Molecular and Structural Basis of Inner Core Lipopolysaccharide Alterations in *Escherichia coli*

**INCORPORATION OF GLUCURONIC ACID AND PHOSPHOETHANOLAMINE IN THE HEPTOSE REGION**

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**Background:** Some of the enzymes that are required for LPS modification(s) are unknown.

**Results:** LPS modifications involving addition of glucuronic acid to heptose III and phosphoethanolamine transfer to heptose I require products of two new genes *waaH* and *epIC*, respectively.

**Conclusion:** Glucuronic acid addition requires PhoB/R activation, and phosphoethanolamine transfer confers detergent resistance.

**Significance:** Nonstoichiometric LPS alterations reflect LPS structural flexibility in response to stress conditions.

It is well established that lipopolysaccharide (LPS) often carries nonstoichiometric substitutions in lipid A and in the inner core. In this work, the molecular basis of inner core alterations and their physiological significance are addressed. A new inner core modification of LPS is described, which arises due to the addition of glucuronic acid on the third heptose with a concomitant loss of phosphate on the second heptose. This was shown by chemical and structural analyses. Furthermore, the gene whose product is responsible for the addition of this sugar was identified in all *Escherichia coli* core types and in *Salmonella* and was designated *waaH*. Its deduced amino acid sequence exhibits homology to glycosyltransferase family 2. The transcription of the *waaH* gene is positively regulated by the PhoB/R two-component system in a growth phase-dependent manner, which is coordinated with the transcription of the *ugd* gene explaining the genetic basis of this modification. Glucuronic acid modification was observed in *E. coli* B, K12, R2, and R4 core types and in *Salmonella*. We also show that the phosphoethanolamine (P-EtN) addition on heptose I in *E. coli* K12 requires the product of the ORF *yijP*, a new gene designated as *epIC*. Incorporation of P-ETN is also positively regulated by PhoB/R, although it can occur at a basal level without a requirement for any regulatory inducible systems. This P-ETN modification is essential for resistance to a variety of factors, which destabilize the outer membrane like the addition of SDS or challenge to sublethal concentrations of Zn2+.

The outer membrane (OM)2 of Gram-negative bacteria such as *Escherichia coli* is an asymmetric bilayer with the lipopolysaccharide (LPS) in the outer leaflet and phospholipids in the inner leaflet (1). LPS are the major amphiphilic constituents of the outer leaflet of the OM. LPS is essential for the bacterial viability and integrity of the OM and provides the permeability barrier function. LPS in general share a common structure composed of an acylated and 1,4’-diphosphorylated β(1→6)-linked glucosamine (GlcN) disaccharide, called lipid A (2, 3). To the lipid A is attached a proximal core oligosaccharide and, in smooth-type bacteria, a distal O-poly saccharide (2). In *E. coli*, the core oligosaccharide can be subdivided into the inner and outer core, and distinct core types have been described (R1, R2, R3, R4, and K12) that differ in the outer core structure (4–6). The inner core is a more conserved structural element of 3-deoxy-α-D-manno-oct-2-ulosylpyranosonic acid (Kdo), L-glycero-α-D-manno-heptopyranosyl (Hep), and phosphate residues. Kdo2-lipid A is considered to be the minimal LPS structure required for viability of bacteria like *E. coli* under optimal growth conditions. The predominant core types among clinical isolates are R1 and R3 (4). Importantly, verotoxigenic isolates belonging to the common enterohemorrhagic *E. coli* such as serogroups O157, O111, and O26 produce LPS of the R3 core type (4, 7). Both of these core types contain structural modifications of the side-chain heptose (HepIII) of the inner core due to a nonstoichiometric incorporation of an α(1→7)-linked GlcN residue (5, 6, 8, 9). This modification is accomplished by a concomitant loss of phosphate at the HepII (10), thereby modifying the charge distribution of the inner core.

The importance of heptose incorporation into the inner core is manifested by our discovery that all genes whose products are required either for heptose biosynthesis or its incorporation are also required for growth at critical high temperatures (11). These include biosynthetic genes for heptose (*gmhA*, *gmhB*, and *gmhD*) and the heptosyltransferases I (*waaC*) and II (*waaF*) (11).
Phosphorylation of the inner core HepI plays a crucial role in the OM stability (12). In the absence of phosphorylation of HepI, HepIII is not incorporated, and HepII phosphorylation is impaired, leading to a truncation of LPS accompanied by permeability and motility defects (deep-rough phenotype) (12). A requirement for the OM permeability/barrier function therefore seems to place structural constraints on the inner core of LPS, accounting for conservation of its base structure (3, 8).

Despite conservation of lipid A and inner core structure, challenges to different stresses, like changes in pH, concentrations of specific ions, and phosphate starvation, are structurally modified by nonstoichiometric substituents. These modifications often result in a modulation of the number of net negative charges. Among the nonstoichiometric substitutions commonly observed in the lipid A are the addition of phosphoethanolamine (P-EtN) and 4-amino-4-deoxy-L-arabinose (Ara4N) (13). Some of these substitutions provide advantages under specific growth niches. For example, the incorporation of P-EtN and Ara4N is known to confer resistance to cationic antimicrobial peptides like polymyxin B (13). Nonstoichiometric structural variations of the E. coli inner core include phosphate, GlcN, rhamnose (Rha), P-EtN, and additional Kdo (8). Some of these substitutions are specific to the individual core type of E. coli. Among these, the physiological significance of P-EtN addition to the second Kdo and phosphorylation of HepI have been addressed to some extent (12, 14, 15).

The nonstoichiometric modifications of the lipid A are positively regulated by the BasS/R two-component system (13). These modifications in the lipid A part arise due to incorporation of P-EtN and Ara4N. In E. coli, EptA is required for the P-EtN transfer to lipid A. The addition of P-EtN to the second Kdo requires EptB (14). The transcription of the eptB gene is induced under conditions of envelope stress and is also negatively controlled by mgrR sRNA (16, 17). The E. coli genome also contains three additional ORFs (ybiP, ybhX, and yijP) whose encoded amino acid sequence bears significant homology to EptA, EptB, and other P-EtN transferases. The nonstoichiometric incorporation of P-EtN to the inner core phosphate of HepI in Salmonella is positively regulated by the PmrA/B two-component system (18). In E. coli, the gene required for this modification was not known, and the corresponding two-component system BasS/R did not seem to be required for changes in the structure of the inner core (15).

We recently addressed the molecular basis of LPS heterogeneity in E. coli K12 (15). During this analysis, we addressed the role of RpoE and its regulators, because RpoE controls and responds to major outer membrane defects (15). Induction of RpoE was found to lead to the increased abundance of glycoforms with a third Kdo accompanied by a truncation in the outer core. This was found to be regulated by the translational repression of WaaR glycosyltransferase due to RpoE-dependent rybB sRNA (15). Furthermore, RpoE induction causes preferential accumulation of glycoform V with Rha attached to the third Kdo and P-EtN on the second Kdo (Fig. 1) (15).

We also showed that LPS heterogeneity is additionally regulated by two-component systems PhoB/R and BasS/R (15). PhoB/R system responds to phosphate limitations, which partially overlaps with BasS/R system. However, the latter was found to be highly inducible under phosphate-limiting conditions when the culture medium was supplemented by submillimolar concentrations of Zn\(^{2+}\) and Fe\(^{3+}\) (17). Under such growth conditions, we showed by mass spectrometry that the LPS of the wild-type E. coli K12 as well as that of E. coli B contains several signals 96 mass units higher than the LPS obtained from bacteria grown under phosphate-rich growth conditions in M9 or LB medium. This modification, specific to the core region, was found to occur in the most common glycoform I as well as in glycoforms IV and V (Fig. 1) and was not induced by the extracytoplasmic function \(\sigma\) factor RpoE or the BasS/R two-component system (15). Also, such modified LPS contained up to three P-EtN residues. One of them was assigned to the lipid A and another to the second Kdo, and the third was predicted to modify phosphorylated HepI (15, 17).

Thus, in this work, we determined the chemical structure of the modified LPS. We describe a novel inner core modification of E. coli LPS arising from the addition of glucuronic acid (GlcUA) to HepIII, with concomitant loss of phosphate on HepII. We also identified the required gene whose product encodes the candidate glycosyltransferase. Mutational analysis and structural examination of LPS allowed us to identify the P-EtN transferase responsible for the addition of P-EtN to phosphorylated HepI. We designated the corresponding gene as eptC and examined the physiological significance of this modification.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Media**—The bacterial strains and plasmids used in this study are described in Table 1. Luria-Bertani (LB) broth, M9, and 121 phosphate-limiting minimal media were prepared as described (15, 19, 20). When necessary, media were supplemented with ampicillin (100 \(\mu\)g ml\(^{-1}\)) and kanamycin (50 \(\mu\)g ml\(^{-1}\)), spectinomycin (50 \(\mu\)g ml\(^{-1}\)), or chloramphenicol (20 \(\mu\)g ml\(^{-1}\)). The indicator dye 5-bromo-4-chloro-3-indolyl-\(\beta\)-galactopyranoside (X-gal) was used at a final concentration of 40 \(\mu\)g ml\(^{-1}\) in the agar medium.

**Generation of Null Mutations and Construction of Their Combinations**—Nonpolar antibiotic-free deletion mutations of various genes were constructed by using the \(\lambda\) Red recombine/FLP-mediated recombination system (21). The coding sequence of each gene was replaced with either the kanamycin (aph) or chloramphenicol (cat) resistance cassette flanked by FRT recognition sequences, using plasmid pKD13 and pKD3 as templates (21). PCR products were used for recombineering on the chromosome of either E. coli K12 strain BW25113 or E. coli B strain BL21 (DE3) containing the \(\lambda\) Red recombinase-encoding plasmid pKD46. Gene replacements and their exact chromosomal locations were verified by PCR and further transduced into either W3110 or in BL21. Multiple null combinations were made through a series of bacteriophage P1-mediated transductions, followed by the removal of the aph or cat cassettes. All the deletions were confirmed to be nonpolar. Construction of deletion derivatives of the eptA, eptB, basR, phoB, and mgrR genes in W3110 were described previously (15, 17).

For protein induction, the minimal coding sequence of the waaH gene of E. coli was cloned in expression vectors pET24b
FIGURE 1. Proposed LPS structures from *E. coli* K12 in phosphate-limiting growth conditions. Schematic drawing of LPS glycoforms I, V, VI, and VII composition with various nonstoichiometric substitutions in the LPS core region is presented. Glycoforms VI and VII have GlcUA addition on the HepIII. The cognate genes, whose products are involved at different steps, are indicated.
TABLE 1

Bacterial strains and plasmids used in this study

| Strains/Plasmids | Relevant characteristic | Reference or source |
|------------------|-------------------------|---------------------|
| W3110            | E. coli Genetic Stock Center, Yale | (21) |
| BL21(DE3)        | Inviromed | (DE3) |
| pBAD-gal-dcm     | E. coli BL21 at an absorbance of 0.1 at 600 nm in a 1-liter culture by the addition of 1 mM IPTG. After induction (4 h at 37 °C), cells were harvested by centrifugation at 7000 rpm for 20 min. The pellet was resuspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole (buffer A)) supplemented with lysozyme to a final concentration of 200 μg/ml. After incubation on ice for 20 min, the mixture was sonicated and centrifuged at 45,000 g for 30 min at 4 °C. Soluble proteins (15 ml) were applied over nickel-nitrilotriacetic acid beads (Qiagen), washed once with ethanol, twice with acetone, and once with diethyl ether and then dried at ambient temperature (yield 654 mg). For the LPS analysis, lyophilized material was dispersed in water by sonication and resuspended by centrifugation (8,671 g, 20 min, 4 °C) and were transferred to the chromosome in single copy by recombination with ARS45, selecting for lysogens as described previously for other promoter fusions (23–25). β-Galactosidase activity was determined as described previously (15).

**Protein Purification**—Expression of hexa-His-tagged WaaH was induced in the strain E. coli BL21 at an absorbance of 0.1 at 600 nm in a 1-liter culture by the addition of 1 mM IPTG. After induction (4 h at 37 °C), cells were harvested by centrifugation at 7000 rpm for 20 min. The pellet was resuspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole (buffer A)) supplemented with lysozyme to a final concentration of 200 μg/ml. After incubation on ice for 20 min, the mixture was sonicated and centrifuged at 45,000 × g for 30 min at 4 °C. Soluble proteins (15 ml) were applied over nickel-nitrilotriacetic acid beads (Qiagen), washed, and eluted with buffer A containing 150 mM imidazole.

**LPS Extraction**—For most of the experiments, cultures of isogenic bacteria were grown in a rotary shaker at 190 rpm in the phosphate-limiting medium with appropriate antibiotic at 37 °C until an absorbance of 0.8–1.0 at 600 nm. In specific cases, LPS from Salmonella and different representative E. coli core types was obtained from cultures grown in LB medium with or without supplementation of 25 mM ammonium metavanadate (NH4VO3). Four hundred-ml cultures were harvested by centrifugation at 7000 rpm for 30 min and dried. LPS was extracted by the phenol/chloroform/petroleum ether procedure (26) and lyophilized. For the LPS analysis, lyophilized material was dispersed in water by sonication and resuspended at a concentration of 2 mg ml−1.

For the LPS analysis by NMR spectroscopy, E. coli BL21 strain was grown in 40 liters of phosphate-limiting 121 medium at 37 °C for 24 h under shaking (150 rpm). Bacteria were harvested by centrifugation (8,671 × g, 20 min, 4 °C) and were washed once with ethanol, twice with acetone, and once with diethyl ether and then dried at ambient temperature (yield 2 g). The LPS was obtained by the phenol/chloroform/petroleum ether procedure (26) and lyophilized. For the LPS analysis, lyophilized material was dispersed in water by sonication and resuspended at a concentration of 2 mg ml−1.

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(yield 195 mg). Of these, 50 mg were separated by high performance anion-exchange chromatography (HPAEC; 5 runs of 10 mg each) using a semi-preparative CarboPak PA100 column (9 × 250 mm) and a DX300 chromatography system ( Dionex). Fractions were analyzed by analytical HPAEC, and those containing the main oligosaccharide were combined and desalted on Sephadex G-10 (GE Healthcare) in 10 mM NH₄HCO₃ buffer (yield 3.6 mg). Conditions for semi-preparative and analytical HPAEC were essentially as described previously (6).

**NMR Spectroscopy**—For NMR spectroscopy, 3 mg of the purified and lyophilized main oligosaccharide were exchanged three times with D₂O by rotary evaporation and finally dissolved in 400 μl of D₂O (99.98%, Deutero GmbH). To ensure a uniformly charged state of the oligosaccharide, NaOD (4 μl of 40%) was added. NMR spectra were recorded at 300 K on a Bruker Avance III 700 MHz ultrashield plus spectrometer equipped with a 5-mm CPQCI ¹H, ³¹C, and ³²P NMR probe head. One-dimensional ¹H, ¹³C, and ³¹P NMR spectra, two-dimensional ¹H, ¹³C-HMQC (hmjghr), NOESY (noesypyr, 200-ms spin lock pulse at 8.43 db), and HSQC-TOCSY (hsqcgpr, 90-ms spin lock, 90° spin lock pulse 26°) were recorded using the indicated ¹H, ¹³C-HMBC optimized for long range coupling constants of 10 Hz (hmbcgpr), NOESY (noesypyr, 200-ms spin lock pulse at 8.43 db), and a ¹H, ¹³C-HMBC optimized for long range coupling constants of 10 Hz (hmbcgplpndqf) were recorded using the indicated Bruker standard pulse programs. The spectra were referenced to the methyl signals of acetone (¹H, 2.225 ppm) (¹³C, 31.5 ppm) and external phosphoric acid (85% in water, ³¹P, 0 ppm) and analyzed using Bruker TopSpin version 3.0 software.

**Mass Spectrometry**—Electrospray ionization Fourier transform ion cyclotron-mass spectrometry was performed on intact and deacylated LPS in the negative ion mode using an APEX QE (Bruker Daltonics, Billerica, MA) equipped with a 7 tesla actively shielded magnet and dual ESI-MALDI. LPS samples were dissolved at a concentration of ~10 ng μl⁻¹ and analyzed as described previously (17, 27). Mass spectra were charge-deconvoluted, and the mass numbers given refer to the monoisotopic peaks. Mass calibration was done externally using well characterized similar compounds of known structure (27).

For gas-liquid chromatography-mass spectrometry (GLC-MS) analysis, authentic glucuronic acid (kindly provided by Tim Steffens, Research Center Borstel), the purified OS1 (100 mg), and native LPS (500 μg) were subjected to methanolysis (0.5 M HCl in methanol, 45 min, 85 °C), dried under a stream of nitrogen, and peracetylated in pyridine/acetic anhydride (1:1). The sample was then carboxyl reduced with NaBD₄ (1 mg of NaBD₄ in 150 μl of MeOH/H₂O, 4 h, room temperature). To convert the remaining disaccharides into the methyl glycosides, a second methanolysis was performed (2 M HCl in methanol, 3 h, 85 °C); the sample was peracetylated, and after hydrolysis with trifluoroacetic acid (4 M TFA, 2 h, 100 °C), carbonyl was reduced with NaBH₄ (1 mg of NaBH₄ in 150 μl of MeOH/H₂O, 4 h, room temperature) and peracetylated. GLC-MS analysis was performed on an Agilent 5975 system equipped with an HP 5890 column. The measurement was performed under S-tune conditions. The samples were applied at an initial temperature of 70 °C, which was held constant for 1.5 min, and a pressure of 10 p.s.i. corresponding to a flow rate of ~1 ml/min. The temperature was raised to 150 °C using a linear gradient of 60 °C/min, held for 3 min at this temperature, and finally increased to 320 °C over 5 min. The spectra were analyzed using Software MSD ChemStation D.02.00.275 (Agilent Technologies).

**RESULTS**

**Structural Analysis of Deacylated LPS**—As shown earlier, the *E. coli* B LPS is less heterogeneous than LPS from *E. coli* K12 because it is mainly composed of a hexaacylated lipid A containing two Kdo, three Hep, two Hex, and a phosphate (15). Therefore, we analyzed *E. coli* B LPS, which, after successive O- and N-deacylation of extracted LPS, yielded one main oligosaccharide and several minor fractions in HPAEC. The major signals of Glc, Hep, Kdo, and a hexuronic acid were identified in addition to minor signals from disaccharides composed of hexuronic acid-Hep, Hep₂, and GlcN₂. The fragmentation pattern of the hexuronic acid in GLC-MS was identical to authentic peracyetylated GlcUA. After carboxyl reduction with NaBD₄, hydrolysis, reduction with NaBH₄, and peracylation, only derivatives of Glc, GlcN, and Hep were present.

The mass spectrometric analysis of the deacylated LPS by electrospray ionization-Fourier transform-ion cyclotron MS showed a major ion signal with 2016.5307 m/z in the charge-deconvoluted mass spectrum. The chemical shift analysis of the isolated main oligosaccharide was determined by one- and two-dimensional ¹H, ¹³C, and ³¹P NMR spectroscopy (supplemental Figs. S1 and S2). The assignment of signals (Table 2) revealed that it was a decasaccharide composed of two αGlc (residues G

| residue | ¹H and ³¹C chemical shift (p.p.m.) of |
|---------|-------------------------------------|
| H-1     | C-1                                 |
| H-2     | C-2                                 |
| H-3     | C-3                                 |
| H-3x    | C-4                                 |
| H-3q    | C-5                                 |
| H-4     | C-6                                 |
| H-6a    | C-7                                 |
| H-6b    | C-8                                 |

a. ³¹P_H11 was 8 Hz.

b. ³¹P_H13 was 10 Hz.

c. ³¹P_H11 was 7 Hz; ³¹P_H13 was 10 Hz; ³¹P_H14 was 10 Hz; and ³¹P_H15 was 7 Hz.
and K, 3\(^{1}J_{\text{H1,H2}}\) 3.6 and 3.8 Hz, respectively), three L-glycero-\(\alpha\)-D-manno-heptoses (Hep, residues E, F, and H), all \(3^{1}J_{\text{H1,H2}} < 2\) Hz, two Kdo (residues C and D), one \(\alpha\)GlcN (residue A), one \(\beta\)GlcN (residue B, \(3^{1}J_{\text{H1,H2}}\) 8 Hz), and one hexuronic acid, which was identified by vicinal \(3^{1}J_{\text{H1,H2}}\) coupling constants as \(\beta\)GlcUA (residue I, \(3^{1}J_{\text{H1,H2}}\) 8 Hz) as depicted in Fig. 2. This composition of \(M_{\text{theor.}}\) 2016.53 was consistent with the mass spectrometric analysis. All residues were present as pyranoses. Only two signals of phosphates appeared in the \(3^{1}P\) NMR spectrum, which were located at O-1 of the \(\alpha\)GlcN (residue A) and at O-4 of Hep (residue E) according to cross-correlation signals in a two-dimensional \(H,^{1}P\)-HSQC spectrum. The 4'-phosphate was according to mass spectrometry almost quantitatively substituted with Ara4N in the LPS (\(m/z\) 3770.7) and therefore eliminated under the strong alkaline conditions used for the N-deacylation. This assignment was indirectly confirmed by additional coupling constants of the proton signals (\(3^{1}J_{\text{H1, H2}}\) 8 Hz (residue A) and \(3^{2}J_{\text{H2, H3}}\) 10 Hz (residue E)) at the substitution sites. The \(\beta\)GlcN was attached to position 6 of the \(\alpha\)GlcNIP, which were thus the residues of lipid A. The chemical shift analysis was consistent with the presence of an \(\alpha\)(2→4)-linked Kdo disaccharide (residues C and D) attached to position 6 of the \(\beta\)GlcN (residue B) (28). This was confirmed by NOE analysis (strong NOE from H-6 of residue D to H-3e of residue C, see Table 3) (29). Low field chemical shifts of \(^{1}H\) and \(^{13}C\) signals at linkage sites and cross-correlation signals in NOESY showed that the Hep residues formed the trisaccharide \(\alpha\)Hep-(1→7)-\(\alpha\)Hep (1→3)\(\alpha\)Hep common to the \(E. coli\) LPS core region, which was connected to position O-5 of the inner Kdo (residue C). The \(\alpha\)Glc residues were the first two (1→3)-linked residues of the outer core connected to position O-3 of the middle Hep (residue F). Finally, the \(\beta\)GlcUA was attached to O-7 of the side-chain Hep (residue H), which was inferred from NOESY correlation signals between the anomeric proton and H-7a and -7b of this Hep and low field chemical shifts of the latter signals. Simultaneously, the phosphate commonly found at position O-4 of the middle Hep (residue F) was absent. Thus, these results for the first time show that \(E. coli\) LPS obtained under phosphate-limiting growth conditions contains GlcUA attached to HepIII, and its presence coincides with the absence of phosphate on HepII.

**Determination of the Minimal in Vivo LPS Structure That Supports the Incorporation of GlcUA**—Previous analyses of LPS composition of \(E. coli\) K12 strains grown in phosphate-limiting growth conditions revealed several new molecules differing from known glycoforms by an additional 96 mass units (15). Such mass peaks with an additional 96 Da were found in glycoforms with either two or three Kdo residues with complete inner core (15), resulting in new glycoforms VI and VII (Fig. 1). Because the same modification was observed in \(E. coli\) B and is shown above to arise due to GlcUA addition, we examined the molecular basis of its incorporation in genetically well defined \(E. coli\) K12. Mass spectrometric analyses of LPS obtained from isogenic strains with in-frame deletions in various structural genes of the \(waa\) region revealed that this structural modification was absent in LPS of \(waaC\) or \(waaO\) mutants (15, 17) as well as in \(\Delta waaP\), \(\Delta waaG\), and \(\Delta waaQ\) derivatives.\(^3\) Confirming our earlier results (15), it was also present in the LPS of \(\Delta waaR\) mutants, which lack the terminal Hex-Hep disaccharide, arguing that this modification should occur proximal to GlcII. Because it was also present in \(\Delta waaB\) mutants, Gal apparently was not required for GlcUA incorporation.

The \(waaQ\) gene encodes heptosyltransferase III, and in its absence phosphorylation of HepII is known to be lacking (12). Comparison of mass spectra of LPS obtained from \(\Delta waaQ\) and isogenic wild-type strains showed the loss of either 272 or 368 mass units from the main peaks (Fig. 3) corresponding to glycoform I or IV/V (Fig. 1). The mass change of 272 units is the result of a concomitant absence of a heptose (192 Da) and a phosphate (80 Da). Whereas \(WaaP\)-dependent Hep phosphorylation is required for the synthesis of the complete core, phosphorylation of HepII is thus dispensable for the biosynthesis of the complete core. The signals shifted to lower mass by 368 units lacked a further 96 mass units. Thus, the mass peak at 3676.7 Da and its derivatives in the LPS of \(\Delta waaQ\) mass spectra (Fig. 3B) corresponded to glycoforms, containing three Kdo and Rha but lacking HepIII and one phosphate of the wild-type LPS (3948.7 Da). Likewise, in the \(\Delta waaQ\) mutant the mass

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3 G. Klein, B. Lindner, N. Kobylak, and S. Raina, unpublished results.
peak at 3930.8 Da arises from the wild-type structure with 4298.9 Da (LAhexa + Kdo3 + Rha + Hep3 + Hex3 + P + P-EtN2 + Ara4N + GlcUA) by the loss of 368 Da (lacking HepIII and GlcUA). Both of them represent derivatives of glycoform V. Other mass peaks in the LPS of ΔwaaQ mutants correspond to glycoform I derivatives lacking either 272 or 368 Da and are represented, for example, by molecules of 3795.7 Da and 4244.9 Da in mass, respectively.
Lack of WaaY Enhances the GlcUA Addition—Because in the absence of WaaQ HepII is not phosphorylated, the role of phosphorylation of HepII for the modification by GlcUA was examined. Thus, LPS from the isogenic derivative carrying a nonpolar deletion in the waaY gene was analyzed. WaaY is the predicted kinase responsible for HepII phosphorylation based on previous mutant analysis in strains synthesizing R1 and R3 LPS core types (12). Interestingly, LPS from ΔwaaY revealed relatively more prominent peaks corresponding to incorporation of GlcUA (Fig. 3C). Examination of mass peaks from LPS of several isogenic E. coli K12 or E. coli B derivatives revealed that the GlcUA addition was always associated with concomitant loss of one phosphate residue. These results argue that GlcUA addition to the side-chain HepIII is incompatible with the presence of phosphate on HepII. This explains the preponderance of mass peaks containing GlcUA in ΔwaaY mutants of E. coli K12 (Fig. 3C). Because among the tested mutants LPS of the ΔwaaY derivative shows predominant presence of GlcUA at the expense of the phosphate residue on HepII, we analyzed if global RpoE-dependent envelope stress response was also induced. Measurement of RpoE-dependent promoter activity revealed that ΔwaaY mutants do not have significant alterations in envelope stress response (supplemental Fig. S4). Thus, the enhanced GlcUA incorporation in a ΔwaaY mutant is due to the presence of a nonphosphorylated acceptor for this modification.

Identification of the Regulatory Gene Responsible for the GlcUA Addition—As GlcUA-containing glycoforms were uniquely observed in LPS obtained from bacteria grown in phosphate-limiting growth conditions, it was reasonable to assume that a gene encoding such a potential glycosyltransferase could be either induced or its product activated upon PhoB/R activation. The PhoB/R two-component system was directly responsible for phosphate sensing and was strongly induced upon phosphate starvation. To confirm a direct role for the positive regulation of the putative gene encoding the GlcUA transferase, a mutational analysis of PhoB/R regulatory genes was carried out. Thus, a nonpolar deletion in the phoB gene was constructed and LPS analyzed from such a derivative. The LPS of ΔphoB was nearly identical to that of the wild-type strain (Fig. 4A), but it did not contain GlcUA (Fig. 4B). The distribution of mass peaks corresponding to glycoforms I as well as glycoforms with three Kdo was unaffected. The mass peaks prominently missing from the spectra of ΔphoB correspond to 4298.8 Da (glycoform V) and 4489.9 and 4612.9 Da (glycoform I). All of these missing peaks are derivatives containing GlcUA. Confirming these results, LPS of all deletion derivatives of phoB such as Δ(phoB basR) and Δ(ephB basR phoB) and similar others were found to lack any mass peaks that can arise due to incorporation of GlcUA (Fig. 4D).

Identification of the waaH Gene Encoding the GlcUA Transferase—Examination of published microarrays from PhoB-inducing conditions led us to study the putative ORF yibD, because its deduced amino acid sequence suggests homology to the glycosyltransferase 2 family (GT-A type). Modeling revealed similarity with members of this family like SpS from Bacillus subtilis, a nucleotide-diphospho-sugar transferase involved in sporulation (30). YibD amino acid sequence also exhibits significant homology (up to 34% in conserved N-terminal domain) to proteins like PgaC biofilm PGA synthase (31) and WcaA protein required for colonic acid synthesis (32). Among the PhoB/R regulon members without any assigned function, the transcription of the yibD gene is strongly induced upon the activation of this two-component system (33). However, a direct in vivo regulation has not been addressed. Furthermore, its predicted promoter region does indeed contain Pho boxes (33). The ORF yibD is located outside the waa locus on the E. coli chromosome at 81.62 min between ORF yibO of unknown function and the tdh gene. The tdh gene encodes threonine 3-dehydrogenase and is transcribed as the tdh kbl operon without any known role for the LPS biosynthesis (34). The minimal coding sequence of the yibD ORF was cloned under the tight ptac promoter and under the T7 polymerase expression system. Based on the presented function (core modification due to the hexuronic acid addition), we have designated the yibD ORF as waaH. The deduced amino acid composition of this glycosyltransferase is predicted to be a polypeptide of 344 amino acid residues. Upon mild induction and cellular fractionation, followed by affinity purification, it was found to be membrane-associated. Homology searches with different databases revealed that its deduced amino acid sequence bears nearly 20% sequence identity with WabO from K. pneumoniae. WabO has been shown to be a glycosyltransferase specific for the GalA incorporation into K. pneumoniae LPS (22).

Next, nonpolar in-frame chromosomal deletion derivatives of the waaH gene were constructed. These mutations were transduced into W3110 using bacteriophage P1. Examination of the LPS obtained from ΔwaaH revealed the absence of mass peaks carrying modification by GlcUA (addition of 96 or 176.1 mass units). The notable mass peaks of 4298.9, 4489.9, and 4612.9 Da corresponding to glycoforms VI and VII, respectively, are missing in LPS of ΔwaaH (Fig. 3D). These are the same mass peaks, which are absent in the LPS of ΔphoB (Fig. 4B), but are present in the LPS from the isogenic wild type (Fig. 4A). MS/MS analysis of isolated mass peaks at 4298.9 and 4489.9 Da revealed fragmentation spectra with predicted hexuronic acid linked to the inner core heptose (data not shown). The absence of GlcUA from the LPS of ΔwaaH was confirmed by GLC-MS as opposed to its presence in the parental wild type. This defect in the ΔwaaH mutant was completely restored when the LPS was examined from the complemented strain using the cloned waaH minimal coding sequence expressed from a tightly controlled ptac promoter.

To further reinforce these results, a ΔwaaH derivative of E. coli B was constructed. The waaH gene is located at the identical chromosomal location between yibO and tdh genes in E. coli B. Analysis of LPS of E. coli B ΔwaaH mutant did not reveal any mass peaks corresponding to GlcUA modification as compared with their presence in the parental wild type (supplemental Fig. S5). Interestingly, unlike E. coli K12, the E. coli B strain BL21 (DE3) was found to have constitutively induced BasS/R and PhoB/R regulons even in LB medium. This is manifested by mass peaks representing the incorporation of Ara4N and P-EtN in the lipid A part. This can explain the occurrence of a mass peak at 3551.7 Da and further derivatives, which have predicted P-EtN and Ara4N substitutions both in the parental...
BL21 strain and its ΔwaaH derivatives. A mild overexpression of the cloned waaH gene (upon the addition of 0.01 mM isopropyl 1-thio-β-D-galactopyranoside) expressed from T7 polymerase in BL21ΔwaaH derivative was used for LPS analysis. LPS from such a strain even without phosphate starvation revealed most prevalent mass peaks with predicted GlcUA incorporation (supplemental Fig. S5). Taken together, these results demonstrate that WaaH is required for the incorporation of GlcUA in E. coli K12 and E. coli B.

GlcUA in Other E. coli Core Types and Salmonella—Examination of the genomic sequence representing different E. coli core types and that of Salmonella revealed that all of them contain the waaH gene. Indeed, the waaH (yibD) gene in Salmonella is part of the Pmr (BasS/R) regulon (35). However, until now no GlcUA has been reported in Salmonella or in E. coli. This can be explained by our results that induction of PhoP/Q and Pmr regulons in low Mg²⁺ conditions did not lead to any detectable levels of GlcUA in the LPS of Salmonella as determined by chemical and mass spectrometric analysis (data not shown). To identify conditions in which incorporation of GlcUA can be observed, we used ammonium metavanadate (NH₄VO₃)-supplemented growth media to grow E. coli and Salmonella derivatives. NH₄VO₃ is a nonspecific phosphatase inhibitor (36), which potentially induces a variety of two-component systems. Thus, LPS was obtained from the wild-type E. coli K12, representative R1, R2, R3, and R4 strains, and Salmonella minnesota R345. Among these, mass peaks indicating the incorporation of GlcUA were observed in all the cases except for the R1 and the R3 core type (Fig. 5 and supplemental Fig. S3). These results were confirmed by a GLC-MS analysis of these LPS, which showed the presence of hexuronic acid (GlcUA) by comparison with a hyaluronic acid standard.

** Regulation of waaH and ugd Genes—** To address the molecular basis of GlcUA incorporation, the putative promoter activities of waaH and ugd genes were monitored from a chromosomal single copy (37). Transcription of the ugd gene was...
**WaaH- and EptC-dependent Modifications in E. coli LPS**

![Image of mass spectra](image)

**FIGURE 5. Addition of ammonium metavanadate inducing the waaH transcription reveals the incorporation of GlcUA in different E. coli core types.** Mass spectra of LPS were obtained from the wild-type E. coli K12 strain W3110 (A), E. coli strain F756 representing R2 core type (B), and E. coli strain F2513, representing R4 core type (C). LPS was extracted from culture grown in LB medium supplemented by 25 mM ammonium metavanadate at 37 °C and incubated with shaking for 24 h. Charge-deconvoluted ESI FT-MS spectra in negative ion mode are presented. The mass numbers refer to monoisotopic peaks with proposed composition.

included because GlcUA incorporation requires conversion of UDP-glucose into UDP-glucuronic acid by the ugd gene product (2). The β-galactosidase activity driven from the waaH promoter was induced up to 200-fold upon the shift to 121 medium (Fig. 6A). This induction was abolished in a ΔphoB derivative but not in a ΔbasR background (Fig. 6C). This strong 200-fold induction was specifically observed in a growth phase-dependent manner (Fig. 6D). No induction was observed in phosphate-rich M9 or LB medium, which are noninducing conditions for PhoB/R regulon. These results strongly argue that the promoter of the waaH gene is specifically positively regulated by PhoB/R and does not require BasS/R. Because 121 medium is inducing for both BasS/R and PhoB/R regulons (17), these results assume significance and unequivocally prove that the WaaH-dependent GlcUA addition primarily requires induction of PhoB/R without any requirement for the BasS/R two-component system in E. coli K12. Thus, the presence of predicted PhoB boxes in the promoter region of the waaH gene (33) supports our results. After the completion of this work, direct PhoB-binding sites have been found upstream of the waaH (yibD) gene, although the function of the gene remained elusive (38).

The activity of the ugd promoter was also induced upon shift to phosphate-limiting growth conditions from 15- to 30-fold in a growth phase-dependent manner (Fig. 7A). Approximately 50% reduction in the basal level activity of the ugd promoter in the phosphate-limiting growth medium is observed in exponential growth phase in a ΔphoB derivative (Fig. 7C). Very low activity of the ugd promoter was observed in a ΔbasR derivative. However, a dramatic increase in the ugd-lacZ activity was observed in a ΔbasR mutant in the stationary phase but not in a ΔphoB. Thus, the transcription of the ugd promoter requires both PhoB/R and BasS/R activation. This dual mode of transcription control by these two-component systems provides precursor UDP-glucuronic acid for GlcUA and Ara4N synthesis. Because BasS/R regulates Ara4N synthesis (lipid A modifications) and PhoB/R regulates core alterations, including GlcUA transfer, the transcription of the ugd promoter is intricately balanced.

eptC Gene Is Required for the P-EtN Addition to HepI in the Inner Core—It is known that the LPS of E. coli K12 often contains nonstoichiometric substitutions by P-EtN residues. These P-EtN substitutions can occur in lipid A, on the second Kdo, as well as in the inner core on HepI. The genes whose products are responsible for the P-EtN addition to Kdo (the eptB gene) and a BasS/R-inducible modification of lipid A (the eptA gene) are known. However, the gene responsible for the P-EtN addition to HepI and its regulation is not known in E. coli. Based on mass spectrometric analysis of LPS obtained from several isogenic strains in phosphate-limiting growth conditions, we could observe mass peaks with a predicted presence of all three P-EtN residues. E. coli contains three additional orthologs of EptA and EptB. These three orthologs are encoded by ORFs designated as yibP, yhjX, and yijP. Deletion derivatives of these three individual ORFs were constructed and combined with deletion mutation of either the eptA gene alone or the eptB gene alone, or their combinations. Among the three deletion derivatives of these ORFs, LPS of ΔyijP alone resulted in the absence of mass peaks with three P-EtN residues (4612.9 Da) (Fig. 8B). Thus, this gene was designated as the eptC gene. Furthermore, accumulation of mass peaks at 3813.7 and 3825.7 Da corresponding to glycoforms I and IV, respectively, were only observed in the LPS of strains lacking either the eptA or eptB or eptC gene (Fig. 8). However, these mass peaks are absent in the
spectra of the wild type (Fig. 8A). These mass peaks, as indicated, lack any substitution of predicted P-EtN addition even in conditions in which the wild type can incorporate all three P-EtN residues.

Next, we analyzed by mass spectrometry the LPS of strains carrying combinations of null mutations in various ept genes and disruptions of the other two ORFs. Again, no differences in mass spectra were observed when \( \text{H}9004\text{ybiP} \) or \( \text{H}9004\text{yhjX} \) was introduced into \( \text{H}9004\text{ept} \) strains (data not shown). However, strains carrying either \( \text{H}9004(\text{eptA eptC}) \) or \( \text{H}9004(\text{eptB eptC}) \) or \( \text{H}9004(\text{eptA eptB}) \) combinations revealed incorporation of only one P-EtN substitution. Data presented here correspond to \( \text{H}9004(\text{eptA eptC}) \) (Fig. 8C). The main striking difference between mass spectra of LPS obtained from \( \text{H}9004\text{eptC} \) and \( \text{H}9004(\text{eptA eptC}) \) mutants is the predicted composition of \( \text{H}9004(\text{eptA eptC}) \) LPS, which contains only one P-EtN substitution, as compared up to two P-EtN substitutions in \( \text{H}9004\text{eptC} \) LPS. This explains the absence of a mass peak at 4489.9 Da in \( \text{H}9004(\text{eptA eptC}) \), which is present in either \( \text{H}9004\text{eptC} \) or the wild type (Fig. 8). These results further show that the eptC gene product is required for the P-EtN incorporation. Consistent with these results, LPS of \( \Delta(\text{eptA eptB eptC}) \) strain did not reveal any mass peaks with a predicted incorporation of P-EtN (Fig. 8D). It should be noted that the LPS obtained from the \( \Delta(\text{eptA eptB eptC}) \) strain lacking all three ept genes reveals mass peaks indicative of an incorporation of Ara4N and GlcUA (Fig. 8D). Thus, in the absence of P-EtN transferases other non-stoichiometric modifications do not seem to be influenced to any significant levels. Thus, LPS of \( \Delta\text{eptC} \) did not exhibit any other major differences either in the accumulation of different glycoforms or the lipid A composition. Hence, EptC function seems to be specific for P-EtN incorporation on phosphorylated HepI, given the known function of the two other P-EtN transferases. Furthermore, unlike EptB, which is required for P-EtN addition to the second Kdo and regulates the shift of Rha to the third Kdo and hence the presence of glycoform V, no such differences in the LPS were observed in \( \Delta\text{eptC} \).

**Regulation of the eptC Gene**—As shown previously, LPS obtained from phosphate-rich M9 growth conditions lack any P-EtN modifications (15). However, mass peaks with up to three predicted P-EtN substitutions seemed to be enriched in the LPS obtained from phosphate-limiting medium. Growth in this medium can induce both BasS/R and PhoB/R two-component systems. Examination of mass spectra of LPS of \( phoB \) revealed incorporation of up to three P-EtN residues indicating that activity/expression of none of the three phosphoethanolamine transferases is abolished (Fig. 4B). This is evident from the presence of the mass peak at 4516.9 Da in the spectra of LPS obtained from the \( \Delta\text{phoB} \) mutant strain. Thus, we addressed the relative role of PhoB/R and BasS/R regulatory systems in the P-EtN addition to HepI. It is known that the transcription of the
eptA gene requires BasS/R induction. Thus, \( \Delta \text{basR} \) mutants lack P-EtN in lipid A (17). Similarly, EptB is required for the P-EtN addition to the second Kdo (14). Thus, a \( \Delta \text{eptB} \Delta \text{basR} \) strain should depict activity of the eptC gene independent of the BasS/R two-component system. As can be seen, most of the assigned mass peaks of LPS obtained under PhoB/R-inducible conditions from the \( \Delta \text{eptB} \Delta \text{basR} \) strain contain one P-EtN (Fig. 4C). This is evident from the predicted composition of main mass peaks at 3948.7 and 4139.8 Da and their derivatives (Fig. 4). These results suggest that induction of PhoB/R must play a major role in the transcriptional activation of the eptC gene. Furthermore, the BasS/R induction does not seem to contribute significantly to the transcription of the eptC gene based on the observed P-EtN modification of phosphorylated HepI in \( \Delta \text{basR} \).

To further investigate the regulation of the eptC gene, a single copy chromosomal eptC-lacZ transcriptional fusion was constructed. Thus, we analyzed in parallel the eptC-lacZ promoter fusion activity in phosphate-rich (M9) versus phosphate-limiting (121) growth conditions at different intervals of growth. As can be seen, \( \beta \)-galactosidase activity was about 6-fold higher in the phosphate-limiting medium (Fig. 9A). Next, we analyzed relative eptC promoter activity in isogenic \( \Delta \text{phoB} \) and \( \Delta \text{basR} \) mutants in phosphate-limiting growth conditions. Because the 121 medium is supplemented with 10 \( \mu \text{M} \) Zn\(^{2+}\) and eptC mutants are sensitive to Zn\(^{2+}\), \( \beta \)-galactosidase assays were performed in the absence or in the presence of Zn\(^{2+}\). It needs to be mentioned that with the supplementation of 10 \( \mu \text{M} \) Zn\(^{2+}\), no growth differences were observed between the wild type and the eptC mutant. Introduction of a \( \Delta \text{phoB} \) mutation led to a dramatic decrease in the eptC-lacZ promoter fusion activity (Fig. 9B). This is more pronounced when Zn\(^{2+}\) was omitted. However, eptC-lacZ activity was only reduced 2-fold in \( \Delta \text{basR} \) mutants as compared with a 4–5-fold reduction in \( \Delta \text{phoB} \) derivatives. Furthermore, the presence or the absence of Zn\(^{2+}\) in a \( \Delta \text{basR} \) background did not significantly reduce the eptC-lacZ promoter fusion activity. These data are consistent with the data from LPS analysis that the major positive regulator of the eptC transcription is PhoB-dependent. As the activity of the eptC-lacZ transcriptional fusion was not totally abolished in \( \Delta \text{phoB} \) mutants, it suggests that a constitutive basal transcriptional activity is present, which is independent of both PhoB and BasR. These results are supported by the presence of few mass peaks with the predicted P-EtN incorporation in a strain derivative \( \Delta \text{eptB} \Delta \text{phoB} \Delta \text{basR} \). Interestingly, these results also show that the induction of the eptC gene of \( E. \text{coli} \) K12 does not require the BasS/R induction.

FIGURE 7. Growth phase-dependent transcriptional activity of the promoter of the ugd gene in phosphate-limiting conditions (A), which requires the functional presence of PhoB/R and BasS/R two-component systems (C). Cultures of \( E. \text{coli} \) wild-type strain carrying single copy chromosomal ugd-lacZ promoter fusion or its isogenic derivative with \( \Delta \text{basR} \) or \( \Delta \text{phoB} \) mutation were grown to early log phase in LB medium at 37 °C, washed, and adjusted to an \( A_{595} \) of 0.02 in either LB medium or phosphate-limiting 121 medium. Aliquots of samples were drawn every 30 min and analyzed for \( \beta \)-galactosidase activity. Data corresponding to phosphate-limiting growth conditions are marked 121 in each case. The experiments were performed on four independent isolates. Error bars represent S.E. of four such cultures. B and D correspond to \( A_{95S} \) indicating growth corresponding to different time intervals in which the \( \beta \)-galactosidase activity assay was performed.

epA gene requires BasS/R induction. Thus, \( \Delta \text{basR} \) mutants lack P-EtN in lipid A (17). Similarly, EptB is required for the P-EtN addition to the second Kdo (14). Thus, a \( \Delta \text{eptB} \Delta \text{basR} \) strain should depict activity of the eptC gene independent of the BasS/R two-component system. As can be seen, most of the assigned mass peaks of LPS obtained under PhoB/R-inducible conditions from the \( \Delta \text{eptB} \Delta \text{basR} \) strain contain one P-EtN (Fig. 4C). This is evident from the predicted composition of main mass peaks at 3948.7 and 4139.8 Da and their derivatives (Fig. 4). These results suggest that induction of PhoB/R must play a major role in the transcriptional activation of the eptC gene. Furthermore, the BasS/R induction does not seem to contribute significantly to the transcription of the eptC gene based on the observed P-EtN modification of phosphorylated HepI in \( \Delta \text{basR} \).

To further investigate the regulation of the eptC gene, a single copy chromosomal eptC-lacZ transcriptional fusion was constructed. Thus, we analyzed in parallel the eptC-lacZ promoter fusion activity in phosphate-rich (M9) versus phosphate-limiting (121) growth conditions at different intervals of growth. As can be seen, \( \beta \)-galactosidase activity was about 6-fold higher in the phosphate-limiting medium (Fig. 9A). Next, we analyzed relative eptC promoter activity in isogenic \( \Delta \text{phoB} \) and \( \Delta \text{basR} \) mutants in phosphate-limiting growth conditions. Because the 121 medium is supplemented with 10 \( \mu \text{M} \) Zn\(^{2+}\) and eptC mutants are sensitive to Zn\(^{2+}\), \( \beta \)-galactosidase assays were performed in the absence or in the presence of Zn\(^{2+}\). It needs to be mentioned that with the supplementation of 10 \( \mu \text{M} \) Zn\(^{2+}\), no growth differences were observed between the wild type and the eptC mutant. Introduction of a \( \Delta \text{phoB} \) mutation led to a dramatic decrease in the eptC-lacZ promoter fusion activity (Fig. 9B). This is more pronounced when Zn\(^{2+}\) was omitted. However, eptC-lacZ activity was only reduced 2-fold in \( \Delta \text{basR} \) mutants as compared with a 4–5-fold reduction in \( \Delta \text{phoB} \) derivatives. Furthermore, the presence or the absence of Zn\(^{2+}\) in a \( \Delta \text{basR} \) background did not significantly reduce the eptC-lacZ promoter fusion activity. These data are consistent with the data from LPS analysis that the major positive regulator of the eptC transcription is PhoB-dependent. As the activity of the eptC-lacZ transcriptional fusion was not totally abolished in \( \Delta \text{phoB} \) mutants, it suggests that a constitutive basal transcriptional activity is present, which is independent of both PhoB and BasR. These results are supported by the presence of few mass peaks with the predicted P-EtN incorporation in a strain derivative \( \Delta \text{eptB} \Delta \text{phoB} \Delta \text{basR} \). Interestingly, these results also show that the induction of the eptC gene of \( E. \text{coli} \) K12 does not require the BasS/R induction.
**EptC Is Required for the Outer Membrane Function**—Because the main function of the LPS is to provide the permeability barrier function, the potential role of LPS modification by nonstoichiometric substitution of LPS with P-EtN was addressed. Thus, a panel of isogenic strains carrying either the deletion of individual ept genes or their combinations was examined for different growth defects related to the membrane permeability. Thus, bacteria were challenged with SDS under phosphate-limiting growth conditions that promote the incorporation of P-EtN residues into the LPS core and lipid A. As shown, *E. coli* eptC mutant bacteria exhibited a severe growth defect in the presence of low concentrations of SDS (0.25%) with a reduction of the colony forming ability by 10^3 (Table 4). At this concentration, the wild type and its isogenic derivatives *E. coli* eptB or *E. coli* eptA showed no growth defects. At higher concentrations of SDS (1%), the colony forming ability was reduced by 10^5 in *E. coli* eptC mutants, although no significant effect on the viability of wild-type or that of other ept mutants was observed (Table 4).

This SDS-sensitive phenotype was not observed in the phosphate-rich medium, consistent with induction of the eptC gene expression primarily in phosphate-limiting growth conditions. Because the expression of the eptC gene is enhanced in the presence of Zn^{2+}, we also tested if EptC is required for the tolerance to its increased concentrations. Although wild-type bacteria can support growth up to a concentration of 200 μM Zn^{2+}, growth of the ΔeptC mutant was highly attenuated even at 150 μM Zn^{2+}. Again, just like in the case of sensitivity to SDS, ΔeptC mutants alone were sensitive to Zn^{2+}, but not the ΔeptB or ΔeptA mutant bacteria. Interestingly, introduction of the ΔeptB mutation suppressed this Zn^{2+}-sensitive phenotype (Table 4). Such a suppression was not observed in Δ(eptA eptC) combination. However, this restoration/suppression by ΔeptB seems to be limited to Zn^{2+} and SDS-sensitive growth defects only. As shown in LPS compositional analysis, introduction of the ΔeptB mutation does not cause any new P-EtN addition in the LPS as seen by the total absence of any mass peaks with predicted P-EtN.

**DISCUSSION**

In this work, we analyzed the genetic and structural basis of inner core nonstoichiometric alterations in the LPS of *E. coli*.

The incorporation of βGlcUA (glucuronic acid) as a novel modification of HepIII is shown here for the first time. Furthermore, a new gene, designated waaH, whose product is responsible for the βGlcUA incorporation into the LPS was identified. In parallel experiments, the modification of phosphorylated HepI by P-EtN and its physiological significance was studied. The responsible P-EtN transferase was identified and according to its function designated EptC.

The definitive assignment of GlcUA was based on the structural analysis of a purified oligosaccharide from an *E. coli* B strain. In GLC-MS, the fragmentation pattern and the retention time of the derivatized hexuronic acid was identical to a standard GlcUA obtained from hyaluronic acid. The NMR analysis...
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FIGURE 9. Induction of transcription of the eptC promoter requires induction of the PhoB/R two-component system and the presence of Zn\(^{2+}\). Cultures of E. coli wild-type strain GK1111 carrying single copy chromosomal eptC-lacZ promoter fusion or its isogenic derivative with \( \Delta \text{basR} \) or \( \Delta \text{phoB} \) mutations were grown to early log phase in LB medium at 37 °C, washed, and adjusted to an \( A_{600} \) of 0.02 in either M9 or phosphate-limiting 121 medium. Aliquots of samples were drawn at 35-min intervals and analyzed for the \( \beta \)-galactosidase activity. Data corresponding to phosphate-limiting growth conditions are shown. A, Samples were analyzed without supplemental Zn\(^{2+}\). B, A representative set of experiments with and without supplementation with Zn\(^{2+}\). (A) The experiments were performed on four independent isolates. Error bars indicate data with and without supplementation by Zn\(^{2+}\) in the phosphate-limiting 121 medium are presented.

TABLE 4

Eptc is required for OM stability

| Strain | 0.25% SDS | 0.5% SDS | 1% SDS | 150 \( \mu \)M Zn\(^{2+}\) |
|--------|-----------|-----------|--------|-----------------|
| Wild type | 3 \( \times \) 10\(^7\) | 3 \( \times \) 10\(^7\) | 3 \( \times \) 10\(^7\) | 3 \( \times \) 10\(^7\) |
| \( \Delta \text{eptA} \) | 2 \( \times \) 10\(^7\) | 2 \( \times \) 10\(^7\) | 3 \( \times \) 10\(^7\) | 2 \( \times \) 10\(^7\) |
| \( \Delta \text{eptB} \) | 2 \( \times \) 10\(^7\) | 3 \( \times \) 10\(^7\) | 2 \( \times \) 10\(^7\) | 3 \( \times \) 10\(^7\) |
| \( \Delta \text{eptC} \) | 2 \( \times \) 10\(^7\) | 7 \( \times \) 10\(^7\) | 3 \( \times \) 10\(^7\) | 3 \( \times \) 10\(^7\) |
| \( \Delta \text{eptB eptC} \) | 6 \( \times \) 10\(^6\) | 2 \( \times \) 10\(^6\) | 5 \( \times \) 10\(^6\) | 2 \( \times \) 10\(^6\) |
| \( \Delta \text{eptC} + \text{vector} \) | 2 \( \times \) 10\(^6\) | 6 \( \times \) 10\(^5\) | 5 \( \times \) 10\(^5\) | 6 \( \times \) 10\(^5\) |
| \( \Delta \text{eptC} + \text{eptC}^+ \) | 3 \( \times \) 10\(^5\) | 3 \( \times \) 10\(^5\) | 3 \( \times \) 10\(^5\) | 3 \( \times \) 10\(^5\) |

showed that the \( \beta \)-GalUA was attached to O-7 of the side-chain Hep. Furthermore, the phosphate residue commonly found at position O-4 of the middle Hep was absent.

To understand structural and regulatory basis of the GlcUA incorporation, LPS was analyzed from strains carrying nonpolar deletions in genes comprising the waa locus and its regulatory systems. These studies revealed that GlcUA is specifically incorporated in phosphate-limiting growth conditions into the LPS of E. coli K12 derivatives, which requires the presence of HepII. This incorporation requires activation of the PhoB/R two-component system without any requirement for BasS/R induction. The minimal in vivo LPS structure supporting the GlcUA incorporation requires the presence of a core oligosaccharide up to GlcII of the outer core. Furthermore, the absence of terminal HepIV-HexIII disaccharide due to a \( \text{waaR} \) mutation or even a \( \text{waaB} \) mutant lacking Gal was found to contain GlcUA.

The gene encoding putative glycosyltransferase for the GlcUA incorporation was identified outside the \( \text{waa} \) locus. Nonpolar deletion derivatives were constructed in E. coli K12 as well as E. coli B. Analysis of LPS of such mutants revealed a unique lack of GlcUA without any other structural alterations. Thus, the gene was designated as \( \text{waaH} \). Hence, the \( \text{waaH} \) gene is not a regulatory gene for GlcUA incorporation but is a structural gene.

A strong induction of the \( \text{waaH} \) transcription was observed upon entry in the stationary phase in PhoB-inducing conditions. The growth phase and PhoB-dependent transcriptional regulation of the \( \text{waaH} \) gene draws an interesting parallel with the observed induction of the promoter activity of the \( \text{ugd} \) gene in stationary phase. The \( \text{ugd} \) gene encodes UDP-glucose dehydrogenase that converts UDP-glucose into UDP-glucuronic acid. The transcriptional regulation of the \( \text{ugd} \) gene has been mainly examined in Salmonella, wherein it is regulated by PhoP/Q, Pmr, and Rcs two-component systems (39). In E. coli, transcription of \( \text{ugd} \) and \( \text{waaH} \) genes seems to respond to similar signals but with interesting differences. Because UDP-glucuronic acid serves as precursor for GlcUA as well as Ara4N synthesis, the transcription of the \( \text{ugd} \) gene in E. coli K12 was found to be controlled by two overlapping two-component systems (PhoB/R and BasS/R), which control GlcUA and lipid A modifications, respectively. The transcription induction in the late stationary phase of both \( \text{ugd} \) as well as \( \text{waaH} \) genes requires PhoB/R induction but not BasS/R. However, the transcription of the \( \text{ugd} \) gene does not require PhoB/R in exponential growth phase but requires BasS/R. Because \( \Delta \text{rpoS} \) mutants contain GlcUA substitutions in the LPS (15), the growth phase-dependent \( \text{waaH} \) and \( \text{ugd} \) regulation is not \( \Delta \text{rpoS} \)-dependent.

We observed that the addition of the nonspecific phosphatase inhibitor \( \text{NH}_4\text{VO}_3 \) to phosphate-rich LB medium also induces the LPS modification by GlcUA substitution. The GlcUA addition upon such treatment can also be ascribed to PhoB/R-dependent transcriptional induction of the \( \text{waaH} \) gene. Previously, it has been shown that \( \text{NH}_4\text{VO}_3 \) induces BasS/R-dependent lipid A modification as well as the \( \Delta \text{rpoE} \)-dependent envelope stress-response regulon (36, 40). Addition of \( \text{NH}_4\text{VO}_3 \) is also known to induce the transcription of the \( \text{waaH} \) gene (41). However, in that work the authors did not study the effects of PhoB/R induction (41). Because we previously observed the presence of mass peaks with GlcUA-modified structures in both \( \Delta \text{basR} \) as well as \( \Delta \text{rpoE} \) mutants (15), we can conclude that this modification is PhoB/R-dependent.

Thus, using growth medium supplemented with \( \text{NH}_4\text{VO}_3 \), GlcUA was also found to be present in the LPS preparations of Salmonella as well as E. coli R2 and R4 core types. Intriguingly, using \( \text{NH}_4\text{VO}_3 \)-supplemented growth medium, no detectable GlcUA was observed in the LPS of R1 and R3 core types. It is possible that under such conditions GlcN incorporation on
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HepIII in R1 and R3 core types is the preferred modification and may preclude GlcUA incorporation. Consistent with such an idea, GlcN incorporation in R3 core type is present in derivatives even with only GlcI in the outer core as shown in the case of E. coli J-5 structure (10).

Although we did not observe GlcUA incorporation in the R3 core type, the waaH gene was also identified in the signaturetagged transposon mutagenesis approach as one of the candidates required for the colonization by E. coli O157:H7 (42). In that study, transposon mutants that were impaired in their ability to colonize host were sought. It is likely that the waaH gene in such cases might be regulated in a different manner than in the case of E. coli K12. At present, no comparative knowledge about regulation of the wabB gene responsible for GlcN addition in the R3 core type vis à vis the waaH gene is available. The plasmid-encoded wabB gene is co-transcribed as the shf-wabB-virk-msbB operon. However, the wabB gene seems to be thermoregulated (43). Until now, the physiological significance of WabB-mediated GlcN incorporation is not known. As waaQ and waaH genes are present in all the E. coli core types and in several Enterobacteriaceae members like Salmonella and Shigella, we speculated that GlcUA-modified LPS must be present in such bacteria. It is quite likely that WaaH-dependent GlcUA incorporation has an adaptive significance under growth conditions like phosphate starvation, which can naturally occur in bacteria growing outside the host. In the case of E. coli K12, ΔwaaH mutants were found to be more sensitive to Fe³⁺, in backgrounds like arrT mutants. It is interesting because both GlcUA and Ara4N syntheses require Ugd-dependent UDP-glucuronic acid synthesis (2, 39).

In this work, it was also observed that the presence of GlcUA is always associated with a lack of phosphate at HepII. This is reminiscent of the situation in the LPS of R1 and R3 core types, wherein nonstoichiometric incorporation of GlcN on HepII is accompanied by the absence of O-4 phosphate of HepII (6). As presented in this work and consistent with earlier results (12), phosphorylation of HepII requires prior WaaQ-mediated incorporation of HepIII. It is likely that the incorporation of GlcUA precludes WaaY-mediated phosphorylation of HepII or that the presence of a negatively charged GlcUA is incompatible with the presence of an additional negative charge at HepII. Furthermore, it seems that nonphosphorylated HepII LPS is a better substrate for the GlcUA incorporation in vivo, because this modification was highly preferred in waaY mutants. This incorporation of GlcUA at the expense of phosphate could be both structural as well as a compensatory mechanism to regain negative charge by the addition of this acidic sugar. Thus, WaaH-mediated GlcUA substitution precluding HepII phosphorylation can be explained on the basis of substrate preference on lines similar to that proposed for GlcN incorporation by WabB on HepIII in E. coli R3 core type (9).

It seems that HepIII in E. coli can accept various substitutions at the expense of phosphate on HepII depending upon the LPS core type. This substitution can be GlcN, GlcNAc, and GlcUA. In K. pneumoniae, which lacks any phosphates in the core region, HepIII can be substituted by GalA (22). Taken together, in all cases such substitutions by uronic acid is suggested to contribute to retaining net negative charges (2). WaaH shares 20% amino acid sequence similarity with WabO of K. pneumoniae, which is responsible for GalA incorporation (22). However, expression of the wabO gene in E. coli K12 or E. coli B strains neither complemented E. coli waaH mutants nor caused any LPS modification.

The biosynthesis of the inner core of LPS, including the phosphorylation of HepI and addition of P-EtN, is critical for the outer membrane permeability. Although the role of WaaP has been addressed, the gene encoding the P-EtN transferase has not been described in E. coli. Phosphorylation of the HepI residue requires WaaP kinase and is also the site of attachment for the third P-EtN in E. coli. In E. coli, the genes encoding P-EtN transferases for the modification of lipid A (eptA) and the second Kdo (eptB) have been identified. The E. coli genome contains three additional distinct ORFs without any demonstrated function, whose deduced amino acid sequence exhibits homology to EptA and EptB. In this work, we showed that the gene encoded by ORF yijP is responsible for the P-EtN transfer to phosphorylated HepI. We designated this gene as the eptC gene. Mass spectrometric analysis of LPS obtained from ΔeptC showed nonstoichiometric incorporation of P-EtN in lipid A, and only the core-specific P-EtN substitution was missing. Furthermore, MS/MS analysis of isolated mass peaks from Δ(eptA eptC) with a predicted P-EtN substitution unequivocally showed that P-EtN was only present on Kdo. The modification of Kdo by P-EtN is solely dependent on EptB, and hence, we can assume that EptC function is dedicated to the modification of P-EtN on HepI. Consistent with such arguments, any mass peaks indicative of such a modification were absent in the spectra of LPS from a Δ(eptA eptB eptC) strain. These results suggest that two other homologs, YbhX and YbiP, may have some other unidentified non-LPS substrate for the incorporation of P-EtN.

We also found that among ept mutants, only an eptC mutant exhibited sensitivity to the mild addition of detergents like SDS or sublethal concentrations of Zn²⁺ in the phosphate-limiting medium. Sensitivity to SDS suggested that the P-EtN addition to the phosphorylated HepI is critical for the outer membrane stability. Thus, the EptC-dependent modification seems to be critical for the permeability function and challenges to sublethal concentrations of Zn²⁺.

Examination of the transcriptional regulation of the eptC gene revealed that it is expressed at the basal level in complex-rich medium like LB medium. However, the transcription of the eptC promoter is induced upon shift to the phosphate-limiting medium supplemented by micromolar concentrations of nontoxic Fe³⁺ and Zn²⁺ salts. This induction was PhoB/R-dependent and did not require the BasS/R activation. Consistent with these results, putative PhoB boxes were found in the promoter region. These boxes are located at −176 and −96, respectively, upstream of the eptC gene (CTCTTCTGCAAACCCTCGTCATTTTATTCAAATTCTG). The observed positive regulation of the eptC gene in E. coli K12 by the PhoB/R two-component system is interesting, because the corresponding modification in Salmonella is Pmr-dependent (18). This induction of EptC is important for bacterial viability under specific growth conditions such as phos-
phosphate starvation and upon challenge with agents that disrupt OM permeability.

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