A Phosphoethanolamine Transferase Specific for the Outer
3-Deoxy-d-manno-octulosonic Acid Residue of
Escherichia coli Lipopolysaccharide

IDENTIFICATION OF THE eptB GENE AND Ca²⁺ HYPERSENSITIVITY OF AN eptB DELETION MUTANT

C. Michael Reynolds‡, Suzanne R. Kalb‡, Robert J. Cotter§, and Christian R. H. Raetz‡¶

From the ‡Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710 and §Middle Atlantic Mass Spectrometry Laboratory, Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Addition of a phosphoethanolamine (pEtN) moiety to the outer 3-deoxy-d-manno-octulosonic acid (Kdo) residue of lipopolysaccharide (LPS) in WBB06, a heptose-deficient Escherichia coli mutant, occurs when cells are grown in 5–50 mM CaCl₂ (Kanipes, M. I., Lin, S., Cotter, R. J., and Raetz, C. R. H. (2001) J. Biol. Chem. 276, 1156–1163). A Ca²⁺-induced, membrane-bound enzyme was responsible for the transfer of the pEtN unit to the Kdo domain. We now report the identification of the gene encoding the pEtN transferase. E. coli yhjW was cloned and overexpressed, because it is homologous to a putative pEtN transferase implicated in the modification of the β-chain heptose residue of Neisseria meningitidis lipo-oligosaccharide (Mackinnon, F. G., Cox, A. D., Pleset, J. S., Tang, C. M., Makepeace, K., Coull, P. A., Wright, J. C., Chalmers, R., Hood, D. W., Richards, J. C., and Moxon, E. R. (2002) Mol. Microbiol. 43, 931–943). In vitro assays with Kdo₂-4[³²P]lipid A as the acceptor showed that YhjW (renamed EptB) utilizes phosphatidylethanolamine in the presence of Ca²⁺ to transfer the pEtN group. Stoichiometric amounts of diacylglycerol were generated during the EptB-catalyzed transfer of pEtN to Kdo₂-lipid A. EptB is an inner membrane protein of 574 amino acid residues with five predicted trans-membrane segments within its N-terminal region. An in-frame replacement of eptB with a kanamycin resistance cassette rendered E. coli WBB06 (but not wild-type W3110) hypersensitive to CaCl₂ at 5 mM or higher. Ca²⁺ hypersensitivity was suppressed by excess Mg²⁺ in the medium or by restoring the LPS core of WBB06. The latter was achieved by reintroducing the waaC and waaF genes, which encode LPS heptosyl transferases I and II, respectively. Our data demonstrate that pEtN modification of the outer Kdo protected cells containing heptose-deficient LPS from damage by high concentrations of Ca²⁺. Based on its sequence similarity to EptA(PmrC), we propose that the active site of EptB faces the periplasmic surface of the inner membrane.

The envelope of Gram-negative bacteria consists of an inner membrane (1, 2), a peptidoglycan cell wall (3), and an outer membrane (4). The latter is an asymmetric bilayer with glycolipids on its inner surface and lipopolysaccharide (LPS) on the outside surface (5, 6). LPS consists of three covalently linked portions (6, 7) as follows: 1) the lipid A moiety, a glucosamine-based saccharolipid that serves as the hydrophobic membrane anchor of LPS; 2) the core region, a non-repeating oligosaccharide modified with phosphate-containing substituents; and 3) the O-antigen, a distal repeating oligosaccharide, which is absent in most strains of Escherichia coli K12 (6). The lipid A moiety and the 3-deoxy-d-manno-octulosonic acid (Kdo) residues of the core are essential for growth of E. coli and most other Gram-negative bacteria (6). Strains lacking all LPS sugars distal to Kdo are termed heptose-deficient or “deep rough” (6, 8). These mutants are viable under laboratory conditions (9), but are hypersensitive to serum and antibiotics (4), and often show reduced virulence in animal models (7).

Under certain conditions, strains of E. coli and Salmonella synthesize LPS molecules modified with a phosphoethanolamine (pEtN) group at position 7 of the outer Kdo residue (9) (Fig. 1). Brabetz et al. (9) have reported that E. coli WBB06, which harbors a deletion spanning the heptosyl transferase genes waaC(rfaC) and waaF(rfaF), contains heptose-deficient LPS, modified with pEtN at position 7 of the outer Kdo sugar. Kanipes et al. (10) later demonstrated that pEtN addition to LPS in WBB06 is a consequence of the presence of Ca²⁺ in the growth medium used by Brabetz et al. (9) and is unrelated to the deletion of the heptosyl transferase genes. A pEtN transferase activity is present in membranes of WBB06 grown in the presence of 5–50 mM Ca²⁺ (10). The enzyme is stimulated by exogenous phosphatidylethanolamine (PE) and is selective for the outer Kdo residue of Kdo₂-lipid A and related substrates (10). Addition of EDTA to the in vitro pEtN transferase assay was found to be inhibitory.

We have now identified the gene that encodes the Ca²⁺-induced pEtN transferase. YhjW is one of six putative pEtN transferases present in E. coli that are homologous to a Neisseria meningitidis gene required for the modification of the

1 The abbreviations used are: LPS, lipopolysaccharide; pEtN, phosphoethanolamine; PE, phosphatidylethanolamine; IPTG, isopropyl 1-thio-b-D-galactopyranoside; Kdo, 3-deoxy-d-manno-octulosonic acid; DTT, dithiothreitol; [16:0,18:2]PE, 1-palmitoyl-2-linoleoyl-glycero-3-phosphoethanolamine; [16:0,18:0]PE, 1-palmitoyl-2-1,1′-dioleoyl-glycero-3-phosphoethanolamine; [16:0,18:2]PE, 1-palmityloyl-2-octadecenoyl-glycero-3-phosphoethanolamine; RBS, ribosome-binding site; MALDI/TOF, matrix-assisted laser desorption ionization/time of flight.

2 The term “saccarolipid” (analogous to “glycerolipid”) has been introduced recently to describe molecules, such as lipid A, in which one or more fatty acyl chains are linked directly to a sugar backbone (57). Other common saccarolipids include trehalose dimycolates of mycobacteria, N-acylated nod factors of rhizobia, and O-acylated glucose derivatives of plants. LPS is classified as a saccarolipid glycan.
Characterization of EptB, a Ca$^{2+}$-induced pEtN Transferase 21003

![Proposed reaction catalyzed by EptB.](image)

**Fig. 1.** Proposed reaction catalyzed by EptB. The position of the pEtN substituent on the outer Kdo unit was determined by Brabetz *et al.* (9) for the pEtN-Kdo$_2$-lipid A isolated from WBB06 grown in the presence of Ca$^{2+}$. The product generated *in vitro* is proposed to have the same structure. The dependence of *in vitro* product formation on PE as the donor substrate is presented. Diacylglycerol is the proposed by-product.

Lipo-oligosaccharide β-chain heptose with pEtN (11). We now demonstrate unambiguously that recombinant YhjW, renamed EptB, utilizes PE as its donor substrate *in vitro*, generates diacylglycerol as a by-product, and depends upon the presence of Ca$^{2+}$ in the assay system for activity. Expression of EptB can be re-engineered to be dependent upon the lac promoter, in which case induction of activity no longer requires the addition of Ca$^{2+}$ to the growth medium. Deletion of *eptB* in the heptose-deficient WBB06 renders this strain strikingly hyper-sensitive to Ca$^{2+}$ at concentrations ≥5 mM, suggesting a function for pEtN modification of the Kdo region in the maintenance of envelope stability. Deletion of *eptB* in wild-type W3110 does not lead to significant Ca$^{2+}$ hypersensitivity, indicating that certain outer core sugars, when present, may provide a similar stabilizing effect.

**EXPERIMENTAL PROCEDURES**

**Materials—**$^{32}$P, and [γ-$^{32}$P]ATP were obtained from PerkinElmer Life Sciences. Silica Gel 60 (0.25 mm) TLC plates were from Merck. Chloroform, ammonium acetate, and sodium acetate were obtained from EM Science. Tryptone and yeast extract were from Difco. Chloroform, ammonium acetate, and sodium acetate were obtained from Avanti Polar Lipids. Phospholipase C from *Bacillus cereus* was purchased from Avanti Polar Lipids. Phospholipase C from *Bacillus cereus* was purchased from Sigma, and 1-palmitoyl-2-[1-$^{14}$C]linoleoylglycero-3-phosphoethanolamine ([116:0, $^{14}$C-18:2]PE) (55 mCi/mmol) was from Amersham Biosciences. The bicinechonic acid protein determination kit and Triton X-100 were from Pierce. All other chemicals were reagent grade and were purchased from either Sigma or Mallinckrodt.

**Bacterial Strains—**The bacterial strains used in this study are described in Table I. Typically, bacteria were grown at 37 °C in LB medium, which consists of 10 g of NaCl, 10 g of tryptone, and 5 g of yeast extract per liter (12). When required for selection of plasmids, cells were grown in the presence of 100 μg/ml ampicillin, 12 μg/ml tetracycline, 25 μg/ml chloramphenicol, or 30 μg/ml kanamycin.

**Construction of eptB Expression Vectors—**The *eptB* (*yhjW*) gene of *E. coli* was cloned into pET28b (Novagen) behind the T7lac promoter. The predicted coding region for *eptB* was amplified by PCR from *E. coli* W3110 genomic DNA. The forward primer contained a clamp region, an NcoI site (underlined), and the coding region with its stop codon (boldface). Sequences of the forward and reverse primers were 5′-GGCGCGGATCC-GTCTTATCACCTGTTTGTCCA-3′ and 5′-GGCGCGATCCCTTGGTACGTTAGCCTTGCCCTC-3′, respectively. The PCR mixture contained 300 ng of genomic DNA as template, 0.2 μg of each primer, 200 μM each of dNTP, 100 mM Tris-HCl, pH 8.8, 35 mM MgCl$_2$, 250 mM KCl, and 5 units of *Pfu* DNA polymerase ( Stratagene) in a reaction volume of 0.1 ml. The reaction mixture was subjected to a 1-min denaturation at 94 °C, followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension for 10 min at 72 °C, using the PerkinElmer Life Sciences GeneAmp PCR System 2400. The PCR product and the pET28b vector were both digested with NcoI and BamHI, ligated together, and transformed into XL-1 Blue cells (Stratagene) for propagation of the desired plasmid, designated pEptB1. In some experiments, pEptB1 was used directly for EptB protein expression in strain C41(DE3) (Table I).

**Molecular Biology Applications—**Protocols for handling of DNA samples were those of Sambrook and Russell (13). Transformation-competent cells of *E. coli* were prepared by the method of Inoue *et al.* (14). When required, *E. coli* cells were prepared for electroporation by the method of Sambrook and Russell (13). Plasmids were prepared using the Qiagen spin prep kit. DNA fragments were isolated from agarose gels using the Qiagen gel extraction kit. Genomic DNA was isolated using the protocol for bacterial cultures in the Easy-DNA$^{TM}$ kit (Invitrogen). T4 DNA ligase (Invitrogen), restriction endonucleases (New England Biolabs), and shrimp alkaline phosphatase (U. S. Biochemical Corp.) were used according to the manufacturer’s instructions. Double-stranded DNA sequencing was performed with an ABI Prism 377 instrument at the Duke University DNA Analysis Facility. Primers were purchased from MWG Biotech.

**Materials—**$^{32}$P, and [γ-$^{32}$P]ATP were obtained from PerkinElmer Life Sciences. Silica Gel 60 (0.25 mm) TLC plates were from Merck. Chloroform, ammonium acetate, and sodium acetate were obtained from EM Science. Tryptone and yeast extract were from Difco. E. coli PE and other PE species were purchased from Avanti Polar Lipids. Phospholipase C from *Bacillus cereus* was purchased from Sigma, and 1-palmitoyl-2-[1-$^{14}$C]linoleoylglycero-3-phosphoethanolamine ([116:0, $^{14}$C-18:2]PE) (55 mCi/mmol) was from Amersham Biosciences. The bicinechonic acid protein determination kit and Triton X-100 were from Pierce. All other chemicals were reagent grade and were purchased from either Sigma or Mallinckrodt.

**Bacterial Strains—**The bacterial strains used in this study are described in Table I. Typically, bacteria were grown at 37 °C in LB medium, which consists of 10 g of NaCl, 10 g of tryptone, and 5 g of yeast extract per liter (12). When required for selection of plasmids, cells were grown in the presence of 100 μg/ml ampicillin, 12 μg/ml tetracycline, 25 μg/ml chloramphenicol, or 30 μg/ml kanamycin.

**Construction of eptB Expression Vectors—**The *eptB* (*yhjW*) gene of *E. coli* was cloned into pET28b (Novagen) behind the T7lac promoter. The predicted coding region for *eptB* was amplified by PCR from *E. coli* W3110 genomic DNA. The forward primer contained a clamp region, an NcoI site (underlined), and the coding region with its stop codon (boldface). Sequences of the forward and reverse primers were 5′-GGCGCGGATCC-GTCTTATCACCTGTTTGTCCA-3′ and 5′-GGCGCGATCCCTTGGTACGTTAGCCTTGCCCTC-3′, respectively. The PCR mixture contained 300 ng of genomic DNA as template, 0.2 μg of each primer, 200 μM each of dNTP, 100 mM Tris-HCl, pH 8.8, 35 mM MgCl$_2$, 250 mM KCl, and 5 units of *Pfu* DNA polymerase ( Stratagene) in a reaction volume of 0.1 ml. The reaction mixture was subjected to a 1-min denaturation at 94 °C, followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension for 10 min at 72 °C, using the PerkinElmer Life Sciences GeneAmp PCR System 2400. The PCR product and the pET28b vector were both digested with NcoI and BamHI, ligated together, and transformed into XL-1 Blue cells (Stratagene) for propagation of the desired plasmid, designated pEptB1. In some experiments, pEptB1 was used directly for EptB protein expression in strain C41(DE3) (Table I).
The eptB gene was also moved from pEptB1 into pWSK29 (15), a lac-inducible, low copy expression vector. The XbaI BamHI-digested fragment, consisting of the eptB gene as well as the P2T28b-derived ribosome binding site (RBS), was ligated to the corresponding restriction sites of pWSK29. This plasmid, designated pEptB2, was then transformed into E. coli WBB06 or electroporated into the RBS-negative E. coli strain WBB06 (9).

Construction of waaC and waaC/waaF Expression Vectors—The waaC gene of E. coli (16) was cloned into pWSK29 behind the lac promoter to generate plasmid pWaaC. The coding region for the adjacent waaC and waaF genes from W3110 genomic DNA was amplified from E. coli W3110 genomic DNA by PCR. The forward primer contained a clamp region, an XbaI site, and an RBS (underlined) immediately in front of the waaC gene, while the reverse primer was the same as that used to amplify pWaaC. A second plasmid (pWaaCF), in which both waaC and waaF were under control of the lac promoter, was created by ligating the corresponding restriction sites of pWSK29. This plasmid, designated pEptB2, was then transformed into E. coli E. coli XL1 Blue-MR (Novagen) to generate pWSK29 Low copy expression vector, pEptB2.

### Relevant bacterial strains and plasmids

| Strain/Plasmid | Description | Source or Ref. |
|---------------|-------------|---------------|
| E. coli strains | merABC recA1 endA1 gyrA96 relA1 supE44 thi-1 lac | Stratagene |
| W3110         | Wild-type, F−, λ− | E. coli Genetic Stock Center (Yale) |
| WBB06         | W3110 Δ(lacO1-165) : tet, heptose-deficient | This work |
| WBB06/pEptB::kan | WBB06 with eptB replaced by kan | This work |
| DY320         | W3330 with eptB replaced by kan | This work |
| DY330/pEptB::kan | W3110 with eptB replaced by kan | This work |
| WBB06/BLR(Δ330)/pEpsS | W3110 ΔrpsL600: Tet1-103 (Δ330), tet(‘em’), kan | Novagen |
| W3110/Δ330 | F−ompT gal hadS2 (R_m) dcm lon ΔDE3 | Avidis SA (France) |

Plasmids
- pET28b: Expression vector, T7lac promoter, kan’
- pEptB1: pET28b expressing eptB (yhiJ)
- pET21a+: Expression vector, T7lac promoter, amp’
- pWSK29: Low copy expression vector, lac promoter, amp’
- pEptB2: pWSK29 expressing eptB
- pKD46: A-RED plasmid, repABC146, parBAD γ β Eko, amp’
- pWaaC: pWSK29 expressing waaC
- pWaaCF: pWSK29 expressing waaC and waaF
- pLpxL: pET21a+ expressing LpxL

#### Preparation of Lipid Substrates—The subunit Kdo-4[^1]Pilepid A was synthesized in three separate steps. First, 100 μC of [γ-[^32]P]ATP, 0.125 mg/ml tetracyclidicarboxylic acid 1-phosphate (21, 22), 1% Nonidet P-40, 5 mM MgCl₂, and 1 mg/ml beef heart cardiolipin were added to a 1.5-mL microcentrifuge tube, and the volume was adjusted to 40 μL with water. Next, 5 μL of E. coli BLR (DE3)/pLysSpJK2 (23) membranes, which express the 4’-kinase LpxK (0.05 mg/mL), were added to the tube, and the mixture was incubated at room temperature for 10 min. A second 5-μL portion of the LpxK-overexpressing membranes was then added and incubated for 10 min at room temperature to generate 4’[^32]Pilepid IV₆ in ~70% yield based on input [γ-[^32]P]ATP.

While the synthesis of the 4’[^32]Pilepid IV₆ was in progress, the components for the second step of the reaction were assembled in a separate 1.5-mL microcentrifuge tube. The reagents for Kdo addition to the 4’[^32]Pilepid IV₆ acceptor consisted of 2 μM carrier lipid IV₆, 0.1% fatty acid-free phosphatidylcholine (22), 20% Unilin (Perkin-Elmer), and 0.03 units of purified E. coli CMP-Kdo synthase (24, 25), and 0.65 μg/ml purified E. coli Kdo transferase (26) in total volume of 50 μL. Upon completion of the 4’[^32]Pilepid IV₆ step, the reaction components needed for the addition of the Kdo residues were added to the 4’[^32]Pilepid IV₆-containing tube, and the mixture was incubated at room temperature for 30 min.

While this was in progress, the components for the third step of the synthesis of Kdo-4[^32]Pilepid A were added. These consisted of 50 mM Hepes, pH 7.5, 0.1% Triton X-100, 50 mM NaCl, 50 mM MgCl₂, 0.1 mg/ml bovine serum albumin, and 2 μM lauryl-acyl carrier protein (27, 28), 0.05 mg/ml E. coli BLR (DE3)/pLysSpLpxL membranes (19), and 0.05 mg/ml E. coli BLR (DE3)/pLysSpLpxLM (27, 28) membranes in a total volume of 50 μL. Upon completion of the Kdo-4[^32]Pilepid IV₆ synthesis step, the above components were added, and the acylation reactions catalyzed by LpxL and LpxM were allowed to proceed at room temperature for 30 min.

The total reaction mixture was then spotted onto a 10 × 20-cm TLC plate and dried under a cold air stream. The plate was developed in the solvent system chlorofrom, pyridine, 88% formic acid, water (30:70:16:10, v/v/v). Following chromatography, the plate was dried under a cold air stream and exposed to x-ray film for 20 s to locate the Kdo-4[^32]Pilepid A. The region of the silica plate containing the product was removed by scraping, transferred to a thick-walled glass tube, and resuspended in 4 mL of acidic single-phase Bligh/Dyer mixture (29, 30) consisting of chloroform, methanol, 0.1 M HCl (1:2:0.8, v/v/v). The suspension was vigorously mixed with the aid of a vortex and subjected to sonic irradiation in a bath for ~30 s. The silica particles were removed with a clinical centrifuge at top speed for 10 min. The supernatant, con-
Characterization of EptB, a Ca\textsuperscript{2+}-induced pEtN Transferase

21205

...ing the 32\textsuperscript{P}-labeled lipid, was removed, and the extraction of the silica was repeated, first with 4 ml and then with 8 ml of the acidic single-phase Bligh/Dyer mixture. The extracts were pooled, passed through a 4 ml glass-wool column to remove remaining silica particles, and placed into glass tubes equipped with Teflon-lined caps. The solution was converted to a two-phase Bligh/Dyer mixture, consisting of chloroform, methanol, 0.1 M HCl (2:2:1.8, v/v). The phases were separated in a clinical centrifuge. The lower phases were transferred to new glass tubes. The upper phases was extracted a second time by the addition of fresh, pre-equilibrated acidic lower phases. The lower phases were pooled, neutralized by addition of pyridine (1 drop per 2-ml lower phase), and dried under a stream of N\textsubscript{2}. The Kdo\textsubscript{2}-4\textsuperscript{IP}lipid A was resuspended in 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. The amount of Kdo\textsubscript{2}-4\textsuperscript{IP}lipid A recovered was typically greater than 50 \muCi. Nonradioactive carrier Kdo\textsubscript{2}-lipid A was prepared from WBB06 in milligram quantities, as described previously (31).

Assay Conditions for Detecting pEtN Transferase Activity—The EptB transferase was assayed under optimized conditions in a 15-µl reaction mixture containing 50 mM Hepes, pH 7.5, 0.1% Triton X-100, 1 mM calcium chloride (CaCl\textsubscript{2}), 1.25 mM dithiothreitol (DTT), 0.6 mM E. coli PE, and 10 µM Kdo\textsubscript{2}-4\textsuperscript{IP}lipid A (50,000 cpm/mmol). Washed membranes were employed as the enzyme. Assay mixtures were incubated at 37 °C with shaking at 250 rpm for predetermined times, and 4-µl portions were spotted onto Silica Gel 60 TLC plates to stop the reactions. Substrate and product(s) were separated using the solvent chloroform/methanol/water/acetic acid (25:15:4:4, v/v). Following chromatography, the plates were dried and analyzed using a Amersham Biosciences PhosphorImager (STORM 840), equipped with ImageQuant software.

Assay for Kdo\textsubscript{2}-Lipid A-dependent Diacyl glycerol Formation—EptB-catalyzed diacylglycerol formation was assayed in a 15-µl reaction mixture containing 50 mM Hepes, pH 7.5, 0.1% Triton X-100, 1 mM CaCl\textsubscript{2}, 1.25 mM DTT, 200 mM Kdo\textsubscript{2}-lipid A, and 100 µM 1-palmitoyl-2-[1-\textsuperscript{14}C]linoleoylglycerol-3-phosphoethanolamine (160.1, 14C-18:2)PE) at 5 × 10\textsuperscript{5} dpm/reaction. Triton X-100-solubilized C41(DE3)/pETB1 membranes (0.1 mg/ml) were used as the enzyme source. Control reactions in which Kdo\textsubscript{2}-lipid A or enzyme was omitted from the assay mixture were performed in parallel. Assay were read as “Control” or “Control—”. Substrate and product(s) were separated using the solvent hexane/diethyl ether/acetic acid (30:70:1, v/v) (32). Following chromatography, the plates were dried and analyzed using a Amersham Biosciences PhosphorImager (STORM 840), equipped with ImageQuant software.

Separation of Inner and Outer Membranes—Membranes of W3110 harboring pEptB2 were separated by isopycnic sucrose gradient centrifugation. First, 120-µl LB cultures of W3110 harboring pEptB2 were grown to an OD of 0.6 at 37 °C, the cells were harvested as described above, and membranes were prepared from a Beckman SW40.1 rotor for 18 h at 4 °C. Each fraction (0.5 ml) was assayed for pEtN transferase activity.

Mild Acid Hydrolysis of pEtN-Kdo\textsubscript{2}-4\textsuperscript{IP} Lipid A Generated in Vitro—Two 10-µl reaction mixtures were prepared in 50 mM Hepes, pH 7.5, and 0.1% Triton X-100, containing either Kdo\textsubscript{2}-4\textsuperscript{IP}lipid A (10,000 cpm) or pEtN-Kdo\textsubscript{2}-4\textsuperscript{IP}lipid A (10,000 cpm). The pEtN-Kdo\textsubscript{2}-4\textsuperscript{IP}lipid A was generated under standard pEtN transerase assay conditions (described above). It was purified by TLC, as described for Kdo\textsubscript{2}-4\textsuperscript{IP}lipid A. Next, 6.5 µl of 200 mM sodium acetate, pH 4.5, and 4 µl of 10% SDS were added to each tube, and the final volumes were adjusted to 40 µl. The reaction mixtures were placed into a heat block at 100 °C. At various times, 4-µl samples were withdrawn and spotted onto a silica TLC plate, which was developed in the solvent chloroform, pyridine, 88% formic acid, water (30:70:16:10, v/v) (10).

In-frame Replacement of eptB with a Kanamycin Resistance Cassette in E. coli WBB06—To create an in-frame replacement of eptB with the kanamycin resistance cassette (kan), WBB06 was transformed by bacteriophage \textsuperscript{32}P-labeled lipid A (10,000 cpm). The pEtN-Kdo\textsubscript{2}-4\textsuperscript{IP}-\textsuperscript{32}P-lipid A (10,000 cpm) was transferred into the \textsuperscript{32}P-labeled lipid A. After growth for 2 h at 37 °C, the cells were plated on LB agar containing kanamycin (20 µg/ml) and incubated overnight at 37 °C. Kanamycin-resistant colonies were re-purified on LB kanamycin plates, which were incubated overnight at 37 °C to cure the strain of pKDa6. The eptB::kan replacement on the chromosome of WBB06 was verified by PCR using external primers located 95 bp upstream (5'-CACACTCTTTCCTCCACATTTTCC-3') and 118 bp downstream (5'-CTCCGACCCCTTCTGGTCCGAAAGAAG-3') of eptB. A single PCR product was resolved on a 1% agarose gel, purified with the QiAquick gel extraction kit, and sequenced using the same primers to confirm the replacement. The resulting strain, designated WBB06::EptB::kan, was made electrodempotent by the method of Simbrock and Russell (33) and subjected to ultracentrifugation at 35,000 rpm in a Beckman SW40.1 rotor—4). Growth was allowed to continue at 37 °C. Whenever the A\textsubscript{600} reached 0.3–0.4, the cultures were diluted 10-fold into 50 ml of prewarmed LB, containing the relevant divalent cations, in order to keep the cells in log phase.

Extraction and Purification of LPS from E. coli WBB06—To determine whether the LPS molecules in WBB06 are covalently modified with pEtN when eptB is expressed under lac control from pWSK29 in WBB06, 1-liter cultures of WBB06 harboring either pWSK29 or pEptB2 were grown in the presence of 100 µg/ml ampicillin and 1 mM IPTG until A\textsubscript{600} = 1.0. The LPS was extracted and purified, as described previously (30).

In-frame Replacement of eptB with a Kanamycin Resistance Cassette in E. coli W3110—The eptB gene was also replaced with kan on the chromosome of E. coli W3110. The purified PCR product used to create the replacement of eptB on the chromosome of WBB06 (see above) was utilized to construct W3110::epetB:kan. First, the PCR product was electroporated into the \textsuperscript{32}P-labeled strain, DY330 (39). After growth for 2 h at 37 °C, the cells were plated on LB agar containing kanamycin (20 µg/ml) and incubated overnight at 37 °C. Kanamycin-resistant colonies were re-purified on LB kanamycin plates. One of the kanamycin-resistant colonies was designated W3110::epetB:kan. A P\textsubscript{1} integrase bacteriophage lysate of donor strain DX330::epetB:kan was made as described by Miller (12) and used to transduce stationary cells of W3110. The transduction mixture was plated onto LB agar, containing 20 µg/ml kanamycin and 5 mM sodium citrate, and then incubated at 37 °C overnight. Kanamycin-resistant colonies were re-purified with selection, and the eptB::kan replacement on the chromosome of W3110 was verified by PCR using the same primers used to confirm the eptB::kan replacement on the chromosome of WBB06 (see above). The PCR product was resolved on a 1% agarose gel, purified with the QiAquick gel extraction kit, and sequenced to confirm the replacement.

Mass Spectrometry of Kdo\textsubscript{2}-Lipid A Samples—Spectra were acquired in the negative-ion linear mode using a Kratos (Manchester, UK) analytical matrix-assisted laser desorption ionization/time of flight (MALDI/TOF) mass spectrometer with a 337-nm nitrogen laser, a 20-kV extraction voltage, and time-delayed extraction. Each spectrum was the average of 50 shots. The lipid A samples were prepared for MALDI/TOF analysis by deposing 0.3 µl of the lipid sample, dissolved in chloroform/methanol (4:1, v/v), followed by 0.3 µl of a saturated solution of 6-aza-2-thiothymine in 50% acetonitrile and 10% trisaccharide ammonium citrate (9:1, v/v) as the matrix. The samples were dried at room temperature before the spectra were acquired.

RESULTS
pEtN Transferase Activity in Membranes of Ca\textsuperscript{2+}-treated WBB06 Versus EptB Overexpressing WBB06—Membranes of the heptose-deficient mutant WBB06, grown in LB broth con-
taining 5–50 mM CaCl₂, exhibit pEtN transferase activity in vitro with the tetra-acylated LPS precursor Kdo₂-4’-[³²P]lipid IV₄ as the acceptor substrate (10). The pEtN transferase activity was also observed when hexa-acylated Kdo₂-4’-[³²P]lipid A was employed as the lipid acceptor (Fig. 2, lanes 3 and 4). To determine whether the pEtN transferase activity seen in Ca²⁺-treated WBB06 is because of EptB, we examined membranes of EptB-overexpressing cells. As shown in Fig. 2, lane 6, the Rₚ of the product generated by membranes of WBB06/pEptB2 was the same as that produced by membranes of Ca²⁺-treated WBB06 (Fig. 2, lanes 3 and 4). However, much more product was formed with WBB06/pEptB2 membranes. No reaction was seen with the vector control WBB06/pWSK29 (Fig. 2, lane 5).

The band labeled x in Fig. 2 reflects the formation of the lipid A 1-diphosphate variant, which is generated from an endogenous, as yet unknown, phosphate donor. This activity is partially suppressed by Ca²⁺ in the assay mixture.

EDTA to the assay inhibits pEtN transferase activity (10). To determine whether or not Ca²⁺ is required for pEtN transfer to Kdo₂-4’-[³²P]lipid A, diluted membranes of WBB06 overexpressing EptB were assayed in the presence or absence of 1 mM CaCl₂. Ca²⁺ greatly stimulated pEtN transferase activity in vitro when 0.025 mg/ml of membranes from EptB-overexpressing cells were employed as the enzyme source (Fig. 3B). The optimal Ca²⁺ concentration in the in vitro system was 1 mM (data not shown); higher concentrations of Ca²⁺ were inhibitory. As noted above, the formation of the lipid A 1-diphosphate (x) in Fig. 3) was suppressed by Ca²⁺. All subsequent assays of EptB therefore included both 0.6 mM PE and 1 mM Ca²⁺, unless otherwise indicated. Production of pEtN-Kdo₂-3⁴⁻lipid A by over-expressed EptB was linear for almost 60 min under the optimized conditions (Fig. 4, A and B), and it was linear with membrane protein up to 0.2 mg/ml at 30 min (data not shown).

**EptB Selectivity for Ca²⁺ Ions**—The induction of pEtN transferase in membranes of WBB06 was strictly dependent upon the addition of ≥5 mM Ca²⁺ to the growth medium; other common divalent cations were not effective (10). To determine whether other divalent cations could replace Ca²⁺ in vitro, we tested several other ions at 1 mM. Ca²⁺ stimulated the greatest conversion of Kdo₂-4’-[³²P]lipid A to pEtN-Kdo₂-4’-[³²P]lipid A. Sr²⁺ also caused a slight stimulation of pEtN transferase activity, but Mg²⁺ and Ba²⁺ were inactive (data not shown).

**Efficacy of PE Molecular Species as Substrates for EptB**—Because *E. coli* PE consists of about 10 different molecular species (2, 42), we tested several commercially available PEs at 500 μM as pEtN donors for EptB. As shown in Fig. 5, PE species (16:0/18:1, 16:0/18:2, 18:0/18:1, or 18:1/18:1) with one or more double bonds were effective pEtN donors, whereas PEs with fully saturated acyl chains (16:0/16:0 and 18:0/18:0) were inactive. Similarly, saturated PEs with shorter acyl chains (10:0/10:0, 12:0/12:0, and 14:0/14:0) were not substrates (data not shown). Fully saturated PEs usually represent less than 5% of the PE molecular species present in the membranes of *E. coli*.

**Stoichiometric Formation of Diacylglycerol and pEtN-Kdo₂⁻⁴⁻**—As shown in Fig. 6A, EptB catalyzed efficient time-dependent conversion of 200 μM Kdo₂-4’-[³²P]lipid A to pEtN-Kdo₂-4’-[³²P]lipid A with 100 μM [16:0/18:2]PE as the donor. At this ratio of concentrations, EptB-dependent diacylglycerol formation could be monitored in parallel by employing...
Characterization of EptB, a Ca\(^{2+}\)-induced pEtN Transferase

**Fig. 4. Time dependence of product formation by EptB.** Membranes of WBB06/pEptB2 were assayed for pEtN transferase activity at 0.1 mg/ml with 10 \(\mu\)M Kdo\(_2\)-lipid A at the indicated times. A, products were separated by TLC in the solvent chloroform/methanol/water/acetic acid (25:15:4:4, \(v/v\)) and analyzed with a PhosphorImager. B, the conversion of Kdo\(_2\)-lipid A to pEtN-Kdo\(_2\)-lipid A is linear with respect to the time for up to 60 min.

unlabeled Kdo\(_2\)-lipid A and [16:0,\(^{14}\)C-18:2]PE as substrates (Fig. 6B). Release of \([^{14}\)C]diacylglycerol (Fig. 6B) followed the same time course as the formation of pEtN-Kdo\(_2\)-[\(^{32}\)P]lipid A (Fig. 6A) with a stoichiometry of 0.9 mol of \([^{14}\)C]diacylglycerol/mol of pEtN-Kdo\(_2\)-[\(^{32}\)P]lipid A at the 60-min time point. The small amount of \([^{14}\)C]diacylglycerol formed by EptB in the absence of added Kdo\(_2\)-lipid A (Fig. 6B, lane 13) may be due to the slow transfer of the phosphoethanolamine residue from [16:0,\(^{14}\)C-18:2]PE to water in the absence of acceptor substrate.

pEtN Is Added to the Outer Kdo Residue of Kdo\(_2\)-lipid A by EptB—The pEtN-Kdo\(_2\)-[\(^{32}\)P]lipid A produced in vitro by membranes of WBB06 cells that overexpress EptB was isolated by TLC, and it was subjected to conditions (pH 4.5 and 100 °C) that slowly cleave the glycosidic linkages of the Kdo moieties (43). By comparing the radioactive products generated by hydrolysis of pEtN-Kdo\(_2\)-[\(^{32}\)P]lipid A to those of Kdo\(_2\)-[\(^{32}\)P]lipid A (Fig. 7A), we showed that EptB adds pEtN mainly to the outer Kdo residue. Hydrolysis of pEtN-Kdo\(_2\)-[\(^{32}\)P]lipid A (Fig. 7B) and Kdo\(_2\)-[\(^{32}\)P]lipid A (Fig. 7A) display the same time course and pattern of product formation, with Kdo\(_2\)-[\(^{32}\)P]lipid A (lacking the pEtN residue) as the apparent intermediate. If the pEtN group had been attached to the inner Kdo residue or to the lipid A moiety, a different product would have been observed. Specifically, pEtN-Kdo\(_2\)-[\(^{32}\)P]lipid A and/or pEtN-4-[\(^{32}\)P]lipid A would have been seen during the hydrolysis of pEtN-Kdo\(_2\)-[\(^{32}\)P]lipid A. The absence of these products indicates that the pEtN unit is attached to the outer Kdo. Whether or not the pEtN moiety is attached to the 7-position of the outer Kdo, as proposed in Fig. 1 (9), remains to be established.

**Inner Membrane Localization of EptB—**Membranes of wild-type E. coli W3110 overexpressing EptB were subjected to isopycnic sucrose gradient centrifugation to separate inner and outer membranes. The EptB activity was found almost entirely within the inner membrane (Fig. 8). As shown in Fig. 8B, the pEtN transferase closely followed the activity of the inner membrane marker NADH oxidase (peak activity at fraction 14). As shown in Fig. 8A, we also determined the membrane protein concentration of each fraction and assayed the outer membrane marker phospholipase A (peak activity in fraction 3).

**MALDI/TOF Mass Spectrometry of LPS Isolated from WBB06/pEptB2—**LPS of WBB06 was shown previously to contain a pEtN substituent attached to its outer Kdo residue when the cells were grown in the presence of 5–50 mM CaCl\(_2\) (10). To confirm that EptB expressed under lac control catalyzes the addition of pEtN to Kdo\(_2\)-lipid A in living cells, we isolated LPS from WBB06 harboring either pEptB2 or the vector control pWSK29, grown with IPTG, but in the absence of added CaCl\(_2\). The LPS was analyzed by MALDI/TOF mass spectrometry in both the negative and positive ion modes. As shown in Fig. 9A, negative ion MALDI/TOF mass spectrometry revealed that DEAE-cellulose purified LPS from WBB06/pWSK29 consisted mostly Kdo\(_2\)-lipid A (\([M - H]\) \(= 2236.4\) atomic mass units). The spectrum also shows the presence of some free lipid A (\(m/z = 1796.6\) atomic mass units), which arises by loss of the Kdo residues during MALDI/TOF analysis. The negative-mode MALDI/TOF spectrum of LPS purified from WBB06/pEptB2 showed the same ions as described above, but in addition also contained a third species at \(m/z = 2360.0\) atomic mass units, interpreted as \([M - H]\) of pEtN-Kdo\(_2\)-lipid A (Fig. 9B). Based upon the mass spectrometry and TLC analysis of LPS isolated from WBB06 harboring pEptB2 (data not shown), we estimate that about one-third of the LPS molecules are modified with pEtN when EptB is expressed from the lac promoter.

Positive-mode MALDI/TOF analysis (not shown) of the LPS from WBB06/pEptB2 confirmed the presence of the pEtN substituent. The spectrum contained peaks at \(m/z = 2362.5\), interpreted as \([M + H]^+\) of pEtN-Kdo\(_2\)-lipid A, and at \(m/z = 2264.5\)
Characterization of EptB, a Ca\textsuperscript{2+}-induced pEtN Transferase

Solvent Front

A

B

Kdo\textsubscript{2}-[\textsuperscript{32}P]lipid A

pETN-Kdo\textsubscript{2}-[\textsuperscript{32}P]lipid A

PE Donor

EptB Extract

Vector Control

Lane 1–7: samples taken at times 0, 20, 40, 60, 80, 100, and 120 min, respectively. Lanes 8–11 are 120-min time points.

FIG. 6. Stoichiometric formation of diacylglycerol during pEtN transfer to Kdo\textsubscript{2}-lipid A. A, EptB-catalyzed formation of pEtN-Kdo\textsubscript{2}-4'-[\textsuperscript{32}P]lipid A was assayed in a 15-\textmu l reaction mixture containing 50 mM Hepes, pH 7.5, 0.1% Triton X-100, 1 mM CaCl\textsubscript{2}, 1.25 mM DTT, 200 \mu M Kdo\textsubscript{2}-4'-[\textsuperscript{32}P]lipid A (5 \times 10\textsuperscript{4} cpm/reaction), and 100 \mu M [16:0,18:2]PE. Triton X-100-solubilized C41(DE3)/pEptB1 membranes (0.1 mg/ml) were used as enzyme. Assays were carried out at 30 °C, and 5-\mu l portions were spotted at various times onto Silica Gel 60 TLC plates to stop the reactions. Substrate and product(s) were separated using the solvent chloroform/methanol/water/acetic acid (25:15:4:4, v/v). Reactions in which PE, enzyme, or both were omitted, and the vector control, are indicated. Lanes 1–7 are samples taken at times 0, 20, 40, 60, 80, 100, and 120 min respectively. Lanes 8–11 are 120-min time points. B, EptB-catalyzed diacylglycerol formation was assayed under the same conditions as in A, but with 200 \mu M Kdo\textsubscript{2}-lipid A and 100 \mu M [16:0,14C-18:2]PE (5 \times 10\textsuperscript{4} dpm/reaction). Substrate and product(s) were separated using the solvent hexane/diethyl ether/acetic acid (30:70:1, v/v) (32). Following chromatography, the plates were dried and analyzed with a PhosphorImager. Reactions in which Kdo\textsubscript{2}-lipid A, enzyme, or both were omitted, and the vector control, are indicated. Lanes 1–7 are samples taken at times 0, 20, 40, 60, 80, 100, and 120 min, respectively. Lanes 8–13 are 120-min time points. A [14C]diacylglycerol standard (lane 10) was generated with B. cereus phospholipase C (PLC) (33).

FIG. 7. EptB adds pEtN to the outer Kdo residue of Kdo\textsubscript{2}-lipid A. A, Kdo\textsubscript{2}-4'-[\textsuperscript{32}P]lipid A, the substrate, was hydrolyzed at pH 4.5 in the presence of SDS at 100 °C over the indicated time course. B, the pEtN4'-[\textsuperscript{32}P]Kdo\textsubscript{2}-lipid A, generated in vitro by membranes of WBB06/pEptB2, was hydrolyzed in parallel. At each time point, 4-\mu l portions of the hydrolysis mixtures were spotted onto a silica gel thin layer chromatography plate, which was developed in the solvent chloroform, pyridine, 88% formic acid, water (30:70:16:10, v/v). The various hydrolysis products were detected with a PhosphorImager.

FIG. 8. Inner membrane localization of EptB. Inner and outer membranes isolated from W3110/pEptB2 were separated by isopycnic sucrose density gradient centrifugation, as described previously, and 0.5-ml fractions were collected. A, the protein concentration (open squares) and the outer membrane phospholipase A activity (closed squares) were determined. B, the pEtN transferase (open circles) and the inner membrane NADH oxidase activity (closed circles) were measured.

(characterized as the B\textsubscript{2} ion of pEtN-Kdo\textsubscript{2}-lipid A) (44). These ions were absent in the positive-mode spectrum of the LPS purified from WBB06/pWSK29.

Ca\textsuperscript{2+} Sensitivity of WBB06eptB::kan and Its Reversal by Excess Mg\textsuperscript{2+}.—To study the function of EptB, we constructed an in-frame replacement of the chromosomal copy of eptB in WBB06 with a kanamycin resistance cassette. The parental strain WBB06 grew normally on LB medium in the presence or absence of added Ca\textsuperscript{2+} (Fig. 10A). The growth rate of WBB06eptB::kan was almost the same as that of WBB06 in the absence of Ca\textsuperscript{2+} (Fig. 10A), but addition of 5 mM (or higher) Ca\textsuperscript{2+} during mid-log phase (Fig. 10A, arrow) rapidly inhibited its subsequent growth.
Neither Mg\(^{2+}\) (Fig. 10B) nor Sr\(^{2+}\) (not shown) had any effect on the growth of WBB06 (not shown) or WBB06epkB::kan. However, inclusion of 50 mM MgCl\(_2\) in the medium prevented the Ca\(^{2+}\)-induced growth arrest of WBB06epkB::kan (Fig. 10B).

Suppression of Ca\(^{2+}\) Sensitivity by Restoration of Either EptB or the Full Core Domain to WBB06—To exclude the possibility that the Ca\(^{2+}\) hypersensitivity was because of a polar effect arising from the replacement of the eptB gene with kan, WBB06epkB::kan was transformed with pEptB2. This construct grew normally in Ca\(^{2+}\)-containing medium (Fig. 10C), whereas the vector control was Ca\(^{2+}\)-sensitive.

Cultures of WBB06epkB::kan harboring either pWaaC or pWaaCF were challenged with 5 mM CaCl\(_2\) to determine whether restoration of a part (or all) of the LPS core could eliminate the Ca\(^{2+}\) sensitivity. As shown in Fig. 10C, supplying waaC in trans, which results in the incorporation of a single heptose residue (6, 8), did not alleviate the Ca\(^{2+}\) hypersensitivity. However, supplying both heptosyl transferase genes (waaC and waaF) in trans, which fully restores the LPS core (6, 8), allowed the eptB knock-out strain to grow in the presence of 5 mM Ca\(^{2+}\). These findings are consistent with the additional observation (data not shown) that deletion of eptB in wild-type E. coli W3110 does not result in Ca\(^{2+}\) sensitivity.

DISCUSSION

Phosphoethanolamine residues are commonly found as substituents of LPS (6–8) and other surface glycoconjugates in
Gram-negative bacteria (45). Very little is known about the enzymes that generate the pEtN-modified molecules present in the cell envelope or about the biological function of pEtN modifications. In order to explore these issues, it is necessary to identify the genes encoding the relevant pEtN transferases and to construct mutants lacking them. In the present work we have found that the eptB gene, formerly yhjW, encodes the Ca\(^{2+}\)-induced pEtN transferase known to modify the outer Kdo residue of E. coli LPS (9, 10). We have constructed a mutant in which eptB is deleted, and we found that it is extremely sensitive to added Ca\(^{2+}\) ions, provided that it is also lacking the heptose residues of the LPS core domain.

The following strategy was used to find eptB. Mackinnon et al. (11) first reported the lpt-3 gene of N. meningitidis, which is required for the modification of the lipo-oligosaccharide core of that organism with a specific pEtN residue. They found the lpt-3 gene by screening random mutants with an antibiotic directed against pEtN-modified lipo-oligosaccharide. However, an in vitro enzymatic assay was not developed to prove that the protein encoded by lpt-3, NMB2010, is an enzyme (11). The N. meningitidis protein, NMB2010, has six significant orthologs in E. coli, five of which are predicted to be inner membrane proteins, containing four or five putative trans-membrane helices near their N termini. We therefore cloned all six of these E. coli NMB2010 orthologs. We found that only yhjW expressed behind the lac promoter on pWSK29-directed massive in vitro overexpression of the Kdo-selective pEtN transferase (10) with Kdo\(_2\)-lipid A as the acceptor substrate (Fig. 2). In this setting, enzyme induction was no longer Ca\(^{2+}\)-dependent. The subcellular fractionation study shown in Fig. 8 confirmed the localization of EptB to the inner E. coli membrane.

Overexpression of the other five E. coli orthologs of NMB2010, using the lac promoter of pWSK29, led to the identification of EptA/PmrC (46), the enzyme that modifies the phosphate groups of lipid A with pEtN residues in polymyxin-resistant mutants (47, 48). The active site of PmrC/EptA has been shown by gene fusion experiments to face the periplasm (48). However, PmrC/EptA has not been purified or characterized enzymatically (48), and our EptA in vitro assay (46) was reported only in abstract form. The full characterization of EptA as an enzyme will be described elsewhere.

In addition to being induced selectively by Ca\(^{2+}\), EptB transferase activity is dependent upon the presence of 1 mM Ca\(^{2+}\) in vitro (Fig. 3). EptB joins a growing list of inner (46, 49–50) and outer (20, 51) membrane-bound enzymes that modify LPS; however, EptB is the first of these to exhibit a requirement for Ca\(^{2+}\). The exact role that Ca\(^{2+}\) plays in the transfer of pEtN from PE to the outer Kdo moiety of LPS remains to be determined. The well characterized outer membrane phospholipase A of E. coli exhibits a similar Ca\(^{2+}\) requirement, which in that case may play a role in dimer formation (52). We found that Sr\(^{2+}\) could partially stimulate the pEtN transferase activity of EptB in our in vitro system. Studies with the phospholipase A likewise revealed that Sr\(^{2+}\) could substitute for Ca\(^{2+}\) to some degree.

Our studies show that EptB utilizes several different PE molecular species (Fig. 7) provided they contain at least one double bond. EptB may have evolved to utilize the most abundant PE species normally present in the E. coli envelope (2). We have also confirmed that recombinant EptB modifies the outer Kdo residue selectively (Fig. 7) but cannot transfer pEtN to the inner Kdo unit or to the lipid A moiety at an appreciable rate. The results of the pH 4.5 hydrolysis of pEtN-Kdo\(_2\)-4\(^{±}\)lipid A are definitive with regard to the outer Kdo (Fig. 7), but they do not establish the proposed localization of the pEtN unit at position 7 (Fig. 1).

The four additional EptB orthologs (besides EptA) encoded within the E. coli genome are likely to include the enzyme responsible for the addition of the pEtN moiety to the outer heptose residue of LPS (6). To date, no in vitro assays with defined acceptor substrates have been described for this heptose modification. Another one of the five EptB orthologs might be required for the transfer of pEtN from PE to a sub-set of the periplasmic membrane-derived oligosaccharides, the biosynthesis of which is induced at low osmolality (45, 53).

EptB generates stoichiometric diacylglycerol as a by-product during the transfer of the pEtN unit from PE to Kdo\(_2\)-lipid A (Fig. 6), consistent with the scheme proposed in Fig. 1. This novel source of diacylglycerol, which is likely also generated by the other members of the EptB family, explains why the diacylglycerol that accumulates in E. coli dgk mutants is not solely due to membrane-derived oligosaccharide biosynthesis (32).

In principle, the EptB reaction should be reversible and share some common mechanistic features with the better characterized eucaryotic phosphoethanolamine transferases that generate PE from CDP-ethanolamine and diacylglycerol (54). However, the EptB family of proteins displays no obvious sequence similarity to the eucaryotic phosphoethanolamine transferases, which generally contain seven predicted trans-membrane segments. Iterative analysis of certain EptB orthologs with the Psi-Blast algorithm does suggest a very distant relationship to the phosphoethanolamine transferases that participate in the assembly of the phosphatidylinositol-linked glycans in eucaryotic cells (55).

The Ca\(^{2+}\)-sensitive phenotype of the heptose-deficient mutant, lacking EptB, is very intriguing, as it suggests a possible function for the pEtN modification of the outer Kdo residue. Based on the observed Ca\(^{2+}\) sensitivity of WBB06eptB::kan (Fig. 10), we propose that pEtN modification of the outer Kdo moiety of LPS is critical for tolerance of elevated levels of Ca\(^{2+}\) in heptose-deficient E. coli. It may be that modification of the outer Kdo residue with pEtN renders the outer membrane less permeable to Ca\(^{2+}\). A reduction in Ca\(^{2+}\) permeability may help maintain the very low level of intracellular Ca\(^{2+}\) (≈0.1 μM or less) normally present in E. coli (56). A dramatic increase in the intracellular Ca\(^{2+}\) concentration might be toxic. The fact that excess Mg\(^{2+}\) can block the growth inhibition produced by Ca\(^{2+}\) (Fig. 10B) suggests that excess Mg\(^{2+}\) may prevent Ca\(^{2+}\) from binding to a critical site that destabilizes the cell envelope, preventing the influx of Ca\(^{2+}\). The observation (Fig. 10C) that eptB mutants are not Ca\(^{2+}\)-sensitive in strains with a complete core suggests that the pEtN units that are normally present on the outer heptose residue of the core may substitute for the Ca\(^{2+}\)-inducible pEtN unit attached to the Kdo region. Further genetic and biochemical characterization of the Ca\(^{2+}\)-sensitive phenotype of WBB06eptB::kan should provide insights into the function of this modification.

Acknowledgment—We thank M. Stephen Trent for constructing the LpxL-overexpressing strain BLR(DE3)pLysS/pLpxL.

REFERENCES

1. Cronan, J. E., Jr., Gennis, R. B., and Maloy, S. R. (1987) in Escherichia coli and Salmonella typhimurium (Neidhardt, F. C., ed) Vol. 1, American Society for Microbiology, Washington, D. C.
2. Cronan, J. E. (2003) Annu. Rev. Microbiol. 57, 203–234
3. Lazar, K., and Walker, S. (2002) Curr. Opin. Chem. Biol. 6, 786–793
4. Nishida, H. (2003) Microbiol. Mol. Biol. Rev. 67, 503–566
5. Raetz, C. R. H. (1990) Annu. Rev. Biochem. 59, 129–170
6. Raetz, C. R. H., and Whitefield, C. (2002) Annu. Rev. Biochem. 71, 635–700
7. Brady, H., Opal, S. M., Vogel, S. N., and Morrison, D. C. (eds) (1999) Endotoxin in Health and Disease, p. 950, Marcel Dekker, Inc., New York.
8. Raetz, C. R. H. (1996) in Escherichia coli and Salmonella: Cellular and Molecular Biology, (Neidhardt, F. C., ed) 2nd Ed., pp. 498–503, American Society for Microbiology, Washington, D. C.
9. Brabetz, W., Muller-Luensies, S., Holst, O., and Brade, H. (1997) Eur. J. Biochem. 247, 716–724
10. Kanipes, M. I., Lin, S., Cotter, R. J., and Raetz, C. R. H. (2001) J. Biol. Chem. 276, 71628–71637
Characterization of EptB, a Ca\textsuperscript{2+}-induced pEtN Transferase

276, 1156–1163
11. Mackinnon, F. G., Cox, A. D., Plesed, J. S., Tang, C. M., Makepeace, K., Coull, P. A., Wright, J. C., Chalmers, R., Hood, D. W., Richards, J. C., and Moxon, E. R. (2002) *Mol. Microbiol.* 43, 931–943
12. Miller, R. J. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
13. Sambrook, J. G., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd Ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
14. Inoue, H., Nogima, H., and Okayama, H. (1990) *Gene* (Amst.) 96, 23–28
15. Wang, R. F., and Kushner, S. R. (1991) *Gene* (Amst.) 100, 195–199
16. Kadrmas, J. L., and Raetz, C. R. H. (1998) *J. Biol. Chem.* 273, 2799–2807
17. Sirisena, D. M., Brozek, K. A., MacLachlan, P. R., Sanderson, K. E., and Raetz, C. R. H. (1992) *J. Biol. Chem.* 267, 18874–18884
18. Clementz, T., Bednarski, J. J., and Raetz, C. R. H. (1996) *J. Biol. Chem.* 271, 12095–12102
19. Tran, A. X., Karbarz, M. J., Wang, X., Raetz, C. R. H., McGrath, S. C., Cotter, R. J., and Brent, M. S. (2004) *J. Biol. Chem.* 279, 55780–55791
20. Trent, M. S., Fabich, W., Raetz, C. R. H., and Miller, S. I. (2001) *J. Biol. Chem.* 276, 9083–9092
21. Ray, B. L., Painter, G., and Raetz, C. R. H. (1984) *J. Biol. Chem.* 259, 4852–4859
22. Radika, K., and Raetz, C. R. H. (1988) *J. Biol. Chem.* 263, 14859–14867
23. Garrett, T. A., Kadmras, J. L., and Raetz, C. R. H. (1997) *J. Biol. Chem.* 272, 21855–21864
24. Goldman, R. C., and Kohlbrenner, W. E. (1985) *J. Bacterial.* 163, 256–261
25. Brozek, K. A., Hoanca, K., Robertson, A. D., and Raetz, C. R. H. (1989) *J. Biol. Chem.* 264, 6956–6966
26. Belumis, C. J., and Raetz, C. R. H. (1992) *J. Biol. Chem.* 267, 9988–9997
27. Vorachek-Warren, M. K., Curtis, S. M., Lin, S., Cotter, R. J., and Raetz, C. R. H. (2002) *J. Biol. Chem.* 277, 14186–14193
28. Vorachek-Warren, M. K., Ramirez, S., Cotter, R. J., and Raetz, C. R. H. (2002) *J. Biol. Chem.* 277, 14194–14205
29. Bligh, E. G., and Dyer, J. J. (1959) *Can. J. Biochem. Physiol.* 37, 911–917
30. Nishijima, M., and Raetz, C. R. H. (1979) *J. Biol. Chem.* 254, 7837–7844
31. Doerrler, W. T., Gibbons, H. S., and Raetz, C. R. H. (2004) *J. Biol. Chem.* 279, 41002–41010
32. Trent, M. S., Ribeiro, A. A., Lin, S., Cotter, R. J., and Raetz, C. R. H. (2001) *J. Biol. Chem.* 276, 43122–43131
33. Bishop, R. E., Gibbons, H. S., Guina, T., Trent, M. S., Miller, S. I., and Raetz, C. R. H. (2000) *EMBO J.* 19, 5071–5080
34. Snijder, H. J., Ubarretxena-Belandia, I., Blauuw, M., Kalk, K. H., Verheij, H. M., Egmond, M. R., Dekker, N., and Dijkstra, B. W. (1999) *Nature* 401, 717–721
35. Miller, K. J., and Kennedy, E. P. (1987) *J. Bacterial.* 169, 682–686
36. Kent, C. (1995) *Annu. Rev. Biochem.* 64, 315–343
37. Ferguson, M. A., Brimacombe, J. S., Brown, J. R., Crossman, A., Dix, A., Field, R. A., Guther, M. R., Milne, K. G., Sharma, D. K., and Smith, T. K. (1999) *Biochim. Biophys. Acta* 1455, 327–340
38. Gangola, P., and Rosen, B. P. (1987) *J. Bacterial.* 262, 12570–12574
39. Faby, E., Subramanian, S., Brown, H. A., Glass, C. K., Merrill, A. H., Murphy, R. K., Raetz, C. R. H., Russell, D. W., Seyama, Y., Shaw, W., Shimizu, T., Spener, F., van Meer, G., VanNieuwenhze, M. S., White, S. H., Wittzum, J. L., and Dennis, E. A. (2005) *J. Lipid Res.*, 46, 839–861