The Activity of a Putative Polyisoprenol-linked Sugar Translocase (Wzx) Involved in Escherichia coli O Antigen Assembly Is Independent of the Chemical Structure of the O Repeat*

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During O antigen lipopolysaccharide (LPS) synthesis in bacteria, transmembrane migration of undecaprenylpyrophosphate (Und-P-P)-bound O antigen subunits occurs before their polymerization and ligation to the rest of the LPS molecule. Despite the general nature of the translocation process, putative O-antigen translocases display a low level of amino acid sequence similarity. In this work, we investigated whether complete O antigen subunits are required for translocation. We demonstrate that a single sugar, GlcNAc, can be incorporated to LPS of Escherichia coli K-12. This incorporation required the functions of two O antigen synthesis genes, ueeA (UDP-GlcNAc:Und-P GlcNAc-1-P transferase) and wzx (O-antigen translocase). Complementation experiments with putative O-antigen translocases from E. coli O7 and Salmonella enterica indicated that translocation of O antigen subunits is independent of the chemical structure of the saccharide moiety. Furthermore, complementation with putative translocases involved in synthesis of exopolysaccharides demonstrated that these proteins could not participate in O antigen assembly. Our data indicate that recognition of a complete Und-P-P-bound O antigen subunit is not required for translocation and suggest a model for O antigen synthesis involving recognition of Und-P-P-linked sugars by a putative complex made of Wzx translocase and other proteins involved in the processing of O antigen.

The biogenesis of complex carbohydrate structures in eukaryotic and prokaryotic organisms generally involves the participation of nucleotide sugar precursors and polyisoprenol lipids. Polyisoprenol-linked sugars are normally available as soluble molecules in cytoplasmic compartments, while polyisoprenol lipids are associated with lipid membrane bilayers. Once assembled, polyisoprenol-linked sugar molecules must have a way to cross the lipid bilayer for further processing. Thus, transmembrane movement of phospholipids and glycolipids, including polyisoprenol-linked sugars, is a process of fundamental biological importance in all types of cells (1, 2). In the case of protein glycosylation in eukaryotes, a topological model has been proposed involving the transmembrane movement of dolichol-linked sugars across the endoplasmic reticulum membrane (3). A similar model involving the transmembrane “flopping” of polyisoprenol-linked sugars has been proposed to explain the synthesis of bacterial cell wall peptidoglycan and lipopolysaccharide (LPS). However, currently it is not known how lipid-linked carbohydrates are translocated from one leaflet of the lipid bilayer to the other. Using several different experimental strategies, it has been shown that the unassisted transbilayer movement of polyisoprenol-linked sugars in liposomes is extremely slow (4, 5), suggesting the need for protein-assisted translocation.

LPS, a major component of the outer leaflet of outer membranes in Gram-negative bacteria, consists of lipid A, core oligosaccharide (OS), and in some microorganisms, O-specific polysaccharide or O antigen that is made of repeating OS subunits (6, 7). LPS biosynthesis involves a large number of enzyme activities, governed by more than 40 genes (7–10). The core OS is assembled on preformed lipid A by sequential glycosyl transfer of sugar components, while the O antigen is assembled on undecaprenylpyrophosphate (Und-P-P) (7). These pathways eventually converge by the ligation of the O antigen onto the lipid A-core OS acceptor, with the concomitant release of Und-P-P (7–11).

At least two different mechanisms for biosynthesis and assembly of O antigens have been described. One of them involves the synthesis of O repeating subunits by the addition of subsequent monosaccharides at the nonreducing end of the molecule, a process that takes place on the cytosolic side of the cytoplasmic membrane (11). These subunits are translocated across the cytoplasmic membrane, and they become polymerized by a mechanism involving the successive addition of the reducing end of the growing polymer to the nonreducing end of undecaprenylpyrophosphate-linked subunits (Fig. 1). The undecaprenyl-linked polymer is then ligated “en bloc” to the lipid A-core OS by reactions occurring on the periplasmic face of the membrane.
membrane (12–14). This pathway, also referred to as the wzy (polymerase)-dependent pathway, occurs in the synthesis of the majority of O antigens, especially in those made of repeating units of different sugars (heteropolymers O antigens) (15). The second mechanism involves the formation of a polymeric O antigen by reactions taking place on the cytosolic face of the cytoplasmic membrane that are mediated by the sequential action of glycosyltransferases elongating the polysaccharide at the nonreducing end (16). The nascent polysaccharide is transported across the cytoplasmic membrane by an ATP-binding cassette transporter (17) and subsequently ligated to lipid A-core OS. This pathway has been observed especially in O antigens made of repeating units of the same sugar (homopolymers O antigens) such as those from *Escherichia coli* O8 and O9 (11), as well as in group 2 and 3 exopolysaccharide capsules (16). In both wzy-dependent and wzy-independent mechanisms, the synthesis of the O subunit is initiated by the formation of a sugar phosphodiester linkage with undecaprenol phosphate. Various studies have recently shown that in most *E. coli* O types, the initiating enzyme is a tunicamycin-sensitive UDP-GlcNAc:Und-P GlcNAc-1-P transferase (18). First, we investigated whether the formation of a complete O subunit is a prerequisite for translocation and ligation to the lipid A-core OS and also determined whether this process requires Wzx. Next, we examined the interchangeability of Wzx proteins from different sources, using a genetic reconstitution system in *E. coli* K12/O16 for ligation of O antigen components to the lipid A-core OS and a nonpolar mutation in the wzx gene of the O7 LPS biosynthesis cluster. In this work, we present genetic, biochemical, and structural data indicating that an incomplete O16 antigen subunit can be processed and assembled onto the lipid A-core OS. We also show that the Wzx translocase encoded by *E. coli* O7, O16, and *Salmonella enterica* LT2 O antigen clusters can complement a wzx gene defect in *E. coli* K12/O16 and *E. coli* O7. Nevertheless, no complementation with *E. coli* K12 or *Rhizobium meliloti* putative translocases involved in exopolysaccharide assembly was observed. Altogether, our data suggest a model for the processing of the O repeating unit that involves the recognition of Und-P-P-linked sugars not only by the translocase Wzx but also by a complex formed by translocase, polymerase (Wzy), and the O antigen ligase or WaaL.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—Unless otherwise indicated all chemicals were from Sigma. A nalidixic acid-resistant derivative of *E. coli* K12 strain W3110 (rph-1 IN(rrnD-rrnE)1) was used in these studies. Strain CLM4 (26) is a recA derivative of S0874 (27) with a large deletion eliminating the genes for synthesis of colanic acid capsule and O16 LPS. CLM20 also derives from S0874, and its construction is described below. *E. coli* DH5α was used as an initial host for the cloning experiments. Cells were generally grown at 37 °C in LB medium consisting of 10 g of NaCl, 5 g of yeast extract, and 10 g of tryptone per liter. Antibiotics were added as needed at final concentrations of 100 μg/ml for ampicillin, 20 μg/ml for tetracycline, 40 μg/ml for kanamycin, 80 μg/ml for spectinomycin, 15 μg/ml for chloramphenicol, and 50 μg/ml for nalidixic acid. In some cases, a final sucrose concentration of 10% (w/v) was also added. The plasmids used in this study are described in Table I.

**DNA Methods**—Restriction enzymes, T4 DNA ligase, polynucleotide kinase, and Klenow DNA polymerase were all purchased from Roche Diagnostics (Laval, Canada), and used according to the conditions recommended by the supplier. Recombinant plasmids were introduced into *E. coli* strains by electroporation using a Bio-Rad Gene Pulser. Isolation of chromosomal DNA was conducted by the miniprep method of Owen and Borman (28). Plasmids were purified as described previously (29). When necessary DNA fragments were isolated from agarose gels using the Qiagick kit (Qiagen Inc., Mississauga, Canada). Oligonucleotides were purchased from Life Technologies, Inc. PCR amplifications were carried out using Pwo and Taq DNA polymerases (Roche Diagnostics).
as recommended by the manufacturer. Recombinant plasmids were verified by DNA sequencing performed using an ABI377 DNA sequencing apparatus (Perkin-Elmer) in the Robarts Research Institute DNA Sequencing Facility (London, Canada).

### Table I  
Characteristics of the plasmids used in this study

| Plasmids          | Relevant properties                                      | Source or reference   |
|-------------------|----------------------------------------------------------|-----------------------|
| pCM206            | 9-kb PCR amplicon containing ΔwecA-C from CLM20, cloned in pMAV3 | This study            |
| pEXT21            | Low copy number expression cloning vector, pSA replicon | Ref. 38               |
| pGEM3             | Cloning vector                                           | Promega               |
| pGEM-T-easy       | Cloning vector                                           | Promega               |
| pGP704            | Cloning vector, suicide plasmid                         | Ref. 60               |
| pJHCV32           | Cosmid clone containing the O7 LPS biosynthesis gene cluster | Ref. 29               |
| pJHCV32 :Tn3HoI-128 | hVax : Tn3HoI-128                                       | Ref. 61               |
| pMAV3             | Cloning vector                                           | Ref. 20               |
| pMAV11            | pACYC184                                                 |                       |
| pMF1              | 1.9-kb amplicon containing the glf gene and flanking sequences cloned in pGEM3 | This study            |
| pMF2              | pMF1, glf - cat, insertion of the cat gene in the unique sty1 site of glf | This study            |
| pMF5              | 0.9-kb PCR amplicon containing the wbbL (rhamnosyltransferase) gene cloned in pGEM-T-easy | This study            |
| pMF9              | 2.5-kb SmaI-SpAI fragment of pMF2 containing glf - cat and flanking sequences, cloned in pGFP704 | This study            |
| pMF10             | pMF9 containing the sacB-npt1 cassette cloned in the unique BglII site | This study            |
| pMF19             | 0.9-kb PCR amplicon containing the wbbL (rhamnosyltransferase) gene cloned in pEXT21 | This study            |
| pMF20             | 1.25-kb PCR amplicon containing the wxc gene of E. coli K-12/O16, cloned in pEXT21 | This study            |
| pMF21             | 1.5-kb PCR amplicon containing the wxc gene of E. coli O7, cloned into pEXT2, SpcR | Ref. 23               |
| pMF24             | 1.2-kb PCR amplicon containing the wxc gene of S. enterica LT2 | This study            |
| pMF26             | 1.5-kb PCR amplicon containing the E. coli K-12 wxcC gene for colanic acid synthesis, cloned in pEXT21 | This study            |
| pMF28             | 1.5-kb PCR amplicon containing the K. melilota exoT gene, cloned in pEXT21 | This study            |
| pPR1474           | 5-kb SphI- EcoRI fragment from E. coli K12 WG1 containing the wbbL (rhamnosyltransferase) gene | Ref. 32               |
| pZY1001           | 22-kb SalI fragment of E. coli W3110 carrying the K12/O16 biosynthesis cluster | Ref. 39               |
| pZY1003           | 12.3-kb XbaI-BamHI deletion of pZY1001 carrying the genes rmlBDAC-wxc-glffwxy from the E. coli K12 O antigen cluster | Ref. 39               |
| pZY1018           | 3.7-kb Stul–XbaI fragment from pZY1001 cloned in pMAV3 | Ref. 38               |
**TRANSLATION OF UND-P-P-BOUND O ANTIGEN UNITS**

**RESULTS**

**An Incomplete O16 Subunit Can Be Attached to Lipid A-Core OS.—** We investigated whether the formation of a complete O antigen subunit is a prerequisite for its assembly onto lipid A-core OS. Our experimental model system was the O polysaccharide biosynthesis cluster of *E. coli K-12*O16 (Fig. 2A). *E. coli* K-12 strains of the W3110 lineage are usually O16-deficient because of an IS5 insertion interrupting the function of *wbbL*, the last gene of the *E. coli* K-12/O16 polysaccharide cluster (32, 39, 46) that encodes a rhomboysferase involved in the addition of rhombose to the second position of the O16 backbone (Figs. 2 and 3). Other investigators have previously shown that complementation in trans with pPR1474, a plasmid encoding a functional rhomboysferase generated by gene disruption in these strains led to the synthesis of O16 biopolymers (32, 46). We reasoned that if the synthesis of a complete O16 subunit is required for its assembly onto lipid A-core OS, a mutation affecting the biosynthesis of the Galf terminal sugar residue (Fig. 3) would result in an O16-deficient LPS phenotype. Furthermore, introduction of pPR1474 or an equivalent plasmid labeled WGA. The presence of bound lectin was determined using anti-digoxigenin antibody conjugated with alkaline phosphatase, followed by developing with 5-bromo-4-chloro-3-indoly-phosphate and 4-nitro blue tetrazolium chloride. Ovalbumin was used as positive controls for lectin binding. In other experiments, LPS samples were transferred to nitrocellulose and reacted with oligo *O*16 and anti-OG polyclonal antibodies. Western blots were developed using protein A-peroxidase as described elsewhere (29). For structural analysis, LPS was isolated by the hot phenol-water extraction procedure (40) and purified by gel permeation chromatography on a column of Bio-Gel-P-2 (1 m x 1 cm) with water as eluent. In all cases, only one carbohydrate-positive fraction was obtained that eluted in the high M_r range (42) and was utilized for chemical analyses.

**Bacterial Slide Agglutination by WGA—** Standardized bacterial suspensions were prepared by growing bacteria to the logarithmic phase (absorbance of 0.4 at 600 nm) and inducing the expression of Wzx proteins with 1-thio-β-D-galactopyranoside for 3 h, 1 ml aliquots were washed once with phosphate-buffered saline, and the absorbance was adjusted to a value of 2. WGA lectin (Roche Diagnostics) was also diluted in phosphate-buffered saline, and 25 μl of the bacterial cell suspension was mixed with 25 μl of serial dilutions of WGA. Agglutination was recorded after 3 min of gentle rotation of the glass plate. The specificity of WGA for GlcNAc was determined by examining the agglutination of bacteria in the presence of glucose or GlcNAc. Differences in concentrations of sugars at 0.1, 0.15, 0.312, 0.625, at sugars at 0.1, 0.312, 0.625 were mixed with 12.5 μg/ml WGA, and the mixture was examined by agglutination as described above. A concentration-dependent agglutination inhibition was only found in the presence of GlcNAc (50% inhibition was obtained with 0.312 μg GlcNAc and 100% inhibition with 1.25 μg/ml).

**Sugar Composition, Methylation Linkage Analyses, and Fast Atom Bombardment Mass Spectrometry—** Sugar composition analysis was performed by the alditol acetate method (43). Hydrolysis of glycolipids was carried out in 4 m trifluoroacetic acid at 100 °C for 4 h followed by reduction in H_2O with NaBD_4 and subsequent acetylation with acetic anhydride, using residual sodium acetate as the catalyst. Alditol acetate derivatives were analyzed by gas-liquid chromatography-mass spectrometry using a Hewlett-Packard 5971 mass spectrometer. GlcNAc standard chromatographed with a 30-m DB-17 capillary column (210 °C (30 min) 240 °C at 2 °C/min). Mass spectra in the electron impact mode were recorded with a Varian Saturn II mass spectrometer. Methylation linkage analysis was carried out by the NaOH/Me.SOCH_2.H_process (44) and with characterization of permethylated aldol acetate derivatives by gas-liquid chromatography-mass spectrometry in the electron impact mode (DB-17 column, isothermally at 190 °C for 60 min).

A fraction of the methylated sample was used for positive ion fast atom bombardment-mass spectrometry that was performed on a Jeol JMS-AX506H mass spectrometer with glycerol/thioglycerol (1:1) as the matrix, and a tip voltage of 3 kV. Product ion scan (B/E) and precursor ion scan (B/E) were performed on metastable ions created in the first field with a source pressure of 5 x 10^-6 torr. The interpretations of positive ion mass spectra of the permethylated LPS derivatives were described previously by Dell et al. (45).

The coding regions of the cloned genes were placed under the control of the *lacZ*-inducible promoter. The copy number of pEXT21 is 3–4 copies per chromosome (38), ensuring a similar level of expression in all constructs. Construction of pMF21 containing the *wzx* was described previously (23). Plasmid pYZ1001 (39) was used as a template to clone *wzx* using primers 5'-ATGGAGCTCTAGATGAGACCCACTGACC-3' and 5'-TAAACCTTGCAACAGGAAAC-3' having BamHI and HindIII sites (underlined) in their 5'-ends. The PCR-amplified fragment containing the coding region of *wzx* was digested with BamHI and HindIII and ligated to pEXT21 digested with the same enzymes, resulting in pMF20. An identical strategy was used to clone *wzx* from *E. coli* K-12 strain W3110, exoT from *R. meliloti* strain B399, and *wzx* from *S. enterica* serovar typhiurum strain LT2. In these cases, the PCR amplifications were from chromosomal DNA. The following primers were used: *wzx*, 5'-ATGGAGCTCTAGATGAGACCCACTGACC-3' and 5'-TAAACCTTGCAACAGGAAAC-3' having BamHI and HindIII sites (underlined) in their 5'-ends. The resulting plasmids were designated as pMF26 (*wzx*), pMF28 (*exoT*), and pMF24 (*wzx*, pMF19, pPR1474, pMF1, and pMF20, as well as the location of PCR primers 99, 100, 85, and 88 are shown. B, partial genetic map of the wec (ECA cluster) was determined by the supplier for application of digoxenin-labeled lectin in glycoconjugate analysis (Roche Diagnostics). Briefly, the membrane was treated with the blocking reagent, washed several times with Tris-buffered saline, pH 7.5, and incubated with 7 μg/ml of digoxin-
would not complement the mutant phenotype. We therefore constructed by gene replacement a nonpolar mutation in the \textit{glf} gene that is located within the \textit{E. coli} O16 LPS biosynthesis cluster (39, 46) and encodes the UDP-Gal\textsubscript{p} mutase catalyzing the interconversion between UDP-Gal\textsubscript{p} and UDP-Gal\textsubscript{f} (47). To ensure expression of the O16 genes downstream of \textit{glf}, we inserted the \textit{cat} gene lacking transcription termination signals in an orientation such that the genes downstream of the mutated \textit{glf} would be transcribed by the \textit{cat} promoter (Fig. 2). The correct insertion in the mutant strain, designated as MFF1, was verified by PCR as described under “Experimental Procedures.”

To investigate the effect of the \textit{glf}:\textit{cat} insertion in O16 LPS synthesis, we complemented the \textit{wbbL}:\textit{IS} mutation with pMF19 (Fig. 2A). This plasmid carries the functional rhamnosyltransferase gene from pPR1474 under the control of the \textit{p_tac} promoter. In the absence of pMF19, both W3110 and its isogenic \textit{glf}:\textit{cat} mutant MFF1 produced only lipid A-core OS (Fig. 4, lanes 1 and 3) that, as expected, did not react with the O16 antiserum (Fig. 4B, lanes 1 and 3). Introduction of pMF19 in the parent strain W3110 resulted in the formation of O polysaccharide, as revealed by typical silver-stained ladder-like bands that also reacted with the O16-specific antiserum (Fig. 4, A and B, lanes 2). In contrast, MFF1 transformed with pMF19 displayed a single LPS band that migrated above the lipid A-core OS band (Fig. 4, lane 4) and also reacted with O16 antibodies (Fig. 4B, lane 4). The LPS phenotype of MFF1(pMF19) was unexpected and resembled that of O antigen polymerase-deficient mutants, where a single O antigen subunit is attached to the lipid A-core OS acceptor (7). However, the O16-reacting LPS band in MFF1(pMF19) has a faster mobility in the gel than the corresponding band in W3110(pMF19), suggesting that the two bands were not identical. The positive reaction with the O16 antiserum indicated that the chemical composition and structure of the novel band in MFF1(pMF19) might be similar to that of the O16 antigen subunit. This was further corroborated by failure to detect this band with a monoclonal antibody that only recognizes high molecular weight O16 polysaccharide but does not react with single O16 subunits (data not shown). Therefore, the novel O16-reactive LPS band expressed by MFF1(pMF19) was consistent with an incomplete O16 subunit that, although it could not be recognized as a substrate for the O16 polymerase, could presumably be translocated across the cytoplasmic membrane and incorporated onto lipid A-core OS.

\textbf{Structural Analysis of Outer Core OS—}Detailed structural analyses of the LPS molecules of strains MFF1 and MFF1(pMF19), indicating the residues (shaded) that correspond to incomplete O subunit components attached to the core OS.

\textbf{O repeating units}

\[
\begin{align*}
\text{E. coli K-12/O16} & : \begin{array}{c}
\text{OAc} \\
\begin{array}{c}
\text{D-Gal} \\
\text{D-Glc} \\
\text{D-Glc} \\
\text{D-GlcNAc}
\end{array}
\end{array} \\
\begin{array}{c}
\text{2} \\
\text{1,6} \\
\text{1,3} \\
\text{1,3} \\
\text{1,6}
\end{array} \\
\begin{array}{c}
\text{D-LRa} \\
\text{D-GlcNAc}
\end{array}
\end{align*}
\]

\textbf{S. enterica LT2}

\[
\begin{align*}
\text{Outer core of E. coli K-12/O16} & : \begin{array}{c}
\text{L-D-Hep} \\
\text{D-Glc} \\
\text{D-Glc} \\
\text{D-Glc} \\
\text{D-Glc}
\end{array} \\
\begin{array}{c}
\beta \text{1,7} \\
\alpha \text{1,6} \\
\alpha \text{1,2} \\
\alpha \text{1,3} \\
\alpha \text{1,3}
\end{array} \\
\begin{array}{c}
\text{D-GlcNAc} \\
\text{D-Gal}
\end{array}
\end{align*}
\]

\text{MFF1} (L-D-Hep)

\[
\begin{align*}
\text{MFF1 (pMF19)} : \begin{array}{c}
\text{L-D-Hep} \\
\text{D-Glc} \\
\text{D-Glc} \\
\text{D-Glc} \\
\text{D-Glc}
\end{array} \\
\begin{array}{c}
\beta \text{1,7} \\
\alpha \text{1,6} \\
\alpha \text{1,2} \\
\alpha \text{1,3} \\
\alpha \text{1,3}
\end{array} \\
\begin{array}{c}
\text{D-GlcNAc} \\
\text{D-Gal}
\end{array}
\end{align*}
\]

\text{Fig. 3.} Chemical structures of the \textit{E. coli} O16 and O7 and \textit{S. enterica} LT2 O antigen repeating units as well as the outer core OS structures in strains MFF1 and MFF1(pMF19), indicating the residues (shaded) that correspond to incomplete O subunit components attached to the core OS.
The type A primary glycosyl oxonium ions (A-type primary ion at the methylated intact MFF1(pMF19) LPS yielded a strong mass-mass spectrometry spectrum of the O16 O-chain polysaccharide region of this strain (Fig. 3). The fast atom bombardment-mass spectrometry spectrum of the methylated intact MFF1 LPS confirmed the presence of the terminal GlcNAc component in this molecule. The sugar linkage types detected in the LPS of MFF1(pMF19) were of the same type as those observed in MFF1 LPS, with the exception that the only GlcNAc residue detected was a 3,6-disubstituted derivative. Furthermore, two additional units not present in MFF1 LPS, terminal Glc and 3-substituted Rha, were determined to be components of the MFF1(pMF19) LPS. The GlcNAc, Rha, and Glc derivatives found in MFF1(pMF19) LPS were of the same type as found in the O16 O-chain polysaccharide region of this strain (Fig. 3). The fast atom bombardment-mass spectrometry spectrum of the methylated intact MFF1(pMF19) LPS yielded a strong A-type primary ion at m/z 842 and a corresponding secondary ion, from β-elimination of Glc-Rha, at m/z 432 (from O-3 position of β-GlcNAc, at m/z 432 (m/z 842 to 410) that represented the following structural saccharide moiety: Glc-(1-3)-Rha-(1-3)/Glc-(1-6)/GlcNAc (Fig. 5). Two additional higher mass primary ions at m/z 1090 (Glc-(1-3)-Rha-(1-3)/Glc-(1-6)/GlcNAc-(1-7)-LDHep) and m/z 1294 (Glc-(1-3)-Rha-(1-3)/Glc-(1-6)/GlcNAc-(1-7)-LDHep-(1-6)-Glc) showed the connection of the Glc-(1-3)-Rha-(1-3)/Glc-(1-6)/GlcNAc O-chain moiety to the core via the 7-substituted LDHep unit. The structural data indicated the presence of an incomplete O16 O-chain repeating block lacking the terminal Gal/g unit (Fig. 3). This incomplete O16 repeating unit, Glc-(1-3)-Rha-(1-3)/Glc-(1-6)/GlcNAc, carried a terminal Glc attached to the O-6 position of the 3,6-disubstituted GlcNAc. The absence of this terminal Glc unit in the MFF1 LPS strongly suggested that this glycosylation required the prior presence of Rha of the repeating unit (Fig. 3). Furthermore, since GlcNAc is a component of the O16 repeating unit, these results show that the GlcNAc-(1-7)-LDHep linkage is the site of attachment between the O-chain and the E. coli K-12 core oligosaccharide (Fig. 3), a function performed by the O-chain ligase WaaL (Fig. 1).

The O Antigen Synthesis Pathway Contributes the GlcNAc Residue in the Outer Core of E. coli K-12—The fact that an incomplete O16 subunit made of three backbone sugars and a glucosyl branch can be ligated to lipid A-core OS raised the question of what is the minimum number of O antigen sugar components required for this process. The presence of a terminal GlcNAc in the structure of MFF1 LPS suggests that this sugar is part of the O16 subunit and may have been donated from Und-P-bound GlcNAc. Therefore, we hypothesized that the terminal GlcNAc in MFF1 LPS arises from the synthesis and translocation pathway of an incomplete O16 subunit. This hypothesis predicts that the presence of a terminal GlcNAc in MFF1 LPS would require the activities of the UDP-GlcNAc:Und-P GlcNAc-1-P transferase (WecA) and the putative O translocase Wzx. To test this prediction, we constructed strain CLM20 that has a deletion eliminating the O16 and colanic acid biosynthesis gene clusters, as well as another deletion eliminating the first four genes of the ECA biosynthesis cluster. This deletion includes part of wccA (Fig. 2B) that is not only needed for the initiation of synthesis of ECA (18) but is also required for the initiation of the synthesis of many O antigens containing GlcNAc (20, 49), including O16 (39, 46). Therefore, CLM20 could be used to reconstruct in a stepwise fashion the synthesis and assembly of the O16 subunit, by the sequential addition of individual components encoded by specific plasmids. pmAV11 and pmF20, carrying wecA and the putative translocase O16 translocase gene wexKo16, respectively, were transformed separately or together into CLM20. LPS from these strains was extracted and examined by Western blot using digoxigenin-labeled WGA, since this lectin has a high specificity for terminal GlcNAc residues (50). In parallel, samples were stained with silver to corroborate that comparable amounts of LPS were loaded in each lane (Fig. 6B). Ovalbumin was used in these experiments as a positive control (Fig. 6A, lane 9). Only LPS from CLM20 carrying both pmAV11 and

Fig. 5. Fast atom bombardment mass spectrum showing mass regions between m/z 400 and 1300 of the methylated intact MFF1 LPS. The type A primary glycosyl oxonium ions (m/z 842, 1090, and 1294) and a secondary ion (m/z 432) from β-elimination of the structure at m/z 842 are shown. *, peak not assigned. The structures represented in the upper part of the spectrum are: (Gal), GlcNAc, and 3-glycerol-o-manno-heptose (LDHep). MFF1(pMF19) LPS was also composed of Glc, Gal, GlcNAc, and LDHep residues and in addition contained rhamnose (Rha). Methylation linkage analysis of intact MFF1 LPS revealed the presence of terminal Gal, LDHep, and GlcNAc units; 2-, 6-, and 3,6-substituted Glc residues; and a linear 7-substituted LDHep component. The inner core LDHep units were not detected in this methylation linkage analysis due to the fact that these sugars were phosphorylated (48). The primary A-type glycosyl oxonium ion at m/z 260 observed in the fast atom bombardment-mass spectrometry spectrum of the methylated MFF1 LPS confirmed the presence of the terminal GlcNAc component in this molecule. The sugar linkage types detected in the LPS of MFF1(pMF19) were of the same type as those observed in MFF1 LPS, with the exception that the only GlcNAc residue detected was a 3,6-disubstituted derivative. Furthermore, two additional units not present in MFF1 LPS, terminal Glc and 3-substituted Rha, were determined to be components of the MFF1(pMF19) LPS. The GlcNAc, Rha, and Glc derivatives found in MFF1(pMF19) LPS were of the same type as found in the O16 O-chain polysaccharide region of this strain (Fig. 3). The fast atom bombardment-mass spectrometry spectrum of the methylated intact MFF1(pMF19) LPS yielded a strong A-type primary ion at m/z 842 and a corresponding secondary ion, from β-elimination of Glc-Rha, at m/z 432 (from O-3 position of β-GlcNAc, at m/z 432 (m/z 842 to 410) that represented the following structural saccharide moiety: Glc-(1-3)-Rha-(1-3)/Glc-(1-6)/GlcNAc (Fig. 5). Two additional higher mass primary ions at m/z 1090 (Glc-(1-3)-Rha-(1-3)/Glc-(1-6)/GlcNAc-(1-7)-LDHep) and m/z 1294 (Glc-(1-3)-Rha-(1-3)/Glc-(1-6)/GlcNAc-(1-7)-LDHep-(1-6)-Glc) showed the connection of the Glc-(1-3)-Rha-(1-3)/Glc-(1-6)/GlcNAc O-chain moiety to the core via the 7-substituted LDHep unit. The structural data indicated the presence of an incomplete O16 O-chain repeating block lacking the terminal Gal/g unit (Fig. 3). This incomplete O16 repeating unit, Glc-(1-3)-Rha-(1-3)/Glc-(1-6)/GlcNAc, carried a terminal Glc attached to the O-6 position of the 3,6-disubstituted GlcNAc. The absence of this terminal Glc unit in the MFF1 LPS strongly suggested that this glycosylation required the prior presence of Rha of the repeating unit (Fig. 3). Furthermore, since GlcNAc is a component of the O16 repeating unit, these results show that the GlcNAc-(1-7)-LDHep linkage is the site of attachment between the O-chain and the E. coli K-12 core oligosaccharide (Fig. 3), a function performed by the O-chain ligase WaaL (Fig. 1).

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Translocation of Und-P-P-bound O Antigen Units

| Plasmid* | WGA μg/ml |
|----------|-----------|
| None     | 250.00 (1) |
| pMAV11 (wecA) | 125.00 (2) |
| pMAV11, pMF20 (wecA; wbbL<sub>EcO16</sub>) | 3.12 (80) |
| pMAV11, pMF21 (wecA; wbbL<sub>EcO16</sub>) | 1.56 (160) |
| pMAV11, pMF24 (wecA; wbbL<sub>LT2</sub>) | 6.25 (40) |
| pMAV11, pMF26 (wecA; exoT<sup>+</sup>) | 15.62 (16) |
| pMAV11, pMF28 (wecA; wbbL<sub>C</sub>) | 125.00 (2) |

* Strain CLM20 was grown as indicated under "Experimental Procedures" and standardized to an absorbance of 2 at a wavelength of 600 nm. 25 μl of the bacterial suspensions were mixed with 25 μl of a WGA dilution. Agglutination was recorded after 3 min.

The values indicate the lowest dilution giving an agglutination reaction comparable with that obtained with the negative control (CLM20 with no plasmid) and represent the means of three independent experiments. The fold reduction in the concentration of WGA to achieve agglutination comparable with that of the negative control is indicated in parenthesis.

pMF20 bound the lectin probe (Fig. 6A, lane 4). Also, the WGA-reacting LPS band was slightly higher in molecular mass than the bands found in the LPS from CLM20 (Fig. 6B, lanes 1 and 4). In some experiments, a faint WGA-reacting band could be observed in CLM20 without any plasmids and in CLM20 with either pMAV11 or pMF20, but only after loading a considerable amount of LPS in the gels (data not shown). This was confirmed by additional experiments involving the agglutination by WGA of standardized bacterial suspensions. Agglutination of CLM20 was possible with 250 μg/ml of WGA, while 3.12 μg/ml was sufficient to detect agglutination of CLM20 containing pMAV11 and pMF20 (Table II). These results were strengthened by the fact that the WGA-reacting band disappeared following the transformation of CLM20 with pZY1003, pMAV11, and pMF19 (Fig. 6A, lane 6). The disappearance of the WGA-reactive band coincided with a new band of higher molecular weight than the lipid A-core OS band of CLM20, and this band was only present in the silver-stained gel (Fig. 6B, lane 6, asterisk). pZY1003 contains the biosynthetic genes for dTDP-rhamnose and the wbbL<sub>EcO16</sub> translocase gene (39), while pMF19 contains the rhamnosyltransferase gene wbbL (Fig. 2A). Therefore, the higher molecular weight band is consistent with the addition of rhamnose to the GlcNAc residue to form an incomplete O16 subunit with two sugar components. As a consequence of the attachment of rhamnose, the GlcNAc residue is no longer terminal (Fig. 3) and therefore cannot bind WGA. The result of the control experiment with CLM20 containing only pMAV11 and pZY1003, where a WGA-reacting LPS band was observed (Fig. 6A, lane 5), confirmed this interpretation. In this case, since the rhamnosyltransferase WbbL is not present, the higher LPS band is absent in the silver-stained gel (Fig. 6B, lane 5). Also, since a WGA-reacting LPS band was not found in CLM20 lacking pMAV11 (Fig. 6A, lanes 1–3), the GlcNAc precursor is probably in the form of GlcNAc-P-P-undecaprenol.

The experiments presented in this section provided strong evidence indicating that the GlcNAc residue in the E. coli K-12/O16 LPS originates from the O antigen biosynthetic and assembly machinery and requires the presence of both WeeA and Wzx protein. At the same time, the results show that a single sugar bound to undecaprenylphosphate can be translocated across the cytoplasmic membrane in a Wzx-dependent fashion.

Specificity in the Translocation of O Antigen—Data above supported the view that an incomplete O subunit can be synthesized and further ligated onto lipid A-core OS. Since this process required the wzx translocase gene product, we explored the specificity of the wzx function. In general, proteins of the Wzx family share very little homology in the primary amino acid sequence, displaying around 20% identity or less. BLAST searches using the E. coli K12/O16 translocase as a query revealed low level homology with several putative O antigen translocases (from E. coli O7, S. enterica, Bacteroides fragilis, Yersinia enterocolitica, Pseudomonas aeruginosa, and Shigella flexneri) and those of E. coli (WzxC) and R. meliloti (ExoT) expolysaccharide synthesis (data not shown). Therefore, we used the CLM20 strain transformed with wzx genes from some of these other O antigen clusters in similar reconstitution experiments to those described above. In the presence of pMAV11 and pMF24, containing the S. enterica LT2 wzx gene cloned under the control of the tac promoter, CLM20 formed WGA-reacting LPS (Fig. 6A, lane 8), not present in the absence of pMAV11 (Fig. 6A, lane 7). A similar result was observed with pMF21, containing the E. coli O7 wzx gene (data not shown). The results of lectin blots were supported by slide agglutination tests, indicating that agglutination of CLM20 containing these wzx genes required a concentration of WGA ranging from 40- to 160-fold lower than the WGA concentration giving a comparable agglutination of CLM20 alone (Table II). We also conducted similar complementation experiments with cloned genes encoding ExoT from R. meliloti and WzxC from E. coli K12. These proteins were postulated to participate in the translocation of Und-P-P-linked sugars involved in the synthesis of exopolysaccharides (16), but their actual function has not been elucidated (51). No WGA-positive LPS band was detected with pMF26 (exoT) and pMF28 (wzxC), and a high concentration of WGA was required to promote the agglutination of CLM20 containing these plasmids (Table II). We concluded from these experiments that the translocases from E. coli and S. enterica, but not those implicated in exopolysaccharide transport, are involved in the transfer of GlcNAc to the lipid A-core OS.

We wanted to also investigate whether these findings hold true for a system where the complete O antigen subunit is made. For this purpose, we turned to the O7 LPS system, since we have available a nonpolar wzx<sub>EcO16</sub> translocase mutant (23). In a previous publication, we have reported that the wzx<sub>EcO16</sub>:Tn3HoHo1–128 mutation expressed a complete O7 LPS in E. coli K-12 strains that contain an intact O antigen biosynthesis cluster but failed to express O7 LPS in derivatives of E. coli S0874 that carry a deletion of the O16 antigen gene cluster (23), suggesting that the E. coli K12/O16 wzx translocase could complement the phenotype of insertion 128. To confirm that wzx<sub>EcO16</sub> can indeed complement the wzx<sub>ExoT</sub>128 mutation, pMF20 was transformed into the strain CLM4 containing pHJC32:Tn3HoHo1–128 (23, 29). A Western blot analysis of LPS using O7-specific antibodies showed that pMF20 (wzx<sub>EcO16</sub>) complemented the formation of O7 LPS at a level similar to that obtained with pMF21 (wzx<sub>ExoT</sub>) (Fig. 7, lanes 3 and 4). A complementation experiment with the S. enterica wzx gene (pMF24) also resulted in the detection of O7-specific LPS by Western blot (Fig. 7, lane 5), although at a lower level than in the case of complementation with the O7 and O16 counterparts. These differences were not due to a loading artifact, since equal concentrations of LPS, as determined by KDO analysis, were loaded in each lane. No complementation was observed in similar experiments with pMF28 (exoT) and pMF28 (wzxC), in agreement with the observations made before with the strain CLM20 (Fig. 7, lanes 6 and 7). Lack of complementation was not due to the absence of expression of cloned exoT and wzxC genes as determined by reverse transcriptase-PCR analysis using RNA prepared from CLM4 cells containing pMF26 and pMF28 (data not shown). These results suggest that Wzx translocases from various sources, but all
involved in O-antigen assembly, may be functionally interchangeable despite their apparent lack of amino acid sequence conservation and act independently from the structure of the saccharide moiety being translocated. In contrast, translocases involved in the synthesis of exopolysaccharides cannot complement an O-antigen translocace null mutation.

DISCUSSION

The classical model for wzy-dependent O antigen synthesis (Fig. 1) assumes the completion of the synthesis of the O subunit prior to its translocation across the cytoplasmic membrane (11, 14, 16). However, our experiments using the O16 synthesis of E. coli K-12 provide conclusive evidence that incomplete O subunits can serve as substrates for the O antigen assembly machinery. We have constructed a derivative of E. coli K-12 devoid of genes for the synthesis of the surface polysaccharide colanic acid, O16 antigen, and ECA. This strain, CLM20, produced a complete lipid A-core OS and permitted us to reconstruct in a stepwise fashion the synthesis and assembly of O16 sugar components onto lipid A-core OS by examining the structure of the outer core OS for the presence of sugar components corresponding to partial O subunits. The presence of these components depended on specific genes that were added as recombinant plasmids.

Structural and biochemical data using WGA binding to terminal GlcNAc show conclusively a terminal GlcNAc residue that can be incorporated into the lipid A-core OS in a wzy-dependent manner. The addition of GlcNAc did not occur in the absence of the wexA function, strongly suggesting that this must be donated to the core OS acceptor as a GlcNAc-P-undeacaprenol intermediate. The unequivocal proof that the GlcNAc residue is transferred into the core OS from GlcNAc-P-undeacaprenol would require a biochemical demonstration of this reaction in a defined in vitro system, currently under development in our laboratories. L-Rhamnose, the next sugar of the O16 repeat, could also be added to GlcNAc-P-undeacaprenol if the genes for the synthesis of dTDP-Rha and the WbbL rhamnosyltransferase were provided. The Rha-GlcNAc disaccharide was also incorporated into the lipid A-core OS in a wex-dependent fashion, as evidenced by abolition of LPS binding to WGA concomitantly with a proportional increase in the molecular mass of the core OS. Finally, a mutation affecting the synthesis of the Galβ terminal residue of the O16 subunit resulted in the formation of a truncated O16 subunit that was incorporated to lipid A-core OS. From these results, we can conclude that (i) the translocation of the O subunits in wzy-dependent systems is independent of the length and completion of the assembly of the O subunit and (ii) undeacaprenyl-GlcNAc is the minimal glycolipid structure that can be recognized as a substrate for the translocation and ligation to the core OS. These conclusions may be applicable to other O types, since GlcNAc is not unique to the O16 subunit. This sugar is not only present in a large number of E. coli O types from enteric bacteria (52) but also is the first residue added to undeacaprenylphosphosphate by WecA in many O antigens (19, 20, 39, 53). In a previous work using a wex mutant in the S. dysenteriae type 1 gene cluster, Klena and Schnaitman (19) reported an extra band in the lipid A-core OS profile that could be attributed to the addition of a partial O subunit. Although these authors did not provide any further evidence, our biochemical and structural data support their conclusions and confirm that our findings can be applied to other O types in addition to O16.

The structural data obtained from the LPS analysis of strain MFF1 with and without pMF19, encoding the WbbL rhamnosyltransferase, confirmed a previous report indicating that there is a GlcNAc residue linked to a terminal L-glycero-d-manno-heptose (HepIV) in the outer core OS of strains W3110 and W3100 (48). More importantly, we determined that the GlcNAc residue is contributed by the O antigen synthesis pathway, as indicated by the studies with WGA. Therefore, we concluded that HepIV is the site of attachment of the O16 antigen to the outer core OS, and the GlcNAc-HepIV β(1→7) linkage defines the specificity of the O antigen ligase of E. coli K-12. The ligase, encoded by the waaL gene, is the only gene product known to be required for the ligation reaction that joins newly synthesized polymeric O antigen to lipid A-core OS (8). WaaL is also an integral membrane protein and acts on the periplasmic face of the cytoplasmic membrane (8). Several lines of evidence suggest that ligase proteins appear to lack any obvious specificity for the structure of the ligated polysaccharide, possibly because it may be presented for ligation in the form of a common undeacaprenol-P-P-linked form (8). Our results confirm this suggestion and also indicate that a similar property exists for Wxx proteins. Furthermore, this also explains why it has been possible to clone and express many different O antigens in E. coli K-12. Based on the results of this study, we can safely conclude that the minimal components required for the processing of the O subunit are Wxx and WaaL. One other interesting finding from our study is the identification of an additional Glc residue attached to GlcNAc in the LPS structure of MFF1(pMF19) (Fig. 3). The presence of this residue could be explained by the activity of a glucosylation gene cluster that is present at a different location than the wb cluster in the E. coli K-12 chromosome and is homologous to bacteriophage-encoded glucosylation systems involved in antigenic changes in S. flexneri (54). The construction of a waaL-deficient mutant derivative of CLM20 as well as of a mutant defective in glucosylation is in progress to investigate in detail the role of these proteins in our experimental system.
Our experimental system permitted us to also explore a second critical question in the O antigen processing regarding the specificity of the Wzx proteins. The addition of GlcNAc to the lipid A-core OS in strain CLM20 occurred not only with O16 Wzx but also with the heterologous proteins from E. coli O7 and S. enterica LT2. These proteins share very little homology in their primary amino acid sequences, and the O antigens in the three cases are structurally different (Fig. 3). In contrast, proteins implicated in the export of capsular polysaccharides such as ExoT and WzxC did not result in a significant addition of GlcNAc to the lipid A-core OS. A semiquantitative analysis by slide agglutination with WGA shows that in the presence of Wzx translocases from E. coli K-12/O16 and E. coli O7 the lipid A-core OS contained higher amounts of GlcNAc residues than in the presence of the translocase from S. enterica. A parallel study with a wzx mutation in the E. coli O7 gene cluster also showed that Wzx from E. coli K-12 or from E. coli O7 complemented the mutation very efficiently, whereas the S. enterica protein also corrected the phenotype but to a lesser extent. These differences cannot be attributed to differences in the expression of the respective genes, since they were cloned in the same vector using the same cloning strategy. One possible explanation for these differences is that the Wzx proteins may be able to recognize the Und-P-P-linked sugar. Thus, Wzx proteins from the E. coli K-12/O16 and O7 would function on the same molecule, Und-P-P-GlcNAc, while the Salmonella LT2 Wzx would interact better with Und-P-P-Gal, since galactose is the first sugar attached to undecaprenylphosphate in this microorganism (21). Alternatively, it is also possible that the process of translocation and ligation requires the efficient interaction among the proteins involved such that the O antigen ligase of E. coli K-12 (WaaL\_E. coli) may function more efficiently with Und-P-P-Gal and Wzx\_E. coli than with Wzx\_S. enterica. The notion that specific interactions between proteins involved in processing and assembly of Und-P-P-linked O subunits is required for proper O antigen biosynthesis is further supported by the fact that proteins responsible for translocation of Und-P-P-linked saccharides involved in exopolysaccharide biosynthesis could not complement an O-antigen translocase mutant, although both types of proteins are predicted to perform the same function. These putative protein interactions may contribute to a fine-tuning in the assembly of O antigen, and they will be further investigated using our experimental reconstitution system in strain CLM20.

There are remarkable similarities in the topology of the wzy-dependent O antigen biosynthesis and the initial steps in the formation of the glycan incorporated to glycoproteins in eukaryotic cells. The glycan, in this case, is assembled onto dolichol phosphate, and its synthesis also involves an initiating enzyme transferring GlcNAc-P, as well as specific glycosyltransferases subsequently adding mannose residues. Furthermore, the dolichol-linked heptasaccharide, Man\_9GlcNAc2-P-P-dolichol, is synthesized on the cytoplasmic face of the endoplasmic reticulum and must also be translocated to the luminal side, where it is further enlarged and finally attached to proteins (55, 56). Therefore, the process of membrane transistion of lipid-linked oligosaccharides represents a basic biological process found in eukaryotic and prokaryotic cells. Recent work using microsomal vesicles suggests that transbilayer movement of dolichol-bound sugars involves protein-mediated transport (57, 58), but at the present time the specific protein(s) has not been identified. Interestingly, alg2 mutants in yeast form truncated oligosaccharides only containing Man\_9GlcNAc2 structures that are transferred in vivo from dolichylphosphatate to proteins, suggesting that a complete dolichol-linked oligosaccharide is not strictly necessary for membrane translo-
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The Activity of a Putative Polyisoprenol-linked Sugar Translocase (Wzx) Involved in Escherichia coli O Antigen Assembly Is Independent of the Chemical Structure of the O Repeat

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