesized lipid A OS's we report here may be useful clinically as vaccine adjuvants. 4′-Monophosphoryl-lipid A, prepared by chemical hydrolysis of LPS, is routinely used as a vaccine adjuvant; *Salmonella* with 1-dephosphorylated LPS were attenuated for virulence, yet their ability to elicit an immune
response was not diminished. Here we demonstrate the ability to produce 1-dephosphorylated lipid A OS's that can be tested for their ability to modulate the immune response.

**ASSOCIATED CONTENT**

* Supporting Information

A list of oligonucleotides used in cloning (Table S1), abbreviations used for lipid A OS's (Table S2), WaaG sugar-nucleotide dependence (Figure S1), Triton X-100 dependence (Figure S2), divalent cation dependence (Figures S3 and S4), WaaB sugar dependence (Figure S5), and Waa UDP-Gal dependence (Figure S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

**ABBREVIATIONS**

LPS, lipopolysaccharide; OS, oligosaccharide; 1,6-Hep, 1-glycerol-6-manno-heptose; KO, knockout; ESI-MS, electrospray ionization mass spectrometry; HEPES, 4-(2-hydroxyethyl)-1-piperazinenuetanesulfonic acid; TLC, thin-layer chromatography; IPTG, isopropyl β-D-1-thiogalactopyranoside; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; UDP-Glc, uridine diphosphate glucose; UDP-Gal, uridine diphosphate galactose; Hep2-1-deP-KLA, heptose2-1-dephospho-Kdo2-lipid A (see Table S2 of the Supporting Information for full abbreviations of other lipid A OS's); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetaacetic acid; Hex, hexose.

**REFERENCES**


