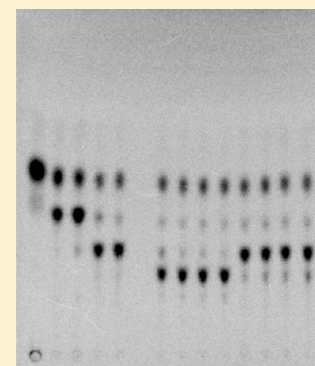


In Vitro Assembly of the Outer Core of the Lipopolysaccharide from *Escherichia coli* K-12 and *Salmonella typhimurium*

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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 $\Delta waaG$ *E. coli* overexpressing the lipid A phosphatase *lpxE* and the lipid A late acyltransferase *lpxM*. The action of *lpxE* and *lpxM* in the $\Delta waaG$ background yielded heptose₂-1-dephospho Kdo₂-lipid A, a 1-dephosphorylated hexaacylated 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 heptose₂-1-dephospho Kdo₂-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 WaaI and WaaO require divalent metal ions for optimal activity; however, WaaO, unlike WaaI, can add several glucose residues to its lipid acceptor. Using the product of WaaG, WaaB, and WaaI, we show that *S. typhimurium* WaaJ and WaaK transfer a glucose and *N*-acetylglucosamine, 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-heptose⁴ 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.² WaaG has been identified as the UDP-glucose:(heptosyl) lipopolysaccharide α -1,3-glycosyltransferase 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:(glucosyl) 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-glycosyltransferase] 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 \rightarrow 6 linkage by WaaB, the UDP-galactose:(glucosyl) lipopolysaccharide α -1,6-galactosyltransferase.⁵ HexIII is appended with an α -1 \rightarrow 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 \rightarrow 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

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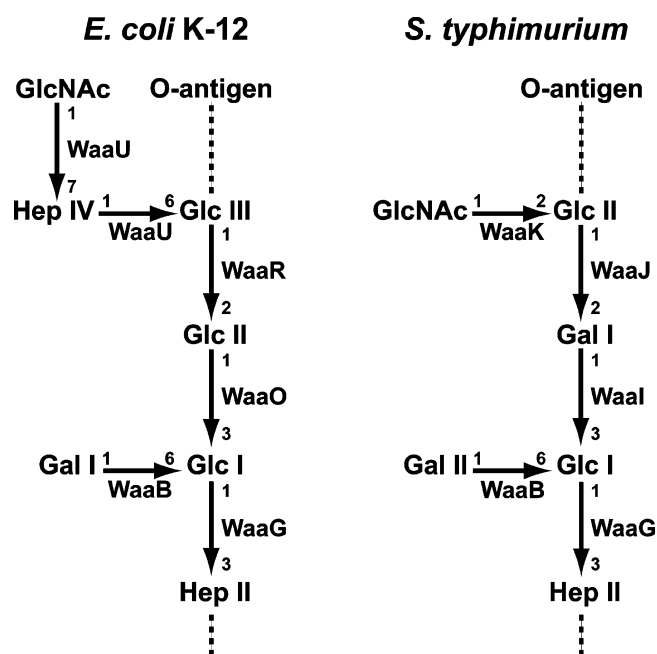


Figure 1. *E. coli* K-12 and *S. typhimurium* LPS outer core oligosaccharides (OS's). The enzymes proposed to catalyze each glycosylation are shown. The linkages among the sugars are indicated;¹ all are α linkages except sugars added by WaaU from *E. coli* K12, which are β linkages. The dashed line attached 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.⁹ In *Salmonella enterica* serovar *Typhi*, the outer core terminal glucose residue (Glc II) is required for the entry of the bacteria into epithelial cells.¹¹ In addition, the inner and outer core OS links lipid A and the O-antigen, both of which are potent modulators of innate immunity.^{1,12}

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.^{13,14} 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 required.^{15,16} In this work, we generated an outer core OS acceptor that is easily isolated by extraction with organic solvents and has a defined structure. The overexpression of the lipid A late acyltransferase *lpxM*¹⁷ and lipid A 1-phosphatase^{18,19} in the $\Delta waaG$ *E. coli* strain yields a lipid A OS, with decreased hydrophilicity due to removal of the 1-phosphate (by *lpxE*), a decreased level of phosphorylation of the inner core heptoses (due to $\Delta waaG$), and a higher proportion of secondary acyl chains (due to *lpxM*). Using this lipid acceptor and commercially available sugar nucleotides, we report the *in vitro* characterization of six outer core glycosyltransferases, *E. coli* WaaG, WaaB, and WaaO and *S. typhimurium* WaaI, WaaJ, and WaaK, and demonstrate the assembly of the entire *S. typhimurium* outer core OS *in vitro*.

■ 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]P_i, 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-acetylglucosamine, 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).²⁰ When required for the selection of plasmids, cells were grown in the presence of 100 $\mu\text{g}/\text{mL}$ ampicillin, 30 $\mu\text{g}/\text{mL}$ chloramphenicol, or 30 $\mu\text{g}/\text{mL}$ 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.²¹ 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.

Cloning, Overexpression, and Purification of *E. coli* K-12 WaaG, WaaB, and WaaO and *S. typhimurium* WaaI, 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 NdeI site was incorporated at the N-terminus and a BamHI or XhoI site was incorporated at the C-terminus. Each PCR product was digested with NdeI and BamHI or XhoI and ligated into a similarly digested pET-21b or pET-28b expression vector as indicated in Table 1. *S. typhimurium waaI*, *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 A_{600} of 0.02 and grown to an A_{600} 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 4000g 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 27000g for 30 min. The final cell pellet was resuspended in 30 mL of buffer

Table 1. Bacterial Strains and Plasmids

strain or plasmid	description	source or reference
strains		
<i>S. typhimurium</i>		
LT2	wild type	Salmonella Genetic Stock Center (University of Calgary, Calgary, AB)
<i>E. coli</i>		
W3110	wild type, F- λ	<i>E. coli</i> Genetic Center (Yale University, New Haven, CT)
BL21(DE3)	F ⁻ <i>ompT hsdSB</i> (r _B ⁻ , m _B ⁻) <i>gal dcm</i> (DE3)	Novagen
CWG303	<i>E. coli</i> F470 (<i>waaG::Gen</i>)	gift from C. Whitfield ⁹
JQ2	W3110 (<i>waaG::Gen</i>) pWFnlpxE, pBAD-lpxM	
plasmids		
pWSK29	low-copy number vector, <i>lac</i> promoter, Amp ^R	38
pWFnlpxE	pWSK29 harboring <i>Francicella novicida</i> <i>lpxE</i>	19
pBAD33	medium-copy number vector, Cam ^R	39
pBAD-lpxM	pBAD33 harboring <i>lpxM</i>	gift from D. Six ^a
pECG28	pET28b harboring <i>E. coli</i> K-12 <i>waaG</i>	
pECB21	pET21b harboring <i>E. coli</i> K-12 <i>waaB</i>	
pECI21	pET21b harboring <i>E. coli</i> K-12 <i>waaO</i>	
pSTI28	pET28b harboring <i>S. typhimurium</i> <i>waaI</i>	
pSTJ28	pET28b harboring <i>S. typhimurium</i> <i>waaJ</i>	
pSTK28	pET28b harboring <i>S. typhimurium</i> <i>waaK</i>	

^a*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 G56²² medium containing 100 μ g/mL ampicillin, 30 μ g/mL chloramphenicol, 1 mM IPTG, and 0.02% arabinose was inoculated with 100 μ L of an overnight culture of JQ2 (Table 1). The culture was incubated at 37 °C while being shaken at 225 rpm until A_{600} 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) CHCl₃/CH₃OH/PBS]²³ 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 CHCl₃ and 1 mL of PBS were added to generate a two-phase neutral Bligh–Dyer extraction mixture [2:2:1.8 (v/v/v) CHCl₃/CH₃OH/PBS].²³ 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 N₂. The dried lipid films were redissolved in a CHCl₃/CH₃OH mixture [4:1 (v/v)] and spotted onto a 10 cm × 20 cm TLC plate. The TLC plate was developed in the CHCl₃/CH₃OH/CH₃COOH/H₂O 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]heptose₂-1-dephosphorylated-KDO₂-lipid A (Hep₂-1-deP-KLA). The region of the silica plate containing the product was dampened with H₂O, 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 CHCl₃ 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 N₂. 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 ³²[P]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 μ Ci 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 μ L. The reaction mixture was converted to a one-phase neutral Bligh–Dyer mixture by the addition of CHCl₃ and CH₃OH. 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 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 N_2 , the lipid films were dissolved in 50 μL of buffer B. WaaB (0.2 mg/mL) and 5 mM UDP-Gal were added to the extracted WaaG 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 \times 20 cm TLC plate and developed in a $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{CH}_3\text{COOH}/\text{H}_2\text{O}$ 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 3 min to locate the [^{32}P]Gal-Glc-Hep₂-1-deP-KLA. The region of product was scraped, extracted, and purified as described above for the purification of [^{32}P]Hep₂-1-deP-KLA, with a yield of ~10% of the starting lipid.

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 A_{600} of 1.8 and harvested by centrifugation at 4000g 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 CHCl_3 and 100 mL of CH_3OH 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 CHCl_3 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 $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ mixture [2:3:1 (v/v/v)] and applied

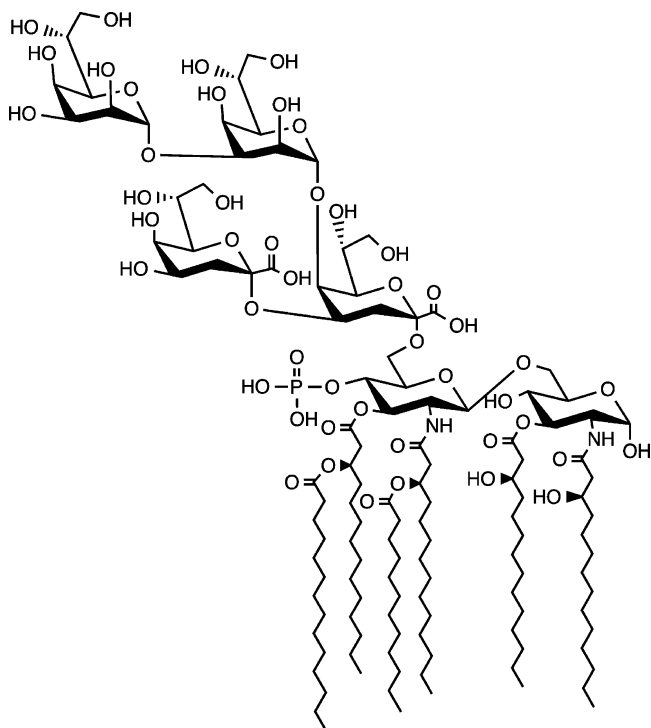


Figure 2. Structure of Hep₂-1-deP-KLA. The structure of the lipid A OS purified from JQ2 is shown.

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 $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ mixture [2:3:1 (v/v/v)]. The bound phospholipids and LPS were eluted using 25 mL steps of the $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{CH}_3\text{COONH}_4$ mixture [2:3:1 (v/v/v)], with successive $\text{CH}_3\text{COONH}_4$ 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 $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{CH}_3\text{COOH}/\text{H}_2\text{O}$ 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 CHCl_3 and H_2O added to generate a 2:2:1.8 (v/v/v) $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{aqueous}$ mixture. The lower phase was dried under a stream of N_2 . The collected lipid was redissolved in a $\text{CHCl}_3/\text{CH}_3\text{OH}$ mixture [4:1 (v/v)] and spotted along the bottom of a 10 cm \times 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 $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{CH}_3\text{COOH}/\text{H}_2\text{O}$ 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.

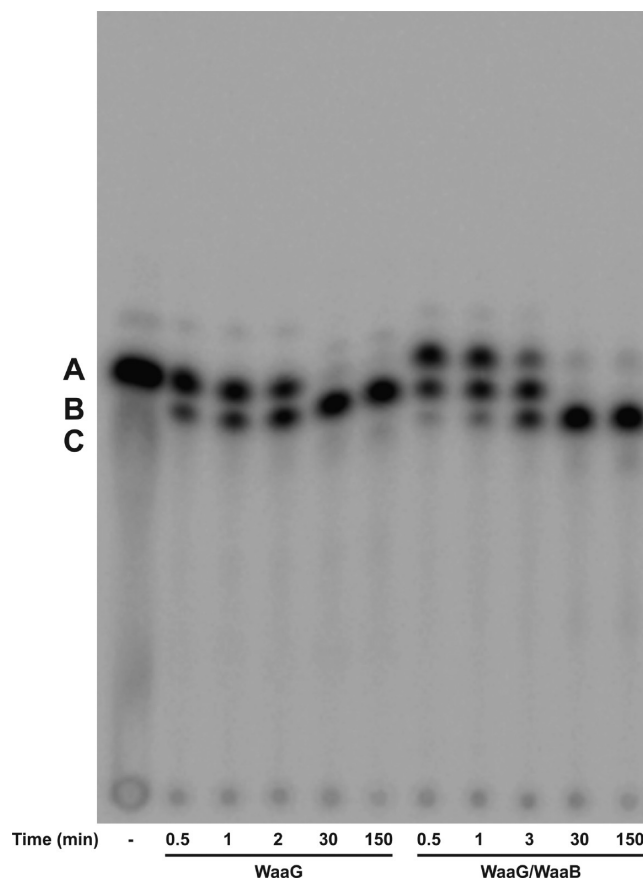


Figure 3. Glycosylations catalyzed by *E. coli* WaaG and WaaB. [^{32}P]Hep₂-1-deP-KLA (marked A) was incubated with WaaG and/or WaaB (0.01 mg/mL each) for the times indicated as described in Materials and Methods. Both UDP-Glc (1 mM) and UDP-Gal (1 mM) were included in the reaction mixture. B marks the location of the WaaG product, Glc-Hep₂-1-deP-KLA. C marks the location of the WaaB product, Gal-Glc-Hep₂-1-deP-KLA.

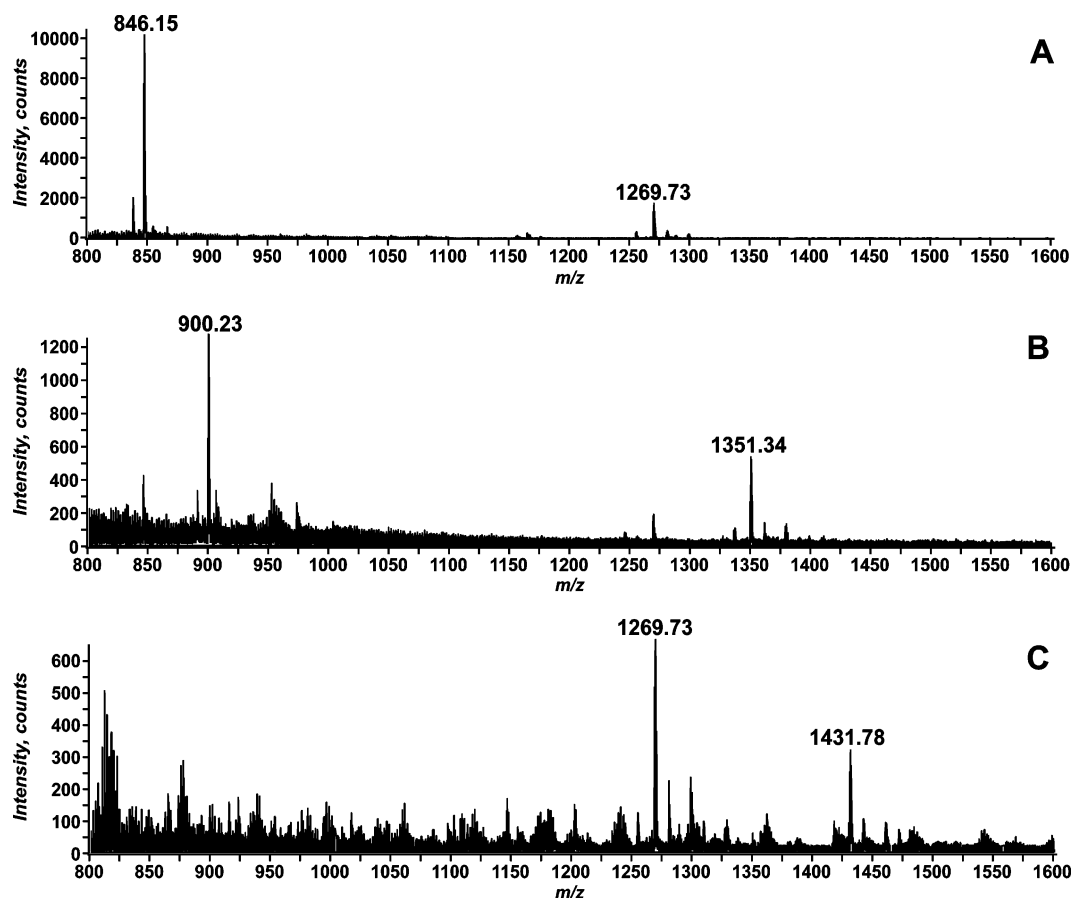


Figure 4. Negative ion ESI-MS of the lipid A OS's, Hep₂-1-deP-KLA, Glc-Hep₂-1-deP-KLA, and Gal-Glc-Hep₂-1-deP-KLA. Panel A shows the mass spectrum from m/z 800 to 1600 for the Hep₂-1-deP-KLA isolated from JQ2. The $[M - 2H]^{2-}$ ion at m/z 1269.73 and the $[M - 3H]^{3-}$ ion at m/z 846.15 correspond by mass to Hep₂-1-deP-KLA (expected values of m/z 1269.7409 and 846.1582, respectively). Panel B shows the mass spectrum from m/z 800 to 1600 for the product isolated from the reaction catalyzed by WaaG using Hep₂-1-deP-KLA and UDP-Glc as substrates. The $[M - 2H]^{2-}$ ion at m/z 1351.34 and the $[M - 3H]^{3-}$ ion at m/z correspond by mass to the expected WaaG product, Glc-Hep₂-1-deP-KLA (expected values of m/z 1351.2690 and 900.1758, respectively). Panel C shows the mass spectrum from m/z 800 to 1600 for the product of the WaaG and WaaB coupled reaction. The product, Gal-Glc-Hep₂-1-deP-KLA, is detected as an $[M - 2H]^{2-}$ ion at m/z 1431.78 (expected value of m/z 1431.7937). Unreacted substrate, Hep₂-1-deP-KLA, is also detected as an $[M - 2H]^{2-}$ ion at m/z 1269.73.

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-^[14C]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 ^[14C]Glc into ^[14C]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 μ M ^[32P]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 μ L). Next,

WaaB (0.1 mg/mL) and 1 mM UDP-Gal were added to the reaction mixture (final volume of 52 μ L) and incubated at 25 °C for 30 min. WaaO (0.1 mg/mL) and 1 mM MgCl₂ were added to the reaction solution after WaaB reacted at 25 °C for 30 min (final volume of 54 μ L).

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 ^[32P]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 MgCl₂. The preparation of ^[32P]Gal-Glc-Hep₂-1-deP-KLA is described above.

Reactions were stopped by spotting 3–5 μ L portions of the reaction mixtures onto a TLC plate. After being dried in a stream of cold air, plates were developed in the CHCl₃/CH₃OH/CH₃COOH/H₂O 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 PhosphoImager.

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

above the predicted K_m . The data were fit to the Michaelis–Menten equation using Kaleidagraph (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 $\text{CHCl}_3/\text{CH}_3\text{OH}$ mixture [2:1 (v/v)], supplemented with 1% piperidine, and immediately infused into the ion source at a rate of 5–10 $\mu\text{L}/\text{min}$. The negative ion ESI was conducted at -4200 V .¹⁸ 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.¹⁰ The lipid A 1-phosphatase *lpxE*¹⁹ and the lipid A late acyl transferase *lpxM*¹⁷ 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 $[\text{M} - 2\text{H}]^{2-}$ and $[\text{M} - 3\text{H}]^{3-}$ ions of Hep₂-1-deP-KLA, respectively (expected values of m/z 1269.7409 and 846.1582, respectively).

WaaG Transfers the Outer Core HexI. 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 is 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 $[\text{M} - 2\text{H}]^{2-}$ and $[\text{M} - 3\text{H}]^{3-}$ 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 $0.162 \pm 0.028\text{ mM}$ and $3.85 \pm 1.80\text{ }\mu\text{M}$, respectively, and the k_{cat} is 0.5 s^{-1} , similar to the values reported previously when WaaG was characterized in crude extracts.^{7,8}

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 Mg^{2+} 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 Mg^{2+} is added to the assay. WaaG was similarly inhibited by other divalent metal ions such as Mn^{2+} , Ca^{2+} , Zn^{2+} , Co^{2+} , Ni^{2+} , and Cu^{2+} (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

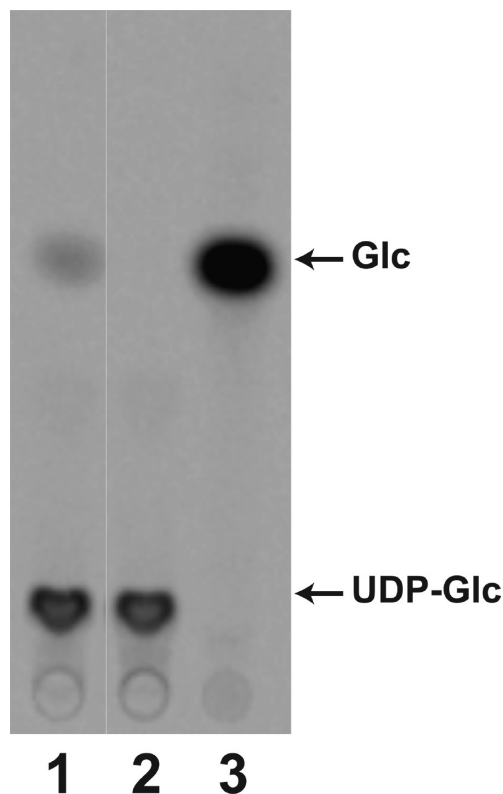


Figure 5. WaaG hydrolyzes UDP-Glc in the absence of a lipid acceptor. UDP-[U-¹⁴C]Glc was incubated in the presence (lane 1) or absence (lane 2) of WaaG (0.25 mg/mL) and then displayed using TLC as described in Materials and Methods. [U-¹⁴C]Glc was spotted as a control in lane 3 to indicate the migration of free glucose.

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 $3.6 \pm 0.8\text{ mM}$ 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).

WaaB Catalyzes the Transfer of Galactose to the *E. coli* K-12 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 coupled *in vitro* with the WaaG reaction, 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 $[\text{M} - 2\text{H}]^{2-}$ 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,

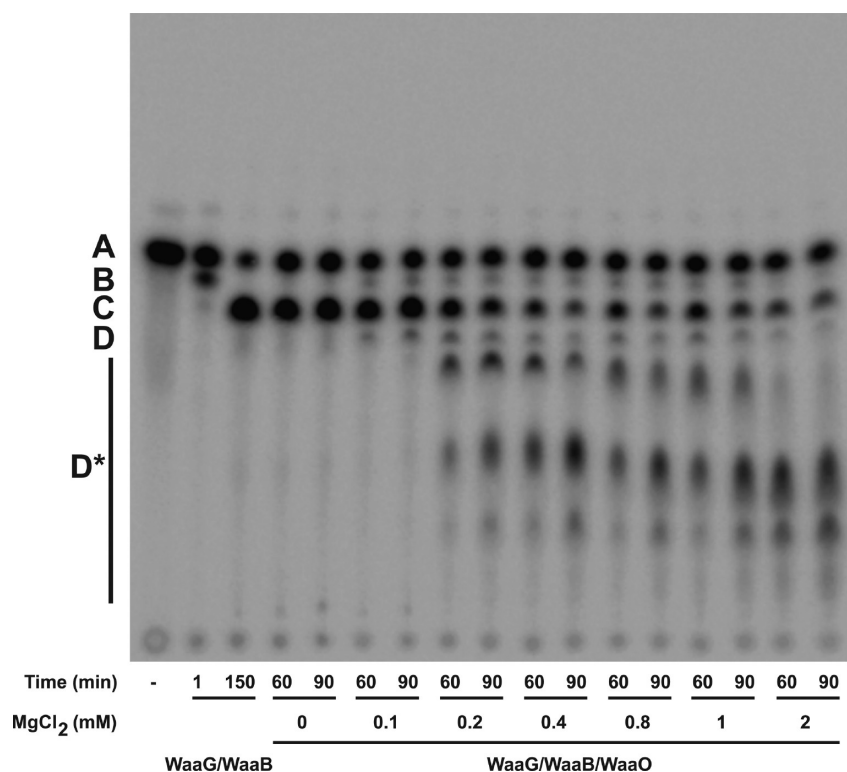


Figure 6. Mg^{2+} concentration affects the activity of *E. coli* K-12 WaaO. Hep₂-1-deP-KLA (labeled A) was reacted with WaaG (0.01 mg/mL) for 15 min to first produce Glc-Hep₂-1-deP-KLA (labeled B). WaaB (0.1 mg/mL) was then added and incubated for an additional 135 min to produce Gal-Glc-Hep₂-1-deP-KLA (labeled C). WaaO was added (0.1 mg/mL) in the presence of various concentrations of Mg^{2+} as indicated after WaaB reacted as indicated. When Mg^{2+} was excluded, no WaaO-dependent glycosylation was detected. However, when Mg^{2+} was included, a product with migration consistent with glucose addition was detected. At a low Mg^{2+} concentration (0.1 mM), one product is detected (labeled D), but as the Mg^{2+} concentration is increased, multiple products (labeled D*) are detected, consistent with the addition of multiple glucoses to Gal-Glc-Hep₂-1-deP-KLA.

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 Mg^{2+} 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 Mg^{2+} is increased, the number of products increased (D*, Figure 6). These glucosylated products (Glc_{*n*}-[Gal]-Glc-Hep₂-1-deP-KLA) were detected only when Mg^{2+} was included in the reaction mixture (Figure 6), indicating that WaaO activity is dependent on divalent metal ions. Taken together, these data strongly suggest that WaaO is capable of adding multiple sugars to the outer core OS. As seen in Figure 7, the formation of these multiglucosylated products, Glc_{*n*}-[Gal]-Glc-Hep₂-1-deP-KLA, increases with time in the presence of WaaO and 1 mM Mg^{2+} .

To determine if the galactose added by WaaB was necessary for the glucosylation catalyzed by WaaO, we coupled the WaaO reaction to only the WaaG reaction. In the presence of WaaO, multiple products whose migration is consistent with glucosylation of the WaaG product Glc-Hep₂-1-deP-KLA to

Glc₂-Hep₂-1-deP-KLA [product C_O (Figure 8)] and Glc_{*n*}-Hep₂-1-deP-KLA [C_O* (Figure 8)] are formed. The glucosylation of Glc-Hep₂-1-deP-KLA by WaaO was similarly dependent on Mg^{2+} (data not shown); multiple glucosylated products were observed only in the presence of Mg^{2+} . 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 K_m for UDP-Glc in this hydrolysis reaction is 1.95 ± 0.4 mM, and the k_{cat} 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

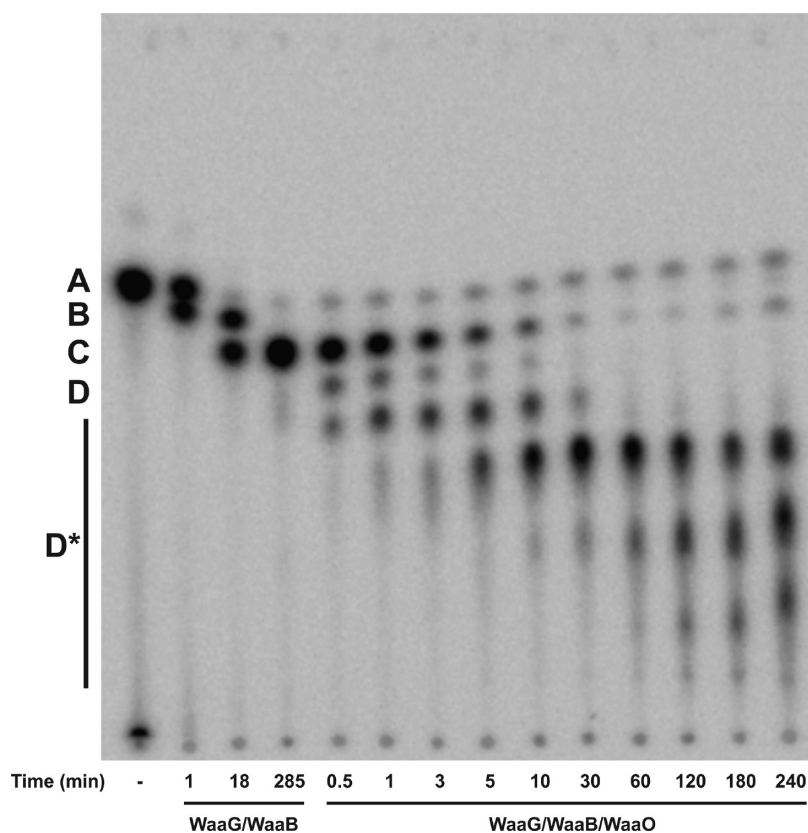


Figure 7. Time dependence of the *E. coli* K-12 WaaO reaction. Hep₂-1-deP-KLA (labeled A) was reacted with WaaG (0.01 mg/mL) and WaaB (0.1 mg/mL) for 285 min as described in the legend of Figure 6. *E. coli* K-12 WaaO (0.1 mg/mL) and 1 mM MgCl₂ were added, and a portion of the reaction mixture was spotted onto a TLC plate at the times indicated. WaaO catalyzes the formation of a single glucose addition (labeled D) and at increased times additional glucoses (labeled D*).

organisms; in *S. typhimurium*, a galactose is added instead of glucose. This suggests the *S. typhimurium* WaaI will preferentially add a galactose to Gal-Glc-Hep₂-1-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-Hep₂-1-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-Hep₂-1-deP-KLA (D_s, 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-Hep₂-1-deP-KLA through coupling only to the WaaG reaction, a faint product consistent with Gal*-Glc-Hep₂-1-deP-KLA (the asterisk indicates this Gal is added in a linkage different from that of the Gal added by WaaB) is observed, suggesting that WaaI does not require the branched α 1 \rightarrow 6 galactose added by WaaB (Figure S6 of the Supporting Information).

The *S. typhimurium* LPS Outer Core HexIII Is Transferred by WaaJ. *S. typhimurium* WaaJ is proposed to add the outer core HexIII. To test this, purified *S. typhimurium* WaaJ was coupled to the reaction of *S. typhimurium* WaaI with Gal-Glc-Hep₂-1-deP-KLA in the presence of UDP-Gal or UDP-Glc (Figure 11). *S. typhimurium* WaaJ shows some promiscuous activity when UDP-Gal was used as the only sugar donor; however, the activity is greatly increased when UDP-Glc is added

as the sugar donor. The enhanced formation of what is presumed to be Glc-Gal-[Gal]-Glc-Hep₂-1-deP-KLA (E_s in Figure 11, last two lanes) is consistent with the structure of the *S. typhimurium* LPS outer core,² though WaaJ likely has some ability to catalyze the addition of HexIII using UDP-Gal as the sugar donor.

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-Hep₂-1-deP-KLA was tested. Gal-Glc-Hep₂-1-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-Hep₂-1-deP-KLA in the presence of UDP-Glc and UDP-Gal to form Gal-[Gal]-Glc-Hep₂-1-deP-KLA (Figure 12, product D_s) and then Glc-Gal-[Gal]-Glc-Hep₂-1-deP-KLA (Figure 12, product E_s). 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 F_s, GlcNAc-Glc-Gal-[Gal]-Glc-Hep₂-1-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

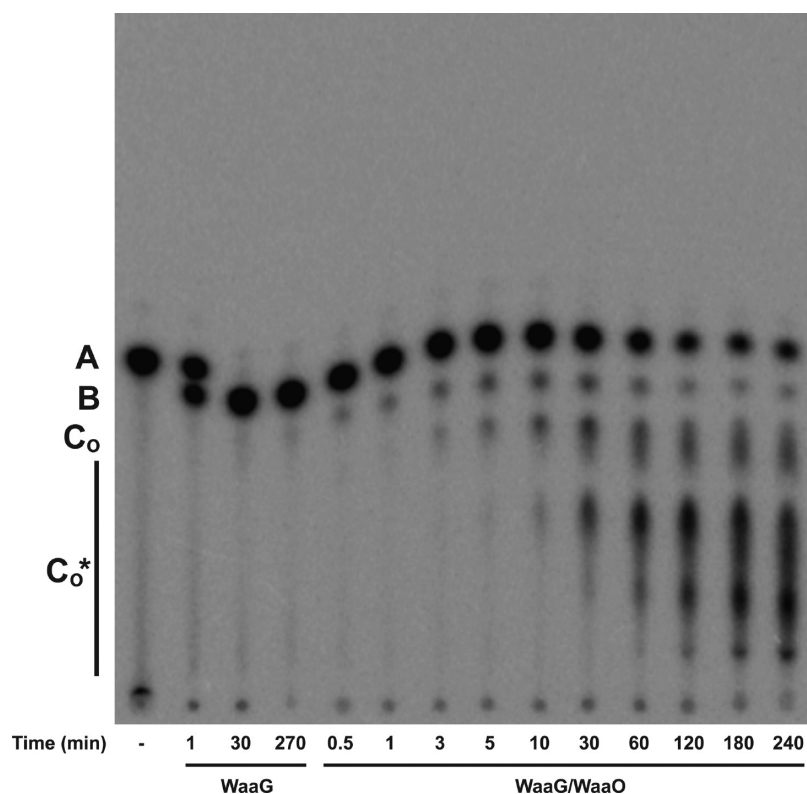


Figure 8. *E. coli* K-12 WaaO does not require the galactose added by WaaB. Hep₂-1-deP-KLA (labeled A) was reacted with WaaG (0.01 mg/mL) for 270 min to produce Glc-Hep₂-1-deP-KLA (labeled B). WaaO (0.1 mg/mL) and 1 mM MgCl₂ were added to the WaaG reaction mixture and incubated for the times indicated. Initially, the product, Glc₂-Hep₂-1-deP-KLA (labeled C₀), is formed. The products labeled C₀* indicate the addition of multiple sugars by Waa.

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.⁹ 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 novocida* lipid A 1-phosphatase,¹⁹ into a strain lacking *waaG* decreases the hydrophilicity of the major LPS species. Furthermore, the incorporation of *lpxM*, the lipid A late acyltransferase,²⁵ 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 ³²P.

Detailed analysis of outer core glycosyltransferases has been limited by the purity of the LPS acceptor and the assay method.¹⁹ Usually, assays depended on following the incorporation of [¹⁴C]glucose or [¹⁴C]galactose with scintillation counting into the LPS acceptor. The availability of Hep₂-1-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 ³²P or by using ¹⁴C-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.^{2,5,13,15} 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, Hep₂-1-deP-KLA (Figures 3 and 4). While *E. coli* WaaG is inhibited by Mg²⁺ (Figure S2 of the Supporting Information), *E. coli* WaaO can add multiple sugars under similar conditions (Figure 6). Because the physiological concentration of Mg²⁺ in *E. coli* could be in the millimolar range, these *in vitro* activities may be physiologically relevant.^{10,16}

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-Hep₂-1-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-Hep₂-1-deP-KLA. The role of WaaB as the second enzyme to act in the LPS outer core assembly in *E. coli* K-12^{10,16} 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-Hep₂-1-deP-KLA than toward Gal-Glc-Hep₂-1-deP-KLA, the product of WaaG and WaaB, suggesting that the addition of HexII occurs after HexI 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).

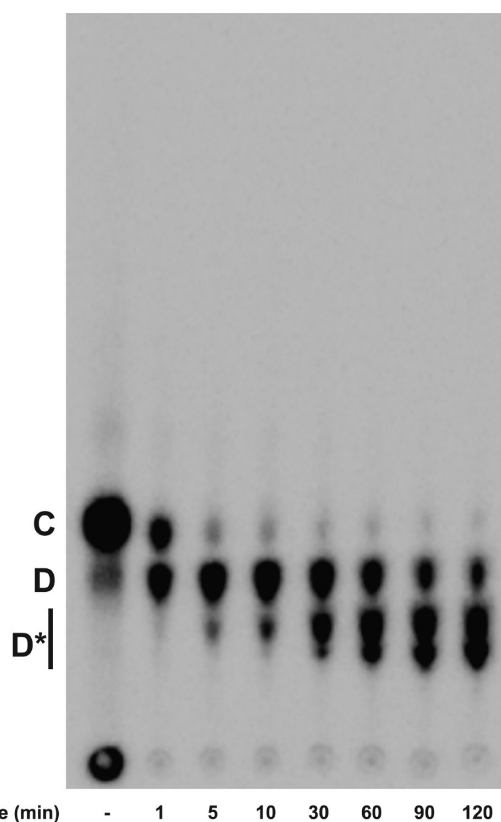
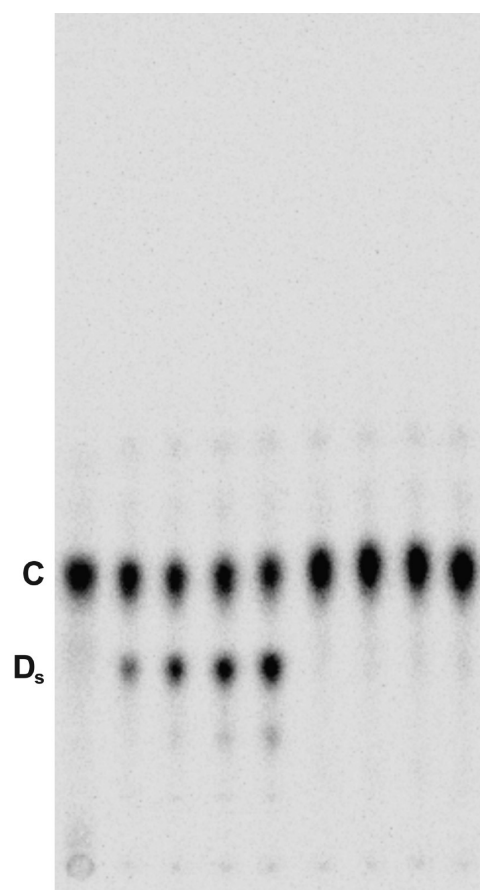


Figure 9. *E. coli* K-12 WaaO glycosylates Gal-Glc-Hep₂-1-deP-KLA, the product of WaaG and WaaB. Purified Gal-Glc-Hep₂-1-deP-KLA (labeled C), produced from Hep₂-1-deP-KLA by WaaG and WaaB as described in Materials and Methods, was reacted with WaaO in the presence of 1 mM MgCl₂ and UDP-Glc to verify that the products attributed to WaaO were not due to WaaG or WaaB. Products consistent with the addition of one glucose (labeled D) and then additional glucoses (labeled D*) are formed.

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). This defined, *in vitro*-assembled *S. typhimurium* LPS outer core on a defined lipid A anchor can serve as a substrate for investigating the ligation of the O-antigen.

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.¹⁶ 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.¹⁶ Our *in vitro* data for *S. typhimurium* WaaI 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 Δgal



Time (min)	-	1	5	10	30	1	5	10	30
WaaI	-	+	+	+	+	+	+	+	+
UDP-Gal	-	+	+	+	+	-	-	-	-
UDP-Glc	-	-	-	-	-	+	+	+	+

Figure 10. *S. typhimurium* WaaI uses UDP-Gal but not UDP-Glc. *S. typhimurium* WaaI (0.1 mg/mL) was incubated with Gal-Glc-Hep₂-1-deP-KLA (labeled C), in the presence of UDP-Gal or UDP-Glc and 1 mM MgCl₂ as indicated. The major product, Gal-[Gal]-Glc-Hep₂-1-deP-KLA, is indicated by D_s. No product was formed when UDP-Glc was used as the sugar donor.

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.²⁶ 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,^{27–29} 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,^{27–29} typified by LgtC being a retaining glycosyltransferase of CAZy family 8 that has been well characterized enzymatically and structurally.^{30,31} 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.^{4,14} One LgtC mechanism invokes an aspartate fairly distant (~9 Å) from the sugar binding site³¹ and

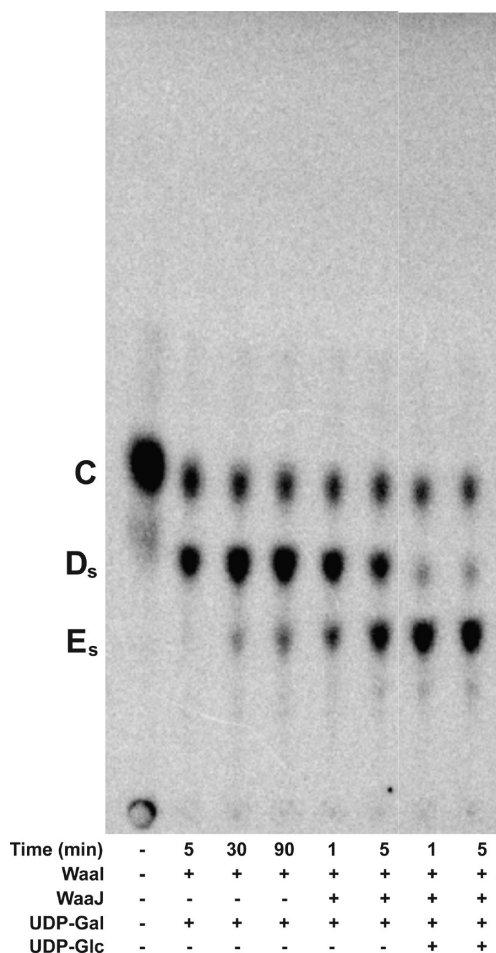


Figure 11. *S. typhimurium* WaaJ preferentially uses UDP-Glc but not UDP-Gal. Gal-Glc-Hep₂-1-deP-KLA (labeled C) and UDP-Gal were reacted with *S. typhimurium* WaaJ (0.1 mg/mL) to produce Gal-[Gal]-Glc-Hep₂-1-deP-KLA (labeled D_s). After 90 min, *S. typhimurium* WaaJ was added and incubated for the times indicated in the presence of UDP-Gal or UDP-Glc. The product labeled E_s is proposed to be Glc-Gal-[Gal]-Glc-Hep₂-1-deP-KLA.

structural dynamics.³² 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.³³ However, we have shown that, *in vitro*, WaaO can add several sugars (at least three) to Gal-Glc-Hep₂-1-deP-KLA and Glc-Hep₂-1-deP-KLA, especially in the presence of MgCl₂. Similar results have been observed previously; Shibayama et al.³³ 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*.³³ In addition, Schnaitman

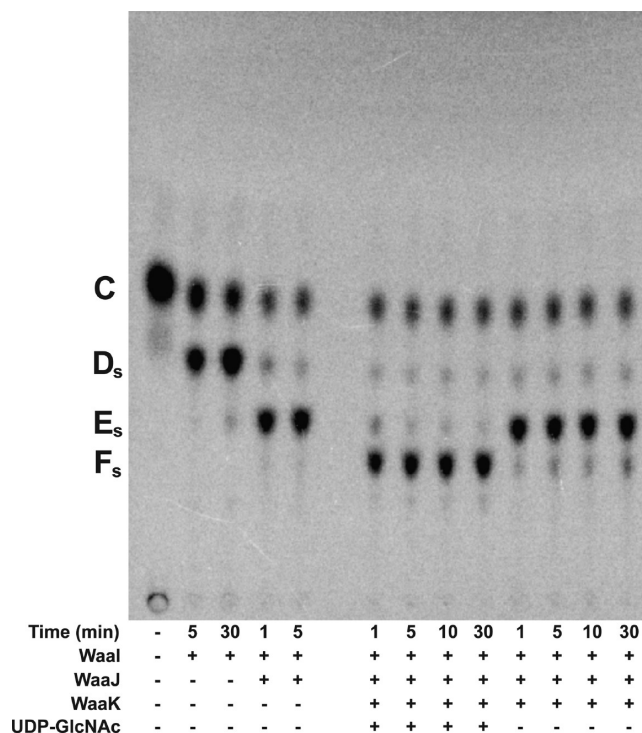


Figure 12. *In vitro* synthesis of the entire *S. typhimurium* outer core OS. Gal-Glc-Hep₂-1-deP-KLA (labeled C) was reacted with *S. typhimurium* WaaI (0.1 mg/mL) for 30 min to generate Gal-[Gal]-Glc-Hep₂-1-deP-KLA (labeled D_s). Next, WaaJ (0.1 mg/mL) was added to generate Glc-Gal-[Gal]-Glc-Hep₂-1-deP-KLA (labeled E_s). *S. typhimurium* WaaK (0.1 mg/mL) was added in the presence or absence of 1 mM UDP-GlcNAc and incubated for the times indicated. Only in the presence of UDP-GlcNAc was the product labeled F_s formed. This product represents the *in vitro* formation of the entire outer core on a defined lipid acceptor.

et al.¹⁰ 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 glucoses, 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-Hep₂-1-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),³⁴ further comparisons will yield insights into the mechanism by which *E. coli* WaaO adds multiple sugars to Gal-Glc-Hep₂-1-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* OS on a defined lipid anchor 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 Hep₂-Kdo₂-lipid A and the various *in vitro*-synthesized lipid A OS's we report here may be useful clinically as vaccine adjuvants.³⁵ 4'-Monophosphoryl-lipid A, prepared by chemical hydrolysis of LPS,³⁶ 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.³⁷ 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 WaaI 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; L_D-Hep, L-glycero-D-manno-heptose; KO, knockout; ESI-MS, electrospray ionization mass spectrometry; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic 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 tetraacetic acid; Hex, hexose.

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