Evidence that the wxe gene of Escherichia coli K-12 encodes a protein involved in the transbilayer movement of a trisaccharide-lipid intermediate in the assembly of enterobacterial common antigen

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Running Title: The Wxe-Translocase of E. coli

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**SUMMARY**

The assembly of many bacterial cell-surface polysaccharides requires the transbilayer movement of polyisoprenoid-linked saccharide and oligosaccharide intermediates across the cytoplasmic membrane. It is generally believed that this transverse diffusion of glycolipid intermediates is mediated by integral membrane proteins called translocases or “flippases.” The bacterial genes proposed to encode these putative translocases have been collectively designated \textit{wzx} genes. The \textit{wzxE} gene of \textit{Escherichia coli} K-12 has been implicated in the transbilayer movement of Fuc4NAc-ManNAcA-GlcNAc-P-P-undecaprenol (lipid III), the donor of the trisaccharide repeat unit in the biosynthesis of enterobacterial common antigen (ECA). Previous studies (Feldman \textit{et al.} 1999. J. Biol. Chem. \textbf{274}, 35129-35138) provided indirect evidence that the \textit{wzx}_{016} gene product of \textit{E. coli} K-12 encoded a putative translocase that was capable of mediating the transbilayer movement of \textit{N}-acetylglucosaminylpyrophosphorylnerol (GlcNAc-P-P-Ner), a water-soluble analogue of GlcNAc-P-P-Und. Previous studies (Feldman \textit{et al.} 1999. J. Biol. Chem. \textbf{274}, 35129-35138) provided indirect evidence that the \textit{wzx}_{016} gene product of \textit{E. coli} K-12 encoded a putative translocase that was capable of mediating the transbilayer movement of \textit{N}-acetylglucosaminylpyrophosphorylnerol (GlcNAc-P-P-Ner), a water-soluble analogue of GlcNAc-P-P-Und. Therefore, genetic and biochemical studies were conducted to determine if the putative \textit{Wzx}_{016} translocase was capable of mediating the transbilayer movement of \textit{N}-acetylglucosaminylpyrophosphorylnerol (GlcNAc-P-P-Ner), a water-soluble analogue of GlcNAc-P-P-Und. Hypothesis: In contrast, \textit{[^3H]}GlcNAc-P-P-Ner was transported into sealed, everted cytoplasmic membrane vesicles of \textit{E. coli} K-12 as well as a deletion mutant lacking both the \textit{wzx}_{016} and \textit{wzxC} genes. In contrast, \textit{[^3H]}GlcNAc-P-P-Ner was not transported into membrane vesicles prepared from a \textit{wzxE} null-mutant, and metabolic radiolabeling experiments revealed the accumulation of lipid III in this mutant. The WzxE-mediated transport of \textit{[^3H]}GlcNAc-P-P-Ner into vesicles did not require a proton-motive force, and it appeared to
occur by facilitated diffusion. In addition, the WzxE translocase exhibited substrate specificity by recognizing both a pryophosphoryl-linked saccharide and an unsaturated α-isoprene unit in the carrier lipid. The results presented here support the conclusion that the wzxE gene encodes a membrane protein involved in the transbilayer movement of lipid III in E. coli.
INTRODUCTION

The biosynthesis of a wide variety of complex glycoconjugates in both eucaryotic and procaryotic cells occurs by a process whereby membrane-bound glycolipids or glycolipid precursors are synthesized on the cytosolic face of a membrane and subsequently translocated to the opposite side of the membrane where they serve as substrates for additional processing reactions. In eucaryotic cells the synthesis of \( N \)-linked oligosaccharides of glycoproteins involves the translocation of dolichyl-linked mono- and pentasaccharide intermediates from the cytosolic leaflet to the luminal monolayer of the endoplasmic reticulum (ER) (1-5). Similarly, the synthesis of glycophosphatidylinositol (GPI) anchors in eucaryotic cells is initiated on the cytoplasmic face of the ER, but the completed anchor structure is linked to protein on the lumenal face (6, 7). The transbilayer movement of phospholipids also occurs during the assembly of the phospholipid bilayers of biogenic membranes in procaryotic and eucaryotic cells (8-11). Studies using synthetic lipid bilayers indicate that the transbilayer migration of di(\( N \)-acetylglucosaminy)pyrophosphoryldolichol (GlcNAc\(_2\)-P-P-Dol) and spin-labeled analogues of polyisoprenyl compounds does not occur spontaneously (12, 13). Rather, it has been proposed that the transbilayer diffusion of polar-lipid head groups is mediated by specific integral membrane proteins called “flippases” or “translocases” (14-17), and a substantial amount of evidence has been obtained which supports this conclusion (17-19).

Flippase proteins are also believed to be involved in the assembly of a large group of bacterial lipopolysaccharide (LPS) O-antigens collectively referred to as Wzy-dependent O-antigens (20). Although these O-antigens are structurally distinct, they are all heteropolysaccharides comprised of oligosaccharide repeat units, and all are assembled by the
same general mechanism (20). The repeat units of these O-antigens are synthesized as undecaprenyl pyrophosphate (Und-P-P)-linked oligosaccharides on the inner face of the cytoplasmic membrane and subsequently translocated en bloc to the periplasmic face where they are utilized as substrates for chain-elongation by a polymerase enzyme (Wzy) (20, 21). Polymerization occurs by the transfer of nascent polysaccharide chains from the carrier lipid to the non-reducing termini of newly synthesized carrier lipid-linked repeat units (22, 23). The assembly of Wzy-dependent O-antigens by this mechanism has been called “block-polymerization.” The O-antigen chains are then transferred to the core-lipid A region of LPS, and the completed LPS molecules are translocated to the exterior leaflet of the outer membrane by an unknown mechanism.

All of the gene clusters involved in the synthesis of Wzy-dependent O-antigens contain a gene, designated \textit{wzx}, that is believed to encode the flippase that mediates the transbilayer movement of the Und-P-P-linked repeat unit (24). Analyses of the predicted structures of the putative O-antigen flippases have revealed that all are hydrophobic proteins with twelve putative transmembrane domains (24). Although these proteins share very little structural homology at the primary amino acid sequence level, all have strikingly similar hydropathy profiles (25). Indirect evidence in support of the proposed functions of the O-antigen flippases of \textit{Salmonella enterica} serovar Typhimurium and \textit{Shigella dysenteriae} was obtained by Liu \textit{et al.}, (26) who demonstrated the accumulation of single lipid-linked O-antigen repeat units on the inner face of the cytoplasmic membrane in \textit{wzx} mutants. However, the mechanism involved in flippase-mediated transbilayer movement of lipid-linked O-antigen repeat units, as well as definitive identification of the proteins involved, remains to be established.
The assembly of phosphoglyceride-linked enterobacterial common antigen (ECA<sub>PG</sub>) of Gram-negative enteric bacteria is also believed to occur by a Wzy-dependent mechanism (27). ECA<sub>PG</sub> is a glycolipid component of the outer membrane of all Gram-negative enteric bacteria (27, 28). The carbohydrate portion of ECA consists of a linear heteropolysaccharide chain comprised of \(N\)-acetyl-D-glucosamine (GlcNAc), \(N\)-acetyl-D-mannosaminuronic acid (ManNAcA), and 4-acetamido-4,6-dideoxy-D-galactose (Fuc4NAc) (29). These amino-sugars are linked to one another to form the trisaccharide repeat unit \(\rightarrow(3)\alpha-D-Fuc4NAc-(1\rightarrow4)\beta-D-ManNAcA-(1\rightarrow4)\alpha-D-GlcNAc-(1\rightarrow\) (29). The ECA trisaccharide repeat unit is synthesized as the Und-P-P-linked intermediate, Fuc4NAc-ManNAcA-GlcNAc-P-P-Und (lipid III) (27, 30-32).

The available data are consistent with the synthesis of lipid III on the inner leaflet of the cytoplasmic membrane followed by its transbilayer movement to the periplasmic face of the membrane where assembly of the polysaccharide chains occurs by a block-polymerization mechanism. The polysaccharide chains are subsequently transferred from the carrier lipid to an as-yet-unidentified glyceride acceptor to yield ECA<sub>PG</sub> molecules in which the potential reducing terminal GlcNAc residue is linked to diacylglycerol through phosphodiester linkage (33, 34). Completed ECA<sub>PG</sub> polymers are then incorporated into the exterior leaflet of the outer membrane.

The synthesis of GlcNAc-P-P-Und is the initial step in the assembly of the ECA trisaccharide repeat unit (30) and the repeat units of many Wzy-dependent O-antigens (20, 35, 36). It is also the first lipid-linked intermediate in the synthesis of several Wzy-independent O-antigens (20). This reaction involves the transfer of GlcNAc 1-P from UDP-GlcNAc to Und-P catalyzed by the enzyme UDP-GlcNAc:undecaprenyl phosphate GlcNAc 1-P transferase (WecA) (31, 37). The \(wecA\) gene (formerly \(rfe\)) is located in the \(wec\) gene cluster (formerly \(rfe\)-
rff) which includes many of the genes involved in the biosynthesis of ECA (27, 38, 39). The wec gene cluster also includes a gene designated wzxE (formerly rfbX) that is believed to encode the flippase that mediates the transbilayer movement of the Und-P-P-linked trisaccharide repeat unit (25). Indeed, the hydropathy profile of the predicted product of the wzxE gene is almost identical to the hydropathy profiles of the putative flippases involved in the assembly of many Wzy-dependent O-antigens (25).

The O16 O-antigen repeat unit of *E. coli* K-12/O16 is a branched pentasaccharide whose assembly is initiated by the synthesis of GlcNAc-P-P-Und catalyzed by WecA, and the assembly of this O-antigen is believed to occur by a Wzy-dependent mechanism (36, 40, 41). Recent studies reported that a complete Und-P-P-linked O16 repeat unit was not required for translocation by the WzxO16-translocase (42). Indeed, the data presented in these studies suggested that the *E. coli* WzxO16-translocase was able to translocate GlcNAc-P-P-Und.

The studies presented here were conducted to demonstrate a role for WzxE in the transbilayer movement of Fuc4NAc-ManNAcA-GlcNAc-P-P-Und in *E. coli*. Attempts to measure the flippase-mediated transbilayer movement of naturally occurring undecaprenyl- or dolichyl-linked substrates are complicated by the extremely hydrophobic nature of these compounds. To circumvent this technical difficulty, we employed a variation of the experimental strategy of Rush *et al.* (18, 19) who used water-soluble citronellyl-based analogues of mannosylphosphoryldolichol (Man-P-Dol) and glucosylphosphoryldolichol (Glc-P-Dol) to assay flippase-mediated transport activities into intact endoplasmic reticulum (ER) vesicles. Bishop and Bell (14) originally used a water-soluble analogue of phosphatidycholine to investigate a phosphatidylcholine flippase(s) in sealed rat liver microsomes. Subsequently, phospholipid analogues with short fatty acid chains have been utilized by several other research
groups to partially purify and characterize membrane proteins involved in the transbilayer diffusion of phospholipids (43-46). Furthermore, water-soluble analogues of glucosylceramide have been used to study the transbilayer movement of glucosylceramide in the Golgi apparatus (47). The results presented here demonstrate the translocase-mediated transport of $[^3$H]$\text{GlcNAc-P-P-Ner}$ into everted membrane vesicles of $E.\ coli$. The data support the conclusion that the observed transport was not mediated by the WzxO16-translocase, but rather by WzxE, the putative flippase involved in the transbilayer movement of lipid III across the cytoplasmic membrane during the assembly of ECA$\text{PG}$. 
EXPERIMENTAL PROCEDURES

Materials – (s)-Citronellol, nerol (cis-3,7-dimethyl-2,6-octadien-1ol), tetramethyl-ammonium-phosphate, trichloroacetonitrile and phosphorous oxy-trichloride were obtained from Sigma-Aldrich (St. Louis, MO). UDP-N-acetyl-D-[6-3H]glucosamine (40-60 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO). [14C]Glucose (360 mCi/mm mol) was obtained from American Radiolabeled Chemicals (St. Louis, MO), and it was adjusted to a specific activity of 94 dpm/pmol by the addition of non-radioactive glucose. [3H]GDP-Mannose was prepared as described previously (48). Selecto Silica Gel was obtained from Fisher Scientific (Pittsburgh, PA). All of other chemicals were reagent grade and were purchased from standard commercial sources.

Bacterial Strains and Plasmids – Escherichia coli K-12 strains used in this study are listed in Table I. Transductions were carried out using phage P1 vir as described by Silhavy et al. (49). Cultures were routinely grown at 37ºC with vigorous aeration in Luria-Bertani (LB) broth (50) supplemented with glucose to give a final concentration of 0.2% or on LB agar containing 0.2% glucose. Tetracycline, ampicillin and chloramphenicol were added to media to give final concentrations of 10 µg/ml, 50 µg/ml and 30 µg/ml, respectively. Plasmid pRL160 was constructed by by digestion pCA32 (39) with HindIII yielding an 8.4 kb fragment containing wzxE as well as additional upstream genes of the wec gene cluster. This fragment was digested with XmaI to yield a 7.65 kb fragment which was subsequently ligated to the HindIII and AvaI sites of pBR322. Plasmid pRL162 was constructed by ligation of the 8.4 kb HindIII-fragment of pCA32, described above, to the corresponding site of the low copy number vector, pWSK29.
Plasmid pRL147 contained the wecA gene under the control of the P_{BAD} promoter, and it was constructed as follows. The wecA gene was obtained by PCR amplification of the DNA sequence from bp 9984 to 11141 (GenBank accession no. AE000454) using genomic DNA from E. coli strain AB1133 as template. The polynucleotides 5'-CTCTGAGAGCATGC-3' and 5'-GCGTCA CGACGTTTCCCAGGCATTGGT-3' were used as forward and reverse primers, respectively, and a SaI restriction site was incorporated into the reverse primer (underlined sequence). PCR amplifications were carried out using Taq polymerase (Sigma-Aldrich Chemicals). The amplified sequence contained 17 bp immediately upstream of the translational start site and 36 bp immediately downstream of the translational termination site, and it was cloned into TA cloning site of the pCR2.1 vector (Invitrogen, Inc.). The resulting construct was digested with EcoRI and SaI, and the 1.1 Kb fragment was subcloned into the expression vector, pBAD18 (52) that was restricted with the same enzymes to yield plasmid pRL147.

**Synthesis and Purification of Radiolabeled GlcNAc-P-P-Ner and Structurally Related Compounds** – Citronellol was phosphorylated using phosphorous oxy-trichloride as described by Danilov and Chojnacki (53). Nerol was phosphorylated with tetrabutylammoniumphosphate and trichloroacetonitrile in anhydrous acetonitrile as described by Danilov et al. (54). The isoprenyl phosphates were purified by ion-exchange chromatography on DEAE cellulose as described previously (48).

[^3H]GlcNAc-P-P-Ner was synthesized enzymatically using the UDP-GlcNAc:undecaprenyl phosphate N-acetylglucosaminyl 1-phosphate transferase (WecA) present in E. coli membranes. Membrane fractions were prepared as previously described (37). Reaction mixtures contained membranes (2.7 mg membrane protein), 0.1 M Tris-HCl (pH 8.0), 10 mM neryl phosphate (Ner-P), 40 mM MgCl₂, 5 mM dithiothreitol, 1 mM sodium
orthovanadate and 0.1 mM UDP-[\textsuperscript{3}H]GlcNAc (18 dpm/pmol) in a total volume of 3 ml. Reaction mixtures were incubated at 21°C for 16 h and then subjected to centrifugation at 100,000 x g for 10 min using a Beckman TL100.3 micro-ultracentrifuge. The supernatant solution was removed and layered on a 15 ml column of Benzyl-DEAE Cellulose (Sigma Chemical Co., St. Louis, MO) equilibrated with 10 mM NH\textsubscript{4}HCO\textsubscript{3}. The column was developed with two column volumes of 10 mM NH\textsubscript{4}HCO\textsubscript{3} followed by a 60 ml gradient of NH\textsubscript{4}HCO\textsubscript{3} (0 to 1 M). Fractions containing [\textsuperscript{3}H]GlcNAc-P-P-Ner were pooled and dried by rotary evaporation under reduced pressure at 30°C. The radiolabeled analogue was then dissolved in H\textsubscript{2}O and desalted by gel-filtration chromatography on a Sephadex G-10 column (1 x 30 cm) equilibrated with H\textsubscript{2}O. Fractions containing [\textsuperscript{3}H]GlcNAc-P-P-Ner were pooled, dried by rotary evaporation, dissolved in a small volume of CHCl\textsubscript{3}/CH\textsubscript{3}OH (2:1, v/v) and layered onto a 15 ml Selecto Silica Gel column equilibrated with CHCl\textsubscript{3}. The column was then eluted with CHCl\textsubscript{3}/CH\textsubscript{3}OH/H\textsubscript{2}O/conc. NH\textsubscript{4}OH (65:35:6:1, v/v/v/v), and fractions of 3.5 ml were collected. Radiolabeled GlcNAc-P-P-Ner eluted in fractions 8 to 15. Fractions containing [\textsuperscript{3}H]GlcNAc-P-P-Ner were pooled, dried by rotary evaporation, dissolved in 2 ml of CH\textsubscript{3}OH and stored at -20°C until used for transport assays. Formation of [\textsuperscript{3}H]GlcNAc-P-P-Ner was strictly dependent upon the addition of exogenous Ner-P to reaction mixtures. The product was detected as a single radioactive compound when analyzed by thin-layer chromatography on silica-gel plates developed with three different solvent systems, and it was detected by a phospholipid specific spray reagent (55) and by an anisaldehyde-based spray reagent for the detection of isoprenoid compounds (56). \textit{N}-Acetyl[\textsuperscript{3}H]glucosamine was the only radioactive product released by mild acid hydrolysis (0.01 N HCl, 100° C, 10 min) as determined by descending paper-
chromatography on Whatman 3MM paper using butanol/pyridine/H₂O (6:4:3, v/v/v) as the developing solvent system.

Reactions mixtures for the enzymatic synthesis of \(^{3}\text{H}\)GlcNAc-P-Ner contained *Bacillus cereus* membranes (6.3 mg membrane protein), 0.04 M Tris-HCl (pH 8.0), 4 mM Ner-P, 20 mM MgCl₂, 2 mM dithiothreitol, 2 mM sodium orthovanadate, 1 mM CDP-choline and 0.1 mM UDP-\(^{3}\text{H}\)GlcNAc (18 dpm/pmol) in a total volume of 5 ml. Reaction mixtures were incubated at 21°C for 2 h and then stopped by the addition of 5 ml of 95% ethanol. The particulate fraction was removed by centrifugation in a clinical centrifuge, and the radiolabeled GlcNAc-P-Ner was purified by chromatography on benzyl-DEAE-cellulose and Selecto silica gel columns and subsequently characterized as described above for \(^{3}\text{H}\)GlcNAc-P-P-Ner. \(^{3}\text{H}\)GlcNAc-P-Ner was stored in CH₃OH at -20°C until used for transport assays.

Enzymatic reactions for the synthesis of \(^{3}\text{H}\)GlcNAc-P-P-citronellol (\(^{3}\text{H}\)GlcNAc-P-P-Cit) and \(^{3}\text{H}\)GlcNAc₂-P-P-Cit contained microsomes from Chinese hamster ovary Tn-10 cells (57) (1 mg membrane protein), 0.1 M Tris-HCl (pH 8.5), 4 mM citronellyl phosphate (Cit-P), 40 mM MgCl₂, 2 mM sodium orthovanadate and 0.5 mM UDP-\(^{3}\text{H}\)GlcNAc (68 dpm/pmol) in total volume of 1 ml. Reaction mixtures were incubated at 30°C for 4 h and then subjected to centrifugation at 100,000 x g for 10 min in a Beckman TL100.3 micro-ultracentrifuge. Radiolabeled GlcNAc₁₂-P-P-Cit was purified as described above for GlcNAc-P-P-Ner. After desalting, the fractions containing GlcNAc₁₂-P-P-Cit were pooled, dried by rotary evaporation at 30°C under reduced pressure and dissolved in a minimal volume of CH₃OH. The sample was then spotted on a sheet of Whatman 3MM paper, and the paper was developed in a descending manner for 14 h using butanol/pyridine/H₂O (6:4:3, v/v/v). Regions of the chromatogram containing \(^{3}\text{H}\)GlcNAc-P-P-Cit and \(^{3}\text{H}\)GlcNAc₂-P-P-Cit were located using a Bioscan System.
200 Imaging Scanner. These regions were cut out, and each compound was eluted with H₂O. The H₂O eluates were dried by rotary evaporation at 30°C under reduced pressure and desalted by gel-filtration on Sephadex G-10 as described above. Fractions containing the products were pooled and dried by rotary evaporation at 30°C under reduced pressure. The compounds were then dissolved in 2 ml of CH₃OH and stored at -20°C until used for transport assays.

(β)-[^3H]Man-P-Cit and (β)-[^3H]Man-P-Ner were synthesized enzymatically using a partially purified preparation of Man-P-Und synthase from *Micrococcus luteus*, GDP[^3H]mannose and either Cit-P or Ner-P as described previously (48).

*Preparation of Sealed Everted Membrane Vesicles* – Everted membrane vesicles were prepared by a modification of the procedure described by Ambudkar *et al.* (58). Cells were grown with vigorous aeration to an absorbance (600 nm) of 1.2 to 1.3 in LB broth supplemented with glucose to give a final concentration of 0.2%. The cells from two 500 ml cultures were harvested by centrifugation at 10,000 x g at 4°C and washed with cold buffer consisting of 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose and 0.14 M choline chloride. The washed cells were resuspended in 20 ml of 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 0.14 M choline chloride and 0.1 mM phenylmethylsulfonyl fluoride and then disrupted by two passages through a cooled French pressure cell at 4,000 p.s.i. Pancreatic DNase was added to the lysate immediately to give a final concentration of 0.1 mg/ml, and the lysate was incubated in an ice bath for 60 min. Intact cells were removed by low-speed centrifugation, and membrane vesicles were harvested by centrifugation of the supernatant solution at 150,000 x g for 60 min at 4°C. The pelleted vesicles were washed with cold buffer consisting of 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose and 0.14 M choline chloride and subsequently resuspended in 1.0 ml of the same buffer. Vesicle preparations were stored at -80°C until used for transport assays.
The orientation of membrane vesicles was determined using the procedure described by Futai (59). This procedure revealed that essentially all of the vesicles were in the everted or “inside-out” orientation. In addition, the integrity of vesicle membranes was verified by assaying the ability of vesicles to maintain an NADH generated ΔpH as determined by monitoring the quenching of acridine orange fluorescence (58).

Assay of Transport Activity by Membrane Vesicles or Human Erythrocytes – The standard assay mixture contained 10 mM Tris-HCl (pH 7.5), 0.14 M choline chloride, 0.125 M sucrose, 2.5 mM MgCl₂, [³H]GlcNAc-P-P-Ner (35 μM, 18 dpm/pmol), and vesicles (120-150 μg) in a total volume of 20 μl. Reaction mixtures were incubated at 37°C for various periods of time and then terminated by the addition of 0.5 ml of cold 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 0.14 M choline chloride and 5 mM CaCl₂. The diluted mixtures were immediately suction-filtered through 0.45 μm HA filter discs, and the discs were washed with 0.5 ml of 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 0.14 M choline chloride and 5 mM CaCl₂. The filters were then added to counting vials and incubated with 0.5 ml of 2% sodium dodecyl sulfate for 1 h at room temperature prior to the addition of 5 ml of Ready Safe liquid scintillation fluid (Beckman). The closed vials were incubated overnight at room temperature, and the radioactivity was quantified using a Beckman LS 6500 liquid scintillation spectrometer.

Transport of either [¹⁴C]glucose or [³H]GlcNAc-P-P-Ner by human erythrocytes was assayed using the same procedure described above for membrane vesicles except that the assays were carried out at 21°C and the cells were harvested by filtration using Whatman GF/C glass-fiber filter discs (Whatman Laboratory Products, Inc., Clifton, NJ). Human erythrocytes were obtained from a donor (J. S. R.) as previously described (18, 19).
Assay for Lipid III Accumulation. Strain PR4180 was grown in 120-ml of proteose peptone beef extract (PPBE) broth (60) containing 0.2% glucose and 50 μg/ml ampicillin at 37°C with vigorous aeration to an optical density (600 nm) = 0.5. The culture was divided into two equal portions, and the cells in each portion were harvested by centrifugation. The cells in each pellet were resuspended in 6 ml of fresh PPBE broth containing either glucose or arabinose at final concentrations of 0.2% and 0.1%, respectively. [3H]GlcNAc (75 μCi, 4.1 Ci/mmol) was then added to both cultures, and the cultures were incubated at 37°C with vigorous aeration for 20 min. Each of the cultures (approximately 4.5 x 10¹⁰ cells/culture) was then poured into a separate beaker containing 5 g of ice, and the cells were harvested by centrifugation at 4°C. The cells were then washed with 6 ml of cold 0.9% saline followed by successive extraction with 6 ml of cold 95% ethanol and 6 ml of cold acetone. The extracted cells were dried under vacuum, and they were then either further processed for the isolation of radiolabeled lipid III or stored at -20°C for later use.

Radiolabeled lipid III was extracted from the dried cells by the addition of 2.5 ml of chloroform:methanol:water (10:10:3, v/v/v) followed by constant stirring for 15 min at room temperature. Particulate matter was removed by centrifugation at room temperature, and the amount of radiolabeled lipid III in each of the extracts was determined by ascending paper-chromatography on EDTA-treated SG-81 paper (Whatman) as previously described (32).

Determination of Cell Viability. Strain PR4180 was grown in PPBE broth containing 0.2% glucose and 50 μg/ml ampicillin at 37°C with vigorous aeration to an optical density (600 nm) = 0.20. The cells were then harvested by centrifugation and resuspended in 100-ml of fresh PPBE broth containing 50 μg/ml ampicillin. The resulting culture was then divided into two equal portions; glucose was added to one portion to give a final concentration of 0.2% whereas
arabinose was added to the other portion to give a final concentration of 0.1%. Both cultures were incubated at 37°C with vigorous aeration, and the number of viable cells per milliliter of each culture was determined at various intervals by plating dilutions of the cultures onto PPBE agar plates and then counting the number of colonies after overnight incubation of the plates at 37°C. In addition, the optical densities of the cultures (600 nm) was monitored throughout the course of the experiment.
RESULTS

_Uptake of[^3]H]GlcNAc-P-P-Ner by Everted Membrane Vesicles_ – GlcNAc-P-P-Und is the first intermediate in the synthesis of the ECA trisaccharide repeat unit (27, 30) as well as the repeat units of many Wzy-dependent O-antigens including the O16 antigen (20, 36, 40, 41). Recent studies provided indirect evidence that the _wzx_ O16 gene-product of _E. coli_ K-12/O16 functions as a translocase (42). These studies also suggested that Wzx O16 was capable of translocating incomplete Und-P-P-linked O16-repeat units including GlcNAc-P-P-Und.

Therefore, we conducted experiments in an attempt to demonstrate translocase activity directly by assaying the transport of[^3]H]GlcNAc-P-P-Ner, a water-soluble analogue of GlcNAc-P-P-Und, by everted membrane vesicles prepared from _E. coli_ K-12/O16. As illustrated in Fig. 1, the isoprenoid moieties of GlcNAc-P-P-Ner and GlcNAc-P-P-Und are fully unsaturated. However, the polyisoprenoid chain of GlcNAc-P-P-Und contains eleven isoprene units, and this compound is extremely hydrophobic. In contrast, the short polyisoprenoid chain of GlcNAc-P-P-Ner contains only two isoprene units thus rendering this compound water-soluble. Incubation of sealed everted membrane vesicles prepared from _E. coli_ S0864 with[^3]H]GlcNAc-P-P-Ner resulted in the time-dependent uptake of the radiolabeled analogue (Fig. 2). In addition, the rate of transport of[^3]H]GlcNAc-P-P-Ner into the lumen of the vesicles increased in linear proportion to the concentration of vesicles in reaction mixtures (Fig. 2, inset).

Extraction of preloaded vesicles with methanol resulted in the recovery of greater than 90% of the vesicle-associated radiolabel. Approximately 50% of this material was intact[^3]H]GlcNAc-P-P-Ner, and the remainder was identified as[^3]H]GlcNAc 1-P. However, it is important to note that[^3]H]GlcNAc 1-P was not detected in reaction mixtures following the
incubation of [³H]GlcNAc-P-P-Ner with membrane vesicles prepared from strains PR4150 or PR4156 (data not shown). These observations support the conclusion that enzymatic hydrolysis of the pyrophosphate linkage of [³H]GlcNAc-P-P-Ner occurred following translocation of the analogue into the vesicle lumen; however, the enzyme responsible for this hydrolysis has not been identified.

The transport of GlcNAc-P-P-Ner was strictly dependent on the integrity of membrane vesicles, and uptake of the analogue was abolished when assay mixtures contained 0.1% Triton X-100 (data not shown). The requirement for an intact permeability barrier was also demonstrated by determining the rate of efflux of [³H]GlcNAc-P-P-Ner from sealed preloaded vesicles following their incubation in isotonic and hypotonic solutions. Dilution of preloaded vesicles into 100 volumes of isotonic buffer (10 mM Tris-HCl, pH 8.0, 0.14 M choline chloride, 0.125 M sucrose, 2.5 mM MgCl₂) resulted in the time-dependent efflux of [³H]GlcNAc-P-P-Ner, and approximately 80% of the analogue was released within 20 min (Fig. 3). In contrast, the release of almost 90% of the [³H]GlcNAc-P-P-Ner from preloaded vesicles occurred within 30 sec when the vesicles were ruptured by dilution into 100 vols of H₂O. The transport of GlcNAc-P-P-Ner into vesicles did not appear to require a proton motive force since it was not affected by the addition of ATP, NADH, D-lactate, carbonyl cyanide m-chlorophenylhydrazone (CCCP) or carbonyl cyanide 4-trifluoro-methoxyphenylhydrazone (FCCP) to assay mixtures (data not shown). These observations suggest that uptake and efflux of [³H]GlcNAc-P-P-Ner by everted membrane vesicles occurs by an equilibrium process. Assuming this to be the case, net transport of the radiolabeled analogue into vesicles was observed until its distribution between the extravesicular solution and the lumen of the vesicles reached equilibrium at 25 min (Fig. 2). Approximately 5% of the [³H]GlcNAc-P-P-Ner was internalized at equilibrium, and from these
data an intravesicular volume of 6.7 μl per mg vesicle protein was calculated. This value is within the range previously estimated for the intravesicular volume of everted bacterial membrane vesicles (61-64).

Transport of $[^3H]GlcNAc-P-P-Ner$ Into Everted Membrane Vesicles is Mediated by the wzxE Gene Product. The results described above suggest that the uptake of $[^3H]GlcNAc-P-P-Ner$ by everted membrane vesicles occurs by a protein-mediated process. Thus, experiments were conducted to determine the possible role of the WzxO16-translocase in this process. *E. coli* S0874 is a derivative of strain S0864 that contains an extended deletion in the region of the chromosome that includes the putative flippase genes involved in the synthesis of the O16 O-antigen (wzxO16) and colanic acid (wzxC) (65). Quite unexpectedly, the kinetics of $[^3H]GlcNAc-P-P-Ner$ uptake by membrane vesicles prepared from strain S0874 (Δ[wzxO16- wzxC]) were the same as those observed using membrane vesicles prepared from strain S0864 (wild-type) (Fig. 2). These results suggested that a membrane protein other than WzxO16 or WzxC was responsible for mediating the transport of the analogue into membrane vesicles. In this regard, it has been suggested that the wzxE gene of the *wec* gene cluster encodes a putative translocase that facilitates the transbilayer movement of the Und-P-P-linked ECA trisaccharide repeat units (25). Thus, experiments were conducted to determine if the observed transport of $[^3H]GlcNAc-P-P-Ner$ was mediated by the wzxE gene-product. As shown in Fig. 2, uptake of $[^3H]GlcNAc-P-P-Ner$ was not detected using everted membrane vesicles prepared from *E. coli* 14.5, a wzxE::Tn10cam insertion mutant derived from *E. coli* HCB33. The same results were obtained using everted membrane vesicles prepared from strains PR4150 and PR4156, two strains that are derivatives of strains S0874 and S0864, respectively, into which the wzxE::Tn10cam-insertion was introduced by transduction (Table II).
These results clearly indicate that the association of $[^3]$H\text{GlcNAc-P-P-Ner}$ with vesicles was the result of transport into the vesicle lumen, and was not due to nonspecific binding of the analogue to the outer surface of the sealed vesicles. Additional support for this conclusion was obtained by the observation that $[^3]$H\text{GlcNAc-P-P-Ner}$ was not transported by human red blood cells under conditions whereby significant facilitated transport of glucose was observed (Fig. 4). All of these results indicate that the ability of vesicles to transport $[^3]$H\text{GlcNAc-P-P-Ner}$ requires a functional $wzxE$ gene product.

The role of WzxE in the transport of $[^3]$H\text{GlcNAc-P-P-Ner}$ into membrane vesicles was further demonstrated by complementation studies. As mentioned previously, no transport of $[^3]$H\text{GlcNAc-P-P-Ner}$ into everted vesicles was observed when the vesicles were prepared from strain PR4150 ($wzxE::Tn10cam$) (Table II). However, wild-type levels of transport were observed using membrane vesicles prepared from strain PR4184, a transformant of strain PR4150 containing plasmid pRL162. Plasmid pRL162 contains several genes of the wec cluster, including the $wzxE$ gene, on an 8.4 kb HindIII insert fragment (Fig. 5). In contrast, transport of $[^3]$H\text{GlcNAc-P-P-Ner}$ into vesicles was not observed using vesicles prepared from strain PR4179, a transformant of strain PR4150 containing plasmid pRL160 (Table II). Plasmid pRL160 contains an insert fragment that was generated by removal of a 745 base pair Xmal-HindIII fragment from the same 8.4 kb HindIII fragment contained in pRL162 (Fig. 5). This truncation resulted in a deletion of 378 base pairs from the 3'-terminus of the $wzxE$ gene. All of the genes on the original 8.4 kb HindIII insert fragment are transcribed in the same direction. In addition, the $wzxE$ gene is the last complete open reading frame in this sequence of genes. Thus, the inability of pRL160 to complement the observed transport defect of membrane vesicles derived from strain PR4150 was attributed to lack of a functional $wzxE$ gene, and it was not due
to a polar effect of the truncated \( wzxE \) gene on downstream genes. Taken together, the above findings support the conclusion that \([^{3}H]\text{GlcNAc-P-P-Ner}\) is transported into everted membrane vesicles, and that the observed transport is mediated by the \( wzxE \) gene product.

**Null Mutations in \( wzxE \) Result in the Accumulation of Lipid III and Cell Death.** The available data indicate that synthesis of carrier lipid-linked ECA trisaccharide repeat units (lipid III) occurs on the inner leaflet of the cytoplasmic membrane. This is followed by the WzxE-mediated transbilayer movement of lipid III molecules to the periplasmic face of the membrane where they are utilized for the assembly of ECA polysaccharide chains by the block-polymerization mechanism. Accordingly, the inability of cells to synthesize a functional \( wzxE \) gene product would be expected to result in the accumulation of lipid III. Indeed, a pronounced accumulation of radiolabeled lipid III was observed in cells when strain PR4180 (\( wecA::\text{Tn10} wzxE::cm/pRL147[\text{wecA under control of the P}_{\text{BAD}} \text{ promoter}] \)) was incubated with \([^{3}H]\text{GlcNAc}\) during the initial 20 min following the addition of arabinose to the growth medium. In contrast, essentially no radiolabeled lipid III was detected when cells were incubated with \([^{3}H]\text{GlcNAc}\) following the addition of glucose to cultures (Table IV).

The arabinose induced overexpression of \( wecA \) in strain PR4180 was accompanied by a loss of cell viability (Fig. 6). In contrast, cell growth and viability was essentially unaffected following the overexpression of \( wecA \) in \( wecA::\text{Tn10} \) mutants possessing a wild-type \( wzxE \) allele. These observations, together with those described above, suggest that the lack of a functional WzxE results in the accumulation of lipid III, and that the accumulation of this intermediate is toxic to cells. In this regard, the accumulation of lipid III and cell death has also been observed in polymerase mutants (WzyE) (P.D. Rick, unpublished results). Thus, the transduction of the \( wzxE::\text{Tn10cam} \)-insertion into recipient strains is essentially precluded unless
the ability of these strains to synthesize lipid III is first abolished. Therefore, strains containing
the \textit{wecA::Tn}$10$-insertion were routinely used as recipients for this purpose. However, the
\textit{wecA::Tn}$10$ insertion-mutation had no apparent effect on the transport of $[^3\text{H}]\text{GlcNAc-P-P-Ner}$
into membrane vesicles as indicated by the results obtained with vesicles derived from strain
21548 (Table II).

\textit{Properties of the WzxE-Mediated Transport System.} The WzxE-mediated transport of
GlcNAc-P-P-Ner into everted membrane vesicles exhibited saturation kinetics (Fig. 7), and the
apparent $K_m$ for GlcNAc-P-P-Ner was approximately 55 $\mu$M.

In order to examine the specificity of the Wzx-translocase, its ability to mediate the
transport of several compounds structurally related to GlcNAc-P-P-Ner was determined. These
studies clearly revealed a marked preference for GlcNAc-P-P-Ner as a substrate (Table III).
Indeed, the rate of transport $\text{N}$-acetyl-glucosaminylpyrophosphorylcitronellol (GlcNAc-P-P-Cit)
was markedly less than that determined for GlcNAc-P-P-Ner despite the close structural
similarity of these compounds. Thus, the WzxE translocase is able to distinguish between
intermediates possessing fully unsaturated polyprenyl moieties and those that contain a saturated
$\alpha$-isoprene unit. A requirement for a pyrophosphoryl-linked saccharide is also indicated by the
inability of the vesicles to transport GlcNAc-P-Ner.
DISCUSSION

Despite the crucial role of “flippases” in membrane biology, very little is known about their structures and the mechanism by which they facilitate the transbilayer movement of polar lipids (17). A critical step in discovering new information concerning this novel class of membrane transporters is the identification of putative proteins that function as flippases. However, the identification of flippase proteins has been hampered by the lack of convenient biochemical assays for their activity.

Water-soluble analogues of a variety of lipids have proven to be useful model compounds for the study of several aspects of glycolipid biochemistry. In most cases, the increased hydrophilic properties of these analogues are due to the presence of shortened hydrocarbon chains relative to those that are present in the naturally occurring compounds. Thus, water-soluble short-chain analogues of Man-P-Dol (18) and Glc-P-Dol (19) containing (C10) isoprenyl chains have been used to investigate the properties of protein(s) that may be involved in the transbilayer movement of dolichol-linked intermediates in the ER of rat liver and pig brain cells. In addition, Cit-P, a 10-carbon analogue of Dol-P, is recognized as a substrate by a wide variety of enzymes involved in the eucaryotic protein \( N \)-glycosylation pathway. These enzymes include Man-P-Dol synthase from pig liver and brain (18), Glc-P-Dol synthase from pig brain (19), Man-P-Dol:Man\(_{5,8}\)GlcNAc\(_2\)-P-P-Dol mannosyltransferase(s) from pig brain (48), Glc-P-Dol:Glc\(_0\)\(_3\)Man\(_9\)GlcNAc\(_2\)-P-P-Dol glucosyltransferase from pig brain (66), UDP-GlcNAc:Dol-P GlcNAc-phosphotransferases from pig brain, CHO cell membranes and hen oviduct (this study), and UDP-GlcNAc:GlcNAc-P-P-Dol GlcNAc-transferase from CHO cell membranes (C.J. Waechter, unpublished results). Furthermore, Man-P-Cit was utilized as a mannose donor for the \textit{in vivo}
synthesis of Man₉GlcNAc₂-P-P-Dol by permeabilized CHO cells (67). Several bacterial enzymes have also been demonstrated to glycosylate Ner-P, a short-chain analogue of Und-P. These include the Man-P-Und synthase of *Micrococcus luteus* (18), the UDP-GlcNAc:undecaprenol phosphate GlcNAc 1-P transferase (WecA) of *E. coli* (this study), and the UDP-GlcNAc:undecaprenol phosphate GlcNAc transferase of *Bacillus cereus* (this study). In addition, ceramide and glucosyl-ceramide analogues with 8-carbon acyl chains are substrates for all of the enzymes involved in higher glycosphingolipid biosynthesis (47). Analogues of phosphatidylcholine (PC) containing short fatty acyl chains (C₄ to C₆) have also been used to partially purify and characterize potential proteins involved in the transbilayer movement of PC (14, 43-46). In this regard, it is significant to note that the properties of the phospholipid flippase(s) present in biogenic membrane bilayers that have been determined using water-soluble diC₄PC are entirely consistent with properties determined using alternative methods for measuring phospholipid “flip-flop” (reviewed in 11).

In this study, the flippase-mediated transport of GlcNAc-P-P-Ner, a water-soluble analogue of GlcNAc-P-P-Und, into sealed everted membrane vesicles of *E. coli* K-12 was investigated in order to assess the role of WzxE in the translocation of the trisaccharide-lipid intermediate involved in ECA assembly. Transport of GlcNAc-P-P-Ner into sealed and everted membrane vesicles of *E. coli* was found to be time-dependent and saturable. In addition, the transport process did not require a proton-motive force, and the data presented here are consistent with the conclusion that entry of GlcNAc-P-P-Ner into the lumen of everted vesicles occurs by the process of facilitated diffusion. Facilitated diffusion is also believed to be the mechanism involved in the flippase-mediated transport of water-soluble analogues of phospholipids (14, 43-46) and polyisoprenyl-linked saccharides and oligosaccharides into
microsomal vesicles (18, 19). Indeed, the \textit{in vivo} movement of lipid III from the inner to the outer leaflet of the cytoplasmic membrane could plausibly be driven by its utilization for the process of ECA polysaccharide chain-elongation catalyzed by WzyE.

Several experimental observations provide strong support for the conclusion that transport of GlcNAc-P-P-Ner into vesicles was mediated by WzxE. Thus, transport of GlcNAc-P-P-Ner was not observed using vesicles prepared from mutant strains possessing a null-mutation in \textit{wzxE}. However, transport was fully restored when vesicles were prepared from transformants of these mutants that expressed the wild-type \textit{wzxE} allele. Furthermore, no transport of $[^{3}\text{H}]$GlcNAc-P-P-Ner into human red blood cells was observed under the same conditions employed for the transport of the analogue into everted membrane vesicles of \textit{E. coli}. Thus, the association of $[^{3}\text{H}]$GlcNAc-P-P-Ner with vesicles was not due to nonspecific binding of the radiolabeled analogue to the vesicles. Indeed, the efflux of approximately 80-90% of the $[^{3}\text{H}]$GlcNAc-P-P-Ner contained in preloaded vesicles was observed following the incubation of these vesicles in isotonic buffer or as a result of their rupture by incubation in water. Finally, $[^{3}\text{H}]$GlcNAc-labeled lipid III was found to accumulate in cells of mutant strains possessing a null-mutation in \textit{wzxE}. All of these data support the conclusion that transport of GlcNAc-P-P-Ner into vesicles was facilitated by WzxE, the putative flippase involved in the assembly of the linear polysaccharide chains of phosphoglyceride-linked ECA. To our knowledge, this is the first direct demonstration of a bacterial flippase activity involved in the transmembrane translocation of a polyisoprenyl-linked saccharide. It may be functionally significant that the hydropathy profile of WzxE is very similar to that of the Rft1 gene-product which is proposed to be a Man$_5$GlcNAc$_2$-P-P-Dol flippase in yeast (68).
A determination of the ability of several water-soluble compounds related in structure to GlcNAc-P-P-Ner to serve as substrates of the WzxE-mediated transport system demonstrated that a pyrophosphoryl-linked GlcNAc substituent and an unsaturated α-isoprene unit are critical structural features required for transport. It seems likely that the rate of WzxE-mediated transport of Fuc4NAc-ManNAcA-GlcNAc-P-P-Ner into vesicles would be considerably faster than that observed for GlcNAc-P-P-Ner. However, these rates have not been determined due to the inability to synthesize the water-soluble analogue of lipid III.

The Wzy-dependent O16 O-antigen of E. coli K-12/O16 is a branched pentasaccharide, and synthesis of this O-antigen is initiated by formation of GlcNAc-P-P-Und catalyzed by WecA (40, 41). Feldman et al. (42) reported that the assembly of the O16 O-antigen involves translocation of Und-P-P-linked O16 repeat units across the cytoplasmic membrane mediated by the putative flippase, WzxO16. However, these studies revealed that the WzxO16 translocase does not appear to require a completed Und-P-P-linked O16 repeat unit, and data were presented which suggested that the WzxO16 translocase was able to mediate the in vivo translocation of GlcNAc-P-P-Und. It has also been suggested that GlcNAc-P-P-Und is the donor of the terminal GlcNAc residue of the outer core region of the K-12 lipopolysaccharide. Therefore, it is somewhat surprising that we failed to detect any WzxO16-mediated uptake of radiolabeled GlcNAc-P-P-Ner into vesicles obtained from E. coli strain 14.5, a wzxE::cm-insertion mutant possessing wild-type wzxO16 and wzxC genes (Table II). Indeed, the data presented in the current study indicate that transport of GlcNAc-P-P-Ner into the lumen of everted vesicles was mediated exclusively by WzxE. Additional work will be required to conclusively establish whether or not WzxE functions for the translocation of GlcNAc-P-P-Und in vivo.
The flippases involved in the translocation of Und-linked repeat units of Wzy-dependent O-antigens appear to exhibit broad substrate specificity. For example, the flippases involved in the translocation of the repeat unit of colanic acid (WzxC), the O-antigen repeat unit of *Salmonella enterica* serovar Typhimurium LT2 (Wzx*SeLT2*), and the O-antigen repeat unit of *E. coli* K-12/O16 (WzxO16) are also able to facilitate the *in vivo* transbilayer movement of the Und-P-P-linked O-antigen repeat unit *E. coli* O7 (42). Indeed, the relaxed specificities of these flippases is quite apparent when one considers the pronounced differences in the structures of their respective natural substrates as well as the differences of these structures to that of the O7 repeat unit. In contrast, it appears that WzxE is unable to mediate the *in vivo* translocation of the Und-P-P-linked O7 O-antigen repeat unit since synthesis of an O7 lipopolysaccharide was not detected in a mutant strain that possessed a wild-type *wzxE* allele but which had an extended deletion that included the *wzxO16* gene (42). Thus, the substrate specificity of WzxE appears to be rather stringent, and the available information suggests that WzxE may only function in the translocation of the Und-P-P-linked trisaccharide repeat unit of ECA. However, as suggested above, WzxE may also function *in vivo* for the translocation of GlcNAc-P-P-Und. The possible roles of WzxE in the translocation of various polyisoprenyl-linked saccharides are summarized in Figure 8.

Null-mutations in *wzxE* result in the accumulation of lipid III and cell death. Accordingly, transduction of the *wzxE::cm* insertion mutation from *E. coli* strain 14.5 into recipient strains resulted in cell death unless the recipient strains were first rendered unable to synthesize lipid III by prior introduction of the *wecA::Tn10* insertion mutation. However, strain 14.5 is not defective in the synthesis of lipid III, and indeed it is able to synthesize ECA*PG*. We believe that strain 14.5 possesses an unlinked suppressor mutation that compensates for the loss
of a functional WzxE; however, the nature of this suppressor mutation has not yet been determined. In this regard, the results of preliminary experiments did not reveal an alteration in the structural gene for WzxO16 in strain 14.5. This finding, in conjunction with the inability of everted vesicles prepared from this strain to function in the transport of GlcNAc-P-P-Ner, further support the conclusion that WzxO16 is unable to mediate the transport of this analogue. These observations also indicate that the putative suppressor mutation is unable to confer the ability to transport the analogue into membrane vesicles prepared from strain 14.5.

No differences in the specific activities of WzxE-mediated transport of GlcNAc-P-P-Ner were found in the current study using membrane vesicles prepared from either wecA::Tn10 insertion mutants or wecA+ strains. These findings suggest that transbilayer translocation of GlcNAc-P-P-Ner is not dependent on the formation of a complex between WecA and WzxE. However, an interaction between WecA, WzxE and perhaps other proteins may be important for the translocation of a complete Und-P-P-linked ECA trisaccharide in vivo, and future experiments will be directed at investigating this possibility.
ACKNOWLEDGMENTS

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REFERENCES


TABLE I

_Escherichia coli_ K-12 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0864</td>
<td><em>F</em> trp lacZ strA thi upp_</td>
<td>(65)</td>
</tr>
<tr>
<td>S0874</td>
<td>Δ(wzx016 wzxC), P2 eductant of S0864 with an extended deletion in the region from <em>udk</em> to <em>his</em></td>
<td>(65)</td>
</tr>
<tr>
<td>21548</td>
<td><em>thr-1 leuB6 Δ(gpt-proA)66 hisG4 argE3 thi-1 rfbD1 lacY1 ara-14 galK2 xyl-5 mtl-1 mgl-51 rpsL31 kgdK51 supE44 wecA::Tn10</em></td>
<td>(39)</td>
</tr>
<tr>
<td>14.5</td>
<td><em>λ</em> <em>F</em> <em>thr-1 leuB6 tonA31 lacY1 tsx-78 supO eda50 his-4 rfbD1 mgl-51 rpsL136 xyl-5 mtl-1 metF159 thi-1 ara-14 wzxE::cm</em></td>
<td>P.N. Danese</td>
</tr>
<tr>
<td>PR4149</td>
<td>As S0874, but <em>wecA::Tn10</em> [21548(P1) x S0874]</td>
<td>This study</td>
</tr>
<tr>
<td>PR4150</td>
<td>As PR4149, but <em>wzxE::cm</em> [14.5(P1) x PR4149]</td>
<td>This study</td>
</tr>
<tr>
<td>PR4151</td>
<td>As S0864, but <em>wecA::Tn10</em> [21548(P1) x S0864]</td>
<td>This study</td>
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<tr>
<td>PR4156</td>
<td>As PR4151, but <em>wzxE::cm</em> [14.5(P1) x PR4151]</td>
<td>This study</td>
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<tr>
<td>PR4179</td>
<td>PR4150/pRL160 (pBR322 containing a 3'-truncation of <em>wzxE</em>)</td>
<td>This study</td>
</tr>
<tr>
<td>PR4180</td>
<td>PR4150/pRL147 (wild-type <em>wecA</em> under control of the <em>P</em>{BAD}_ promoter)</td>
<td>This study</td>
</tr>
<tr>
<td>PR4184</td>
<td>PR4150/pRL162 (pWSK29 containing wild-type <em>wzxE</em>)</td>
<td>This study</td>
</tr>
<tr>
<td>PR4189</td>
<td>PR4149/pRL147 (wild-type <em>wecA</em> under control of the <em>P</em>{BAD}_ promoter)</td>
<td>This study</td>
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TABLE II

Transport of GlcNAc-P-P-Ner into everted membrane vesicles obtained from wild-type and mutant strains of Escherichia coli K-12

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Transport pmol/min/mg vesicle protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0864</td>
<td>Wild-type</td>
<td>11.3</td>
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<tr>
<td>S0874</td>
<td>Δ(wzx016 wzxC)</td>
<td>9.2</td>
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<tr>
<td>21548</td>
<td>wecA::Tn10</td>
<td>10.9</td>
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<tr>
<td>14.5</td>
<td>wzxE::cm</td>
<td>1.0</td>
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<td>Δ(wzx016 wzxC) wecA::Tn10 wzxE::cm</td>
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<td>PR4156</td>
<td>wecA::Tn10 wzxE::cm</td>
<td>0.6</td>
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<tr>
<td>PR4179</td>
<td>PR4150/pRL160 (3'-truncation of wzxE)</td>
<td>0.3</td>
</tr>
<tr>
<td>PR4184</td>
<td>PR4150/pRL162 (wild-type wzxE)</td>
<td>11.5</td>
</tr>
</tbody>
</table>
TABLE III

Transport of GlcNAc-P-P-Ner and related compounds into everted membrane vesicles prepared from E. coli S6864<sup>a</sup>

<table>
<thead>
<tr>
<th>Compound</th>
<th>Transport (pmol/min/mg vesicle protein)</th>
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<tr>
<td>GlcNAc-P-P-Ner</td>
<td>7.90</td>
</tr>
<tr>
<td>GlcNAc-P-Ner</td>
<td>2.00</td>
</tr>
<tr>
<td>GlcNAc-P-P-Cit</td>
<td>0.30</td>
</tr>
<tr>
<td>(GlcNAc)&lt;sub&gt;2&lt;/sub&gt;-P-P-Cit</td>
<td>0.10</td>
</tr>
<tr>
<td>Man-P-Ner</td>
<td>1.04</td>
</tr>
<tr>
<td>Man-P-Cit</td>
<td>1.09</td>
</tr>
</tbody>
</table>

<sup>a</sup> All assays were conducted using the conditions described under “Experimental Procedures.”
TABLE IV

Accumulation of $[^3\text{H}]\text{GlcNAc}$-lipid III in *E. coli* 4180 in the presence and absence of *wecA* expression$^a$

<table>
<thead>
<tr>
<th>Additions to growth medium</th>
<th>Expression of <em>wecA</em></th>
<th>$[^3\text{H}]\text{GlcNAc}$-lipid III recovered in extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$dpm/4.5 \times 10^{10}$ cells</td>
</tr>
<tr>
<td>Glucose</td>
<td>-</td>
<td>0.30 x 10$^6$</td>
</tr>
<tr>
<td>Arabinose</td>
<td>+</td>
<td>4.94 x 10$^6$</td>
</tr>
</tbody>
</table>

$^a$Experimental details are provided under “Experimental Procedures.”
**FIGURE LEGENDS**

**FIG. 1.** Structural relationship between GlcNAc-P-P-Undecaprenol (GlcNAc-P-P-Und) and GlcNAc-P-P-Nerol (GlcNAc-P-P-Ner).

**FIG. 2.** Uptake of $[^3H]$GlcNAc-P-P-Ner by everted membrane vesicles. Incubation mixtures contained 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 0.14 M choline chloride, 2.5 mM MgCl$_2$, 35 µM $[^3H]$GlcNAc-P-P-Ner (18 dpm/pmol) and membrane vesicles (120-150 µg protein) in a total volume of 20 µl. After incubation at 37°C for the indicated periods of time, the amount of radiolabel transported into the vesicles was determined as described under *Experimental Procedures*. Data were obtained using membrane vesicles were prepared from the following strains: SØ864 (wild-type) (●); SØ874 (Δ[wzxO16 wzxC]) (□); 14.5 (wzxE::cm) (○).

**FIG. 3.** Efflux of $[^3H]$GlcNAc-P-P-Ner from preloaded membrane vesicles. Incubation mixtures contained 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 0.14 M choline chloride, 2.5 mM MgCl$_2$, 1.6 µM $[^3H]$GlcNAc-P-P-Ner (1,800 dpm/pmol), and 600 µg membrane protein in a total volume of 65 µl. The reaction mixture was incubated at 37°C for 5 min, and a 10 µl aliquot (zero time sample) was removed and assayed for the amount of radiolabel transported into the vesicles as described under “*Experimental Procedures*.” Aliquots of 10 µl were then removed and added to either 1-ml of assay buffer (●) or water (○), and they were further incubated at 37°C for the indicated periods of time. The amount of radiolabel retained by the vesicles was then determined using the filtration assay as described under *Experimental Procedures*.

**FIG. 4.** Uptake of $[^14C]$glucose and $[^3H]$GlcNAc-P-P-Ner by human red blood cells. Incubation mixtures contained 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 0.14 M choline
chloride, 2.5 mM MgCl₂, freshly obtained human red blood cells (containing the indicated amount of phospholipid), and either 0.08 μM [¹⁴C]glucose (94 dpm/pmol) or 0.08 μM [³H]GlcNAc-P-P-Ner (18 dpm/pmol) in a total volume of 50 μl. The mixtures were incubated for 1 min at 21°C, and the amount of radiolabel transported into the red blood cells was determined by filtration on Whatman GF/C glass fiber filter discs as described under Experimental Procedures.

FIG. 5. Physical map of the region of the wec gene cluster between genes wzxE and wecF. The regions of the gene cluster contained in plasmids pRL160 and pRL162 are indicated by the open rectangles.

FIG. 6. Effect of a wzxE null-mutation on cell viability. Strain PR4180 (Δ[wzx₀₁₆ wzxC] wecA::Tn10 wzxE::cm/pRL147 [wild-type wecA under control of the P_BAD promoter]) was grown at 37°C to an optical density (600 nm) = 0.2 in PPBE broth containing 0.2% glucose. The cells were then harvested, resuspended in fresh broth lacking glucose, and then divided into two equal portions. Glucose was added to one portion to give a final concentration of 0.2% (○) whereas arabinose was added to the other portion to give a final concentration of 0.1% (■). Both cultures were incubated with shaking at 37°C, and the number of viable cells in each culture was determined at the indicated times. As a control, the effect of arabinose induced expression of wecA on the cell viability of strain PR4189 (Δ[wzx₀₁₆ wzxC] wecA::Tn10/pRL147 [wild-type wecA under control of the P_BAD promoter]) was determined using the same conditions as described above (▲). Additional details are provided in the Experimental Procedures section.

FIG. 7. Uptake of [³H]GlcNAc-P-P-Ner by everted membrane vesicles is saturable. Incubation mixtures contained 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 0.14 M choline chloride, 2.5 mM MgCl₂, and the indicated concentration of [³H]GlcNAc-P-P-Ner (18
dpm/pmol), and 60 µg membrane protein in a total volume of 20 µl. Reaction mixtures were incubated at 37°C for 10 min, and the amount of radiolabel transported into the vesicles was determined as described under Experimental Procedures.

FIG. 8. **Proposed roles of WzxE in the translocation of polyisoprenyl-linked saccharides across the cytoplasmic membrane.** It is proposed that WzxE participates in the transbilayer movement of Fuc4NAc-ManNAcA-GlcNAc-P-P-Und (translocation 1) and GlcNAc-P-P-Und (translocation 2) *in vivo*. The WzxE-mediated transbilayer movement of GlcNAc-P-P-Ner across the cytoplasmic membrane (dashed arrow) was demonstrated in this study using sealed everted cytoplasmic membrane vesicles.
GlcNAc-P-P-Undecaprenol

GlcNAc-P-P-Nerol
[3H] GlcNAc-P-P-Ner Uptake
pmol/mg Vesicle Protein

Time (min)

0 5 10 15 20 25 30

Rick et al., Fig. 2.
Rick et al., Fig. 3.

![Graph](image-url)
Rick et al., Fig 4.

![Graph showing transport against phospholipid concentration](image-url)
Rick et al., Fig. 8.

Diagram showing the process of glycine betaine biosynthesis involving enzymes WecA, WecG, and WecF. The pathway starts with Und-P and proceeds through GlcNAc-P-P-Ner to GlcNAc-P-P-Und and finally to cyanamide. The process involves two main stages and involves the transfer of glycine betaine from the cytoplasm to the periplasm.
Evidence that the wzxE gene of Escherichia coli K-12 encodes a protein involved in the transbilayer movement of a trisaccharide-lipid intermediate in the assembly of enterobacterial common antigen

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