Biosynthesis of a Novel 3-Deoxy-D-manno-oct-2-ulosonic Acid-containing Outer Core Oligosaccharide in the Lipopolysaccharide of Klebsiella pneumoniae*

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The core oligosaccharide region of Klebsiella pneumoniae lipopolysaccharide contains some novel features that distinguish it from the corresponding lipopolysaccharide region in other members of the Enterobacteriaceae family, such as Escherichia coli and Salmonella. The conserved Klebsiella outer core contains the unusual trisaccharide 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo)-(2,6)-GlcN-(1,4)-GalUA. In general, Kdo residues are normally found in the inner core, but in K. pneumoniae, this Kdo residue provides the ligation site for O polysaccharide. The outer core Kdo residue can also be non-stoichiometrically substituted with an 1,2-glycero-D-manno-heptopyranosyl (Hep) residue, another component more frequently found in the inner core. To understand the genetics and biosynthesis of core oligosaccharide synthesis in Klebsiella, the gene products involved in the addition of the outer core GlcN (WabH), Kdo (WabI), and Hep (WabJ) residues as well as the inner core HepIII residue (WaaQ) were identified. Non-polar mutations were created in each of the genes, and the resulting mutant lipopolysaccharide was analyzed by mass spectrometry. The in vitro glycosyltransferase activity of WabI and WabH was verified. WabI transferred a Kdo residue from CMP-Kdo onto the acceptor lipopolysaccharide. The activated precursor required for GlcN addition has not been identified. However, lysates overexpressing WabI were able to transfer a GlcNAc residue from UDP-GlcNAc onto the acceptor GalUA residue in the outer core.

Klebsiella pneumoniae is an important nosocomial pathogen and is implicated in diseases including urinary tract infections, pneumonia, and bacteremia (reviewed in Ref. 1). It is second only to Escherichia coli as the most common cause of Gram-negative sepsis. The lipopolysaccharide (LPS)† of K. pneumoniae serves as an important virulence factor. It has been shown that isolates of serotypes O1 and O2 during growth release an extracellular toxic complex composed of LPS, capsule, and a small amount of protein. The extracellular toxic complex is associated with the extensive lung damage and high lethality typically seen in mice with pneumonia derived from Klebsiella (2, 3). The degree of virulence and pathology can be directly correlated with the amount of LPS in the complexes, whereas an increase in the amount of extracellular capsule has no effect (4).

As might be expected for a member of the Enterobacteriaceae, K. pneumoniae LPS shares significant similarity with the well characterized LPS structures of E. coli and Salmonella (5, 6). In all three species, LPS is subdivided into three regions: 1) lipid A, the hydrophobic membrane anchor; 2) a core oligosaccharide (OS); and 3) a polymer of glycosyl (repeat) units known as O polysaccharide (O-PS). The core OS region can be further subdivided on the basis of sugar composition into two regions, the inner and outer core (see Fig. 1B) (5). The inner core region, which is generally well conserved in the Enterobacteriaceae, contains 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and 1,2-glycero-D-manno-heptopyranosyl (Hep). In contrast, the outer core region shows more diversity and is usually composed of hexose and acetamidohexose sugars in E. coli and Salmonella. The structures of the core OS of prototype isolates representing the different serotypes of K. pneumoniae have been determined (7). The basic core structure is identical in all the serotypes examined; they differ only in the amounts and positions of non-stoichiometric sugar substitutions (7). The core OS of K. pneumoniae does have some features that distinguish it from the E. coli and Salmonella paradigms (see Fig. 1B).

The inner core of K. pneumoniae LPS is made up of the Hep-Kdo backbone seen in other members of the Enterobacteriaceae. In E. coli and Salmonella, the Hep residues are further substituted with phosphate and phosphorylethanolamine (5). The negative charge provided by these residues plays an important role in maintenance of the barrier function of the outer membrane by providing sites for cross-linking of adjacent LPS molecules with divalent cations (reviewed in Ref. 6). K. pneumoniae LPS lacks these phosphate residues, a feature also seen in the LPS of Rhizobium etli and Rhizobium leguminosarum (8) and Plesiomonas shigelloides O54 (9). It is thought that the carboxyl groups of the GalUA sugars in the core may provide the negative charge needed for outer membrane stability. The first residue of the outer core of K. pneumoniae is GalUA. The gene required for the addition of this GalUA residue (wabG)

2-(hydroxymethyl)propane-1,3-diol; Tricine, N-[2-hydroxyethyl]glycine; ESI, electrospray ionization; d,D-Hep, 1,2-glycero-D-manno-heptopyranosyl.
has been identified, and mutants lacking wabG show enhanced sensitivity to hydrophobic compounds (10). The outer core backbone contains another unusual structural motif, the trisaccharide Kdo-(2,6)-GlCN-(1,4)-GalUA. Kdo is generally confined to the inner core, but in K. pneumoniae, it provides the ligation site for O-PS and can also be further substituted with a non-stoichiometric Hep residue (11). Hep residues are typically confined to the inner core. As a result, the enzymology of K. pneumoniae core OS biosynthesis is considerably different from that of the well characterized E. coli and Salmonella systems. These novel characteristics may be important for the development of novel therapeutic strategies or vaccines against this prevalent nosocomial pathogen. Furthermore, elucidation of the biosynthesis pathway may provide insight into the LPS assembly of other bacteria with outer cores containing Kdo.

The genes responsible for core OS biosynthesis in K. pneumoniae are encoded by the waa gene cluster, whose sequence has been reported (12). The transferases of known function are shown in Fig. 1. Genes responsible for inner core synthesis are readily identified by conserved sequences shared with E. coli and Salmonella, and the enzymology of the biosynthesis of the K. pneumoniae Hep-Kdo inner core backbone has been verified (13). Preliminary structural analysis of a non-polar waaE mutant indicates that WaaE is involved in the addition of the inner core β-Glc residue (14) and that WabG is required for the addition of the initial GalUA residue of the outer core (10). The functions of the remaining transferases have not been assigned. The objective of this study was to resolve the biosynthesis of the outer core OS and to specifically identify the transferases involved in the addition of the novel GlcN, Kdo, and Hep residues.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in this study are summarized in Table I. Bacteria were grown in LB broth at 37 °C (15). The growth media were supplemented with ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), gentamicin (30 μg/ml), kanamycin (50 μg/ml), or tetracycline (15 μg/ml) as necessary. YEG-CI (0.5% yeast extract, 1% NaCl, 0.4% glucose, 0.2% p-chlorophenylalanine) plates were used as a counterselection method when using the suicide vector pWQ173 to construct allelic exchange mutants (16, 17).

DNA Methods—Plasmid DNA was isolated using the Sigma GenElute plasmid miniprep kit, and chromosomal DNA was prepared by the method of Hull et al. (18) or by using DNAzol reagent (Invitrogen) in a modified protocol for bacteria. Briefly, the cells from 1.5 ml of an overnight culture were harvested by centrifugation and resuspended in 0.03 ml of distilled water. The cell suspension, and the mixture was incubated for 1 h at room temperature. Cell debris was removed by two 10-min centrifugation steps at 16,100 × g, and then 0.5 ml of cold 100% ethanol was added to the lysate and mixed by inversion. After a 0.5–1 h incubation at room temperature, the precipitated DNA was collected by centrifugation for 30 min at 16 100 × g. The DNA pellet was washed twice with cold 70% ethanol, air-dried, and dissolved overnight in ~0.03 ml of distilled H2O. PCR amplification was performed in 0.05-ml volumes with either Pfu Turbo DNA polymerase (Roche Applied Science) or PrimersExpress PCR DNA polymerase (Invitrogen) under conditions optimized for the primer pair. All PCR products were sequenced to verify that they were error-free. Restriction endonuclease digestions and ligation reactions were performed using standard methods as directed by the manufacturer. Plasmids were maintained in E. coli DH5α, except for pWQ173 derivatives, which were transformed into E. coli JM103. DNA formation was carried out by electroporation following methods described elsewhere (19). For some K. pneumoniae strains, a modification of this method was required (20). Briefly, colonies were scraped off an LB plate (after overnight growth) and resuspended in 0.04 ml of cold distilled H2O. Plasmid DNA was added to the cells, and the DNA-cell suspension was then transferred to an electroporation cuvette for electroporation as described above.

In Vitro Mutagenesis and Allelic Exchange—Individual genes were mutated by insertion of a non-polar antibiotic resistance cassette into the target open reading frame (ORF). The same strategy was used for each mutation. The non-polar cassette used was the aphA-3 (kanamycin resistance cassette) from plasmid pYAS265 (21), and it was cloned in the same orientation for transcription as the target gene. The mutated gene was transferred to the chromosome by homologous recombination using the temperature-sensitive suicide delivery vector pWQ173 containing the counterselectable marker pheS as described previously (17). Mutations were made in waaQ, wabH, and wabJ. The plasmid containing waaQ::aphA-3 (pWQ47), wabH::aphA-3 (pWQ39), wabJ::aphA-3 (pWQ33), and wabJ::aphA-3 (pWQ36) were transformed into K. pneumoniae CWK2 or CWG399 (waaL) by electroporation, as appropriate, for allelic exchange. Mutants were selected on kanamycin resistance and chloramphenicol sensitivity, and the chromosomal mutations were confirmed by sequencing of the insertion junction of an amplified PCR product. One representative mutant for each gene was selected for further analysis (Table I).

The waaQ::aphA-3 mutant was constructed by first PCR-amplifying waaQ from K. pneumoniae CWK2 chromosomal DNA using KPwaa9 (5'-GGCCCTCGTCATCCATGCAGT-3') and KPwaa10 (5'-GGCGGCAAGTTACGGCTCG-3'). The primers include KpnI sites (underlined). The 1876-bp PCR product was ligated using blunt ends to HindII-digested pGEM-SZ(+) (18). A recombinant plasmid was selected with the waaQ gene inserted in the same orientation as the TT promoter of pGEM-SZ(+) to form pWQ45. A HindII fragment carrying the aphA-3 cassette was inserted into HindII- and Smal-digested pWQ45. The waaQ::aphA-3 gene was then removed as a 1834-bp KpnI fragment (from the sites introduced by the PCR primers) and cloned into pWQ173 to form pWQ47.

To construct the wabH::aphA-3 mutant, the wabH coding region was PCR-amplified as a 1614-bp fragment from K. pneumoniae CWK2 chromosomal DNA with primers KPwaa35 (5'-GGCCCTCGAGACAGTCCGATG-3') and KPwaa37 (5'-GGGCGGTAGCTACGGCTCAG-3'). The PCR product was digested with BamHI and KpnI at sites designed into the primers (underlined) and ligated to the similarly digested pBCSK(+) vector. This plasmid was digested with BglII at a single site within the wabH ORF and ligated to the aphA-3 cassette digested from pYA3265 with BamHI. The 2473-bp wabH::aphA-3 gene was then removed as a BamHI-KpnI fragment and ligated to the suicide delivery vector pWQ173 using the same sites to construct pWQ48.

To construct the wabJ::aphA-3 mutant, the coding region was first PCR-amplified from K. pneumoniae CWK2 chromosomal DNA using primers KPwaaCl (5'-GGGCGGCAAGTCTTGCTCGTGTG-3') and KPwaa25 (5'-GGGGCAACGGTACCCGTCGAG-3'). The 2588-bp PCR product was digested with XbaI and KpnI at sites introduced by the primers (underlined) and ligated to the similarly digested pBCSK(+) vector. The resulting plasmid was digested with EcoRV at a single site within the wabJ ORF and ligated to the HindII fragment containing the aphA-3 cassette from pYA3265. The wabJ::aphA-3 gene was removed from pBCSK(+) as a 3425-bp XbaI-KpnI fragment and ligated to the equivalent sites in the suicide delivery vector pWQ173, generating pWQ49.

For the wabJ::aphA-3 mutant, wabJ was PCR-amplified from K. pneumoniae CWK2 chromosomal DNA using KPwaa35 (5'-GGCCCTCGAGACAGTCCGATG-3') and KPwaa4 (5'-GGGGCAACGGTACCCGTCGAG-3'). The 1485-bp PCR product was digested with HindII and ligated to HindII-digested pBCSK(+) vector. The resulting plasmid was digested with EcoRV at a single site within the wabJ ORF and ligated to the HindII fragment containing the aphA-3 cassette from pYA3265. The wabJ::aphA-3 gene was removed as a 3425-bp XbaI-KpnI fragment and ligated into the equivalent sites in the suicide delivery vector pWQ173, generating pWQ53.

Plasmid Constructs for Mutant Complementation Studies—For complementation, each gene was expressed using a pBAD vector derivative in the relevant mutant strain. Plasmid pBAD18-Cm belongs to a family of expression vectors that uses the arabinose-inducible and glucose-repressible promoter (22). Repression from the araC promoter was achieved by growth in medium containing 0.4% (w/v) glucose, and induction was obtained by adding l-arabinose to a final concentration of 0.2% (v/v). L-Arabinose was then added, and the 0.2% (v/v). L-Arabinose was then added, and the culture was grown for another 2 h. Repressed controls were maintained in glucose-containing medium.

For expression of the waaQ coding region, the waaQ ORF was removed from pWQ45 as a KpnI fragment (KpnI sites were introduced into the primers used to PCR-amplify waaQ) and ligated to pBAD18-Cm to form pWQ48.

The wabJ gene was PCR-amplified from K. pneumoniae CWK2 chro-
Strain or plasmid & Genotype, serotype, or description & Ref. or source \\
--- & --- & --- \\
**E. coli strains** & & \\
BL21 (DE3) & F^− ompt^− hsdS2(rK mK^−) gal dcm (DE3) & Novagen \\
DH5α & K12 d800 deo lacZAM15 endA1 recA1 hsdR17(rK mK^−) supE44 thi-1 gyrA96 relA1 Δ(lacZYA-argF) U169 F^− & Ref. 52 \\
HB101 & hsdS2(rK mK^−) recA1 ara-14 proA2 lacY1 galK2 rpsL20 sly-5 metI-1 supE44 λ^- F^− & Ref. 52 \\
LE392 & hsdR514 (rK mK^−) supE44 supF58 lacY1 galK2 galT2 metB1 trpR55 λ^- F^− & Ref. 52 \\
**K. pneumoniae strains** & & \\
CWK2 & O1K^−, derivative of CWK1 (O1:K20); Str^r Ap^r & Ref. 53 \\
CWG399 & waaL::wac1-1 derivative of CWK2; Km^r & Ref. 11 \\
CWG600 & wabI::aphA3 derivative of CWK2; Km^r & This study \\
CWG601 & wabI::aphA3 derivative of CWK2; Km^r & This study \\
CWG602 & wabI::aphA3 derivative of CWK399; Km^r, Km^r & This study \\
CWG603 & wabI::aphA3 derivative of CWK399; Km^r, Km^r & This study \\
CWG628 & waaQ::aphA3 derivative of CWK2; Km^r & This study \\
CWG629 & waaQ::aphA3 derivative of CWK399; Km^r, Km^r & This study \\
**Plasmids** & & \\
pBAD18-Cm & Arabinose-inducible expression vector; Cm^r & Ref. 22 \\
pBCSK (+) & Cloning vector, ColE1 origin; Cm^r & Stratagene \\
pET28a (+) & IPTG-inducible expression vector; Km^r & Novagen \\
pGEM5-zF (+) & Cloning vector, pBlueScript origin; Ap^r & Promega \\
pKBT2 & Expression plasmid for C-terminal His^6^-KdsB in pCB20 shuttle vector; Km^r & Ref. 23 \\
pYAA285 & Source of non-polar aphA3 cassette conferring kanamycin resistance; Km^r & Ref. 21 \\
pWQ173 & Counterselectable (pheS), temperature-sensitive suicide vector based on pKO3; Cm^r & Ref. 17 \\
pWQ33 & pWQ173 derivative containing wabI gene interrupted by aphA3 cassette used to construct CWG600; Km^r, Km^r & This study \\
pWQ36 & pWQ173 derivative containing wabI gene interrupted by aphA3 cassette used to construct CWG601 and CWG602; Cm^r, Km^r & This study \\
pWQ39 & pWQ173 derivative containing wabI gene interrupted by aphA3 cassette used to construct CWG603; Cm^r, Km^r & This study \\
pWQ40 & pET28a (+) derivative expressing WabI with N-terminal His^6^-tag; Km^r & This study \\
pWQ42 & pBAD18-Cm derivative expressing His^6^-WabI; Cm^r & This study \\
pWQ43 & pBAD18-Cm derivative expressing WabI; Cm^r & This study \\
pWQ44 & pBAD18-Cm derivative expressing WabH; Cm^r & This study \\
pWQ45 & pGEM5 derivative expressing WaaQ; Cm^r & This study \\
pWQ47 & pWQ173 derivative containing waaQ gene interrupted by aphA3 cassette used to construct CWG628 and CWG629; Cm^r, Km^r & This study \\
pWQ48 & pBAD18-Cm derivative expressing WaaQ; Cm^r & This study \\
pWQ161 & pRK404 derivative carrying waaL coding region of CWK2; Tet^r & Ref. 11
were harvested, washed once with 50 mM Tris-HCl (pH 8.0), and then frozen until needed. Lysates were prepared as described for WabI.

Purification of CMP-Kdo Synthetase (KdsB)—Plasmid pJB72 (23) was transformed into E. coli LE392 for overexpression of KdsB. The purification procedure was a modification of that described elsewhere (23). The strain was grown for 18 h at 37 °C in LB medium supplemented with kanamycin. The culture was then diluted 1:100 in fresh medium until it reached an 

\[
\text{OD}_{600} = 0.6.
\]

Expression of KdsB was induced by adding isopropyl-1-thio-

\[
\beta-
\text{Galactoside.}
\]

The culture was incubated for another 3.5 h. The cells were harvested and washed once with 50 mM NaH₂PO₄ (pH 8.0) containing 300 mM NaCl (buffer B), and then the pellet was frozen until required. The frozen pellet was thawed on ice and resuspended in lysis buffer (buffer B containing 20 mM imidazole). RBCs were removed by ultracentrifugation for 90 min at 100,000 × g. Proteins were precipitated by the addition of 10% trichloroacetic acid and then washed twice with 10 column volumes of buffer B containing 20% acetone. The pellet was then washed 2 times with 10% trichloroacetic acid and resuspended in lysis buffer (buffer B containing 20 mM imidazole). MgCl₂ was then added to the lysate at a final concentration of 1 mM. Unbroken cells and large cell debris were removed from the lysate by high speed centrifugation for 10 min at 20,000 × g. Cell membranes were removed by ultracentrifugation for 90 min at 100,000 × g. Proteinase inhibitor mixture tablets (Complete Mini, EDTA-free, Roche Applied Science) were added to the supernatant and dissolved. Next, 1 ml of 50% nickel-nitrilotriacetic acid mixture suspension (Qiagen Inc.) was added, and the mixture was incubated for 90 min at 4 °C on a rotary shaker. The lysate/nickel-nitrilotriacetic acid mixture was then loaded onto a disposable plastic column (5 ml) with elution by gravity flow. The column was washed twice with 10 column volumes of buffer B containing 500 mM imidazole, and ~2 ml of eluate was collected in 0.25-ml fractions. The fractions were examined by SDS-PAGE, and those with the highest amount of protein were pooled and dialyzed against 50 mM Tris-HCl (pH 8.0). Protein quantitation was done using the Bio-Rad Bradford assay (typically, preparations contained 3 mg/ml). The CMP-Kdo synthetase activity was confirmed according to Roy and Benedict (24).

Kdo Transferase Activity of WabI—The enzymatic activity of WabI was assayed in a standard reaction adapted from the procedure of Gronow et al. (25). The 0.05 ml reaction mixture comprised 50 mM HEPES (pH 7.5) containing 10 mM MgCl₂, 2 mM KCl, 5 mM MgCl₂, 0.3 mg of CGW600 (wabI) mutant LPS, 0.007 mg of KdsB, and either 0.2 mg (protein) of the soluble cell-free lysate from the BL21 (DE3) (pET28) control or BL21 (DE3) (pET28c, His₆-WabI) or 0.004 mg of purified His₆-WabI. KdsB was added to start the reaction, and the mixture was incubated at 37 °C for 2 h. To stop the reaction, proteinase K was added to a final concentration of 0.8 mg/ml, and the samples were incubated for 18 h at 55 °C. For SDS-PAGE analysis, 2 × 10^6 pl reactions were run on a 10% polyacrylamide gel. The proteins were silver stained (29) or silver stained and then placed into a scintillation vial with 4 ml of EcoLite scintillation mixture (ICN). The amount of [¹⁴C]GlcNac label incorporated into the LPS was measured in a scintillation counter.

PAGE Analysis—For PAGE of LPS, the LPS was isolated on a small scale from proteinase K-digested whole cell lysates as described by Hitchcock and Brown (28). The LPS was then separated on 4–12% BisTris NuPAGE gels or 10–20% Tricine gels (Invitrogen) and visualized by silver staining (29). For SDS-PAGE of proteins, the protein samples were solubilized in SDS-containing sample buffer (30) by boiling at 100 °C for 15 min. Separation of protein was achieved using 12% SDS-polyacrylamide gels. Proteins were visualized by Coomassie Brilliant Blue staining or by Western immunoblotting. For Western immunoblotting, proteins were transferred to nitrocellulose membranes (Pall Life Sciences) and probed with mouse anti-pentahistidine monochlonal antibodies (Qiagen Inc.) according to the manufacturer’s instructions. Colorimetric detection was performed with a secondary goat anti-mouse antibody conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories, Inc.), visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

LPS Isolation—K. pneumoniae mutant strains were grown in a fermenter (2 × 10 liters) in LB medium for 21 h, harvested, and then lyophilized. The LPS was isolated from the dried cells by the phenol/chloroform/light petroleum method (26).

Desacetylation of LPS—LPS was desacetylated, and individual oligosaccharides were isolated by procedures described elsewhere (31, 32). Briefly, the LPS (100 mg) was dissolved in anhydrous hydrazine (3 ml), incubated for 1 h at 40 °C, and then poured into cold acetone. The precipitated O-deacylated LPS was collected by centrifugation, washed with acetone, and lyophilized. The O-deacylated LPS was then N-deacylated by dissolving it in 4 N KOH. The solution was incubated at 120 °C for 16 h, cooled, and neutralized with 2 x HCl. The precipitate was removed by centrifugation, and the supernatant was desalted by gel chromatography on Sephadex G-50.

Mass Spectrometry—Electrospray ionization (ESI) mass spectra were obtained using a Micromass Quattro spectrometer in 50% MeCN with 0.2% HCOOH at a flow rate of 15 μl/min with direct injection.

RESULTS

WabI Is Required for Addition of the Outer Core Kdo Residue—The wabI gene product is encoded in the waa gene cluster of K. pneumoniae. It shares sequence similarity with the Smb20805 ORF of Sinorhizobium meliloti, which is encoded on the pSymB megaplasmid in a cluster involved in LPS biosynthesis (Table II) (33) and with several hypothetical proteins in other organisms. WabI also shares limited amino acid similarity with WaaZ in E. coli core type K12 and R2 and in Salmonella species. The function of WaaZ has been established; it is required for the addition of a KdoIII residue to KdoII in the inner core of some E. coli core type 34. WabI therefore provided a candidate for the unidentified outer core Kdo transferase that forms the O-PS ligation site (Fig. 1B). As an initial step to determine the function of WabI in K. pneumoniae core biosynthesis, a non-polar aphA-3 cassette was inserted into the wabi gene of K. pneumoniae CWK2. The LPS of the mutant, CGW600 (wabI), was examined by PAGE and was found to migrate slightly faster than the wild-type (CWK2) LPS, indicative of the possible loss of a residue from the core (Fig. 2, third lane). In addition, the wabI mutant lacked high molecular mass LPS species, consistent with the loss of the O-PS ligation site. Wild-type core OS migration and O-PS ligation were restored when pWQ42 (expressing His₆-WabI) was introduced into the mutant strain (Fig. 2, fourth lane).

The structure of the CGW600 (wabI) mutant LPS was determined by ESI-MS after O-deacylation with hydrazine (structures 5 and 6) (Fig. 3B). The CGW600 (wabI) LPS showed two main peaks whose predicted structures lack the Kdo and Hep residues that are linked to the outer core GlcN in the complete core of CGW399 (waaI) (Fig. 3A). The two oligosaccharides from CGW600 (wabI) differ in the amount of non-stoichiometric 3-β-GalUA residues present in the inner core. The collective data
**K. pneumoniae Outer Core Biosynthesis**

**TABLE II**

| K. pneumoniae ORF | Homologous protein | Organism | Function | Identity/ similarity | E-value (54) | Accession no. | Ref. to function |
|-------------------|--------------------|----------|----------|----------------------|--------------|--------------|----------------|
| WabI              | RfaZ               | *Photobacterium luminescens* | LPS core biosynthesis protein | 30/54 | 1e-36 | NP_929281 |
| WabI              | Hypothetical protein | *Pseudomonas syringae* | LPS core biosynthesis protein | 26/45 | 9e-12 | ZP_00123800 |
| WabI              | Smb20805 (on megaplasmid pSymB) | *S. meliloti* | Putative glycosyltransferase | 24/42 | 3e-11 | NP_745160 |
| WabI              | WaaZ               | *Salmonella enterica sv. typhimurium* | KdoII transferase | 23/43 | 3e-05 | NP_462615 |
| WabI              | WaaZ (RfaZ)        | *E. coli* K12 core type | KdoII transferase | 20/44 | 0.023 | AAA24522 |
| WabI              | WaaZ               | *E. coli* R2 core type | KdoII transferase | 21/42 | 0.024 | AAC69650 |
| WabJ              | WaaU (RfaK)        | *E. coli* K12 | α-1,6-Hep transferase | 25/46 | 6e-18 | AAB18600 |
| WabJ              | LbgB               | *H. ducreyi* | α-1,6-Hep transferase adding HEP to Glcα | 26/48 | 2e-17 | AAB49624 |
| WaaQ              | WaaQ               | *Serratia marcescens* | LPS core HepIII transferase | 64/78 | 1e-124 | AAL23760 |
| WaaQ              | WaaQ (RfaQ)        | *P. luminescens* | LPS core HepIII transferase | 56/73 | 1e-107 | CAE17225 |
| WaaQ              | WaaQ (RfaQ)        | *Yersinia pestis* KIM | LPS biosynthesis protein | 47/62 | 3e-79 | AAM87308 |
| WaaQ              | WaaQ (RfaQ)        | *E. coli* CFT073 (R1 core type) | LPS core HepIII transferase | 44/61 | 1e-75 | AAN82892 |
| WaaQ              | WaaQ (RfaQ)        | *E. coli* K12 core type | LPS core HepIII transferaseα | 42/60 | 2e-71 | AAC76656 |
| WaaQ              | WaaQ               | *S. enterica sv. typhimurium* | LPS core HepIII transferaseα | 42/58 | 9e-71 | AAC16416 |
| WaaH              | Putative glycosyltransferase | *S. marcescens* | LPS core glycosylation | 59/71 | e-117 | AAD28802 |
| WaaH              | WaiW               | *P. luminescens* | Probable LPS biosynthesis protein | 45/67 | 4e-93 | NP_932005 |
| WaaH              | WbbB               | *E. coli* O104 | Putative GOS or GOSN transferase | 26/47 | 3e-22 | AAK64373 |
| WaaH              | WlaE               | *Campylobacter jejuni* | Putative glycosyltransferase (possibly GalNAc) | 27/45 | 1e-17 | CAA72354 |

a Function was established by direct experimentation rather than by sequence homology.

indicate that Wabl is required for the addition of the Kdo residue to the outer core OS.

In Vitro Kdo Transferase Activity of Wabl—Nucleotide sequence analysis predicts that wabl encodes a soluble protein of 34,163.78 Da with a pl of 9.45. Wabl was cloned into a pET28a(+) vector to express a protein with an N-terminal His6 tag (His6-WabI), producing a protein with a predicted size of 36,756.93 Da. A protein of the expected size was visible by Coomassie Blue-stained SDS-PAGE (Fig. 4A) as well as by Western immunoblotting with antibodies specific to the His6 tag (data not shown). When comparing equivalent amounts of soluble and membrane fractions, it was apparent that the majority of the protein was membrane-associated (Fig. 4A, compare lanes 2 and 4), despite no obvious transmembrane helices in the predicted Wabl protein. Similar results have been obtained with other “soluble” core OS biosynthesis enzymes. His6-Wabl was purified from the soluble fraction to near homogeneity by nickel chelation (Fig. 4B), but was found to be stable in solution only at very low concentrations.

The soluble fraction of a cell-free lysate from BL21(DE3)(pWQ40) overexpressing His6-Wabl or purified His6-Wabl was used as the enzyme source in an in vitro Kdo transferase assay. The CWG600 (wabl) LPS provided the acceptor. Because of its instability, the CMP-Kdo substrate was produced in situ using KdsB (CMP-Kdo synthetase). Assay reactions were first analyzed by PAGE. Both the BL21(DE3)(pWQ40(His6-WabI)) extract and purified His6-Wabl enzyme modified the acceptor LPS to generate a product with decreased migration relative to control assays (Fig. 4, A, lanes 2; and D, lane 5). The altered migration is consistent with the addition of one sugar residue.

Structural analysis was performed on the LPS reaction products using the BL21(DE3)(pWQ40(His6-WabI)) extract as the enzyme source to determine what sugar was added to the CWG600 (wabl) LPS acceptor. In addition to the two peaks from the acceptor (CWG600) LPS (Fig. 3B), two novel peaks were seen upon ESI-MS analysis of O-deacylated LPS (Fig. 3E). These two peaks at m/z 2889.4 and 2865.6 reflect the addition of the outer core Kdo residue, but differ in a non-stoichiometric β-GalUA residue in the inner core. Attempts to replicate this experiment with purified His6-Wabl failed because of the poor stability of the purified enzyme and an inability to scale up the reactions to give sufficient products for MS.

WabH Is Required for Addition of the Outer Core GlcN Residue—The wabH gene product shares similarity with numerous putative glycosyltransferases implicated in the transfer of hexose or N-acetylhexosamine sugars, although none have verified functions (Table II). WabH provided a candidate for the GlcN transferase. A non-polar wabH mutant (CWG603) produced LPS with faster migration upon PAGE relative to both the parent CWG399 (waaL) and CWG600 (wabl) (Fig. 2). This increase in migration is consistent with the loss of two or more sugars from the core. When the wabH coding region was intro-

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6 E. Frirdich, E. Vinogradov, and C. Whitfield, unpublished data.
reduced into CWG603 (waaL wabH) on a plasmid (pWQ44), wild-type LPS migration was restored (Fig. 2, sixth lane).

ESI-MS analysis of O-deacetylated LPS from CWG603 (waaL wabH) showed two major peaks (structures 7 and 8) (Fig. 3C) that differ in the amount of non-stoichiometric β-GalUA residues in the core. Both peaks lack the outer core GlcN, Kdo, and Hep residues. These structural data are consistent with WabH being the outer core GlcN transferase since GlcN and all residues dependent on its presence are missing in the mutant.

In Vitro GlcNAc Transferase Activity of WabH—The sugar nucleotide precursor for GlcN has not yet been identified (35). An in vitro assay was developed to determine whether WabH could use UDP-GlcNAc as a substrate. Two assays were carried out: (i) analyzing by PAGE whether WabH could add a residue from UDP-GlcNAc to the lipid A core of the wabH mutant (CWG600) LPS and (ii) measuring the amount of radio-label from UDP[14C]GlcNAc incorporated into the CWG603 (waaL wabH) LPS by WabH-containing lysates. The enzyme sources used in the assays were cell-free lysates from CWG603(pBAD18-Cm) (control) and CWG603(pWQ44/14C/WabH)). Lysates from the E. coli DH5α(pBAD18-Cm) control showed some uncharacterized incorporation of UDP-GlcNAc into the K. pneumoniae lipid A core acceptor (data not shown), so the reactions were performed in a CWG603 (waaL wabH) background devoid of any background activity. PAGE analysis (Fig. 5A) of reaction products formed by CWG603/pWQ44/WabH) lysates reduced lipid A core migration in comparison with those from control reactions containing CWG603(pBAD18-Cm). The controls showed no alteration of the acceptor LPS. The WabH-mediated modification is consistent with the addition of a single residue. Fig. 5B shows the time-dependent incorporation of [14C]GlcNAc into the CWG603 (waaL wabH) acceptor LPS mediated by CWG603(pWQ44/WabH) lysates; no incorporation was detected in the control lysate from CWG603(pBAD18-Cm). The wabH gene of K. pneumoniae is a UDP-galacturonate 4-epimerase involved in the conversion of UDP-GlcUA to UDP-GalUA, producing the precursor required for GalUA addition to the core OS (36). When LPS from a wabH mutant lacking the GalUA residue in the outer core was used as the acceptor to confirm the acceptor site, no [14C]GlcNAc incorporation was seen with CWG603/pWQ44/WabH) lysates (data not shown). This indicates that WabH can use UDP-GlcNAc as a substrate, specifically transferring a residue to the GalUA residue of the lipid A core acceptor.

Identification and Assignment of the Core Heptosyltransferases: wabJ and waaQ—The products of the genes wabJ and waaQ share sequence similarity with two different classes of heptosyltransferases (Table II). BLASTP and PSI-BLAST searches with WabJ showed that it shares sequence similarity with WaaU from E. coli K12 and LbgB from Haemophilus ducreyi. WaaU is a putative α-1,6-L-D-Hep transferase adding a Hep residue to a Glc residue (37), and LbgB is an α-1,6-D-glycero-D-manno-heptopyranose (D-D-Hep) transferase with a biochemically established activity involved in lipooligosaccharide biosynthesis of H. ducreyi (38–40). WabJ shares only limited local similarity with the known inner core heptosyltransferases (WaaC, WaaF, and WaaQ) from a variety of Gram-negative bacteria. WaaC is the HepI transferase (adding HepI to Kdo1); WaaF is the HepII transferase (adding HepII to HepI); and WaaQ is the HepIII transferase (adding HepIII to HepII) (Fig. 1B) (6). In contrast to WabJ, WaaQ shares high similarity with WaaQ enzymes from several organisms and weak similarity with heptosyltransferases WaaF and WaaC (Table II). These similarities are distributed throughout the entire length of the waaQ polypeptide sequence. WabJ provides the most likely candidate for the outer core Hep transferase because it shares homology with transferases that add a Hep residue to a non-heptosyl acceptor (i.e. WaaU and LbgB). WaaQ was considered the more likely candidate for the inner core HepIII transferase (equivalent to the E. coli WaaQ enzyme) because it shares higher similarity with WaaQ and WaaF.

**Fig. 1.** Genetic organization of the core OS biosynthetic cluster and the core OS structure of K. pneumoniae. A shows the inner gene cluster of K. pneumoniae (12). Genes involved in inner core backbone synthesis are shown in gray. Genes of unknown function that are characterized in this work are highlighted in black. The K. pneumoniae core OS structure (7, 11) is shown in B. Dashed arrows indicate non-stoichiometric substitutions with GalUA and Hep residues, and the various combinations identified in LPS preparation are given below the structure (7). The dashed lines indicate the known or predicted gene products involved in the indicated linkages. In the original report of the inner gene cluster sequence, wabI, wabH, and wabJ were designated as ORF4, ORF9, and ORF6, respectively (12). GalA, GalUA.

**Fig. 2.** Polyacrylamide gels showing the migration of the LPS from mutants CWG600 (wabI) and CWG603 (waaL, wabH). The upper panel shows LPS samples separated on 10–12% NuPAGE gels, which provide separation of the full spectrum of LPS molecules, including those containing O-PS. The lower panel shows the lipid A core molecular species separated on 10–20% Tricine gels, which can better resolve the low molecular mass LPS to show subtle changes. Gene expression from complementing plasmids was induced with the addition of 0.02% arabinose.

K. pneumoniae Outer Core Biosynthesis

Identification and Assignment of the Core Heptosyltransferases: wabJ and waaQ—The products of the genes wabJ and waaQ share sequence similarity with two different classes of heptosyltransferases (Table II). BLASTP and PSI-BLAST searches with WabJ showed that it shares sequence similarity with WaaU from E. coli K12 and LbgB from Haemophilus ducreyi. WaaU is a putative α-1,6-L-D-Hep transferase adding a Hep residue to a Glc residue (37), and LbgB is an α-1,6-D-glycero-D-manno-heptopyranose (D-D-Hep) transferase with a biochemically established activity involved in lipooligosaccharide biosynthesis of H. ducreyi (38–40). WabJ shares only limited local similarity with the known inner core heptosyltransferases (WaaC, WaaF, and WaaQ) from a variety of Gram-negative bacteria. WaaC is the HepI transferase (adding HepI to Kdo1); WaaF is the HepII transferase (adding HepII to HepI); and WaaQ is the HepIII transferase (adding HepIII to HepII) (Fig. 1B) (6). In contrast to WabJ, WaaQ shares high similarity with WaaQ enzymes from several organisms and weak similarity with heptosyltransferases WaaF and WaaC (Table II). These similarities are distributed throughout the entire length of the waaQ polypeptide sequence. WabJ provides the most likely candidate for the outer core Hep transferase because it shares homology with transferases that add a Hep residue to a non-heptosyl acceptor (i.e. WaaU and LbgB). WaaQ was considered the more likely candidate for the inner core HepIII transferase (equivalent to the E. coli WaaQ enzyme) because it shares higher similarity with WaaQ and WaaF.
To test these sequence-based assignments, non-polar chromosomal insertions were introduced into \( \text{wabJ} \) and \( \text{waaQ} \) in wild-type \( \text{K. pneumoniae} \) CWK2 and the O-PS-deficient mutant, CWG399 (\( \text{waaL} \)). The effect of the mutations on LPS core structure was assessed. PAGE analysis showed that the \( \text{wabJ} \) (CWG601) and \( \text{waaQ} \) (CWG628) mutants in CWK2 were both still capable of ligating O-PS to the core OS (Fig. 6, \( \text{A}, \text{third lane} \) 3; and \( \text{B}, \text{third lane} \)). To simplify structural analysis, the \( \text{wabJ} \) (CWG602) and \( \text{waaQ} \) (CWG629) mutants were constructed in CWG399 (\( \text{waaL} \)). The LPS profiles of these mutants were indistinguishable from those of the parents (Fig. 6), consistent with the non-stoichiometric nature of the Hep residues in wild-type \( \text{K. pneumoniae} \) core OS.

The structure of the \( \text{O-deacylated CWG602 (waaL wabJ)} \) LPS was analyzed by ESI-MS and showed two main peaks at \( m/z \) 2689.4 and 2865.6. These peaks differ in a non-stoichiometric \( \beta\)-GalUA residue (structures 1 and 2) (Fig. 7A). The structure for both of these molecular species contains three Hep residues, indicating that either the outer core HepIV residue or the inner core HepIII residue is absent. To differentiate between the two Hep residues, hot KOH treatment was used. Hot KOH is used to \( \text{N-deacylate O-deacylated LPS}. \) However, this treatment also results in \( \beta\)-elimination in the presence of 4-substituted uronic acids (like GalUA in the \( \text{K. pneumoniae} \) outer core), introducing a double bond between C-4 and C-5 to produce a \( \text{threohex-4-enuronopyranosyl residue} \) (11, 32). This results in the release of all sugar residues linked to O-4 of GalUA and therefore distinguishes structures with a HepIV residue on Kdo in the outer core (which would be released by KOH treatment, as it is distal to the GalUA residue) from those with a HepIII residue in the inner core (which would not be released) (Fig. 1). After hot KOH treatment, the outer core Hep residue was absent (structures 9 and 10) (Fig. 7A), indicating that all three Hep residues in the LPS from CWG602 (\( \text{waaL wabJ} \)) are indeed located in the inner core Hep residue. Therefore, by process of elimination, \( \text{WabJ} \) must be the outer core heptosyltransferase.

![FIG. 3. Structural analysis of the LPS from mutants CWG600 (wabI) and CWG603 (waaL wabH). A shows the ESI mass spectrum obtained from O-deacylated LPS of \( \text{K. pneumoniae} \) CWG399 (waaL) and the corresponding structures of the main peaks (as reported previously) (11). The ESI mass spectra from O-deacylated LPS of mutants CWG600 (wabI) and CWG603 (waaL wabH) and the structural assignments of the main peaks in those spectra are shown in B and C, respectively. Lipid A* in these structures consists of a \( \beta\)-Glc(acyl)-4-P-(1,6)-\( \beta\)-Glc(acyl)-1-P backbone, where acyl represents a 3-hydroxytetradecanoyl residue. Non-stoichiometric substitutions are represented by residues J and P as reported previously (7). GalA, GalUA.](image-url)
The O-deacylated LPS of CWG629 (waaL waaQ) showed four peaks upon ESI-MS. The peaks at m/z 2497.3 and 2673.4 (structures 11 and 12, respectively) (Fig. 7B) have a similar composition, with only two Hep residues present. They differ in the presence of a non-stoichiometric β-GalUA substitution. The peaks at m/z 2689.4 and 2865.6 (structures 13 and 14, respectively) (Fig. 7B) are distinguished from structures 11 and 12 by the possession of an additional Hep residue. After hot KOH treatment, MS of the CWG629 (waaL waaQ) LPS showed peaks with only two Hep residues remaining, Hep1 and HepII of the inner core (structures 15 and 16). The third Hep residue seen in structures 13 and 14 must therefore be, by process of elimination, the terminal HepIV residue. Since the third branching Hep residue in the core OS of this mutant was absent, WaaQ must be the K. pneumoniae equivalent of the E. coli WaaQ transferase.

Complementation of CWG602 (waaL wabJ) with pWQ43 (wabJ+) expressing the wabJ ORF, resulted in lipid A core species that migrated more slowly than wild-type CKW2, possibly because of the addition of an extra sugar to the wild-type core (Fig. 6A, fifth lane). Structural analysis by ESI-MS of O-deacylated LPS from CWG602(pWQ43(WabJ+)) resulted in five components (structures 1–4 and 7) (Table III). Structures 1–4 are also present in the wild-type core and differ in the amounts of non-stoichiometric Hep and β-GalUA residues. Structure 7 at m/z 2308.1 represents a highly truncated molecule, missing all residues distal to the GalUA residue in the outer core. The molecular species at m/z 2689.4 and 2865.6 correspond to structures 1 and 2, respectively. Similar molecules are also present in the CWG602 (waaL wabJ) mutant and are devoid of the terminal outer core Hep residue (Table III). The molecular species at m/z 2881.6 (structure 3) and 3057.7 (structure 4) are predicted to contain four Hep residues, indicating the restoration of the full spectrum of heptosyltrans-
The objective of this study was to examine the biosynthesis of this novel region of the core OS. The gene product involved in the addition of the GalUA residue (WabG) was previously identified by others (10), and the remaining transferases were established in this present study.

The wabf gene product was first identified as the putative Kdo transferase since it shares similarity with the characterized KdoIII transferase of E. coli and Salmonella (Table II). Structural analysis of the CWG600 (wabf) mutant LPS and ESI-MS analysis of the products of in vitro enzymatic assays are entirely consistent with the assignment of Wabf as the outer core Kdo transferase. Wabf shows similarity to the Smb20805 ORF of S. meliloti. The complete structure of the S. meliloti core OS has not yet been determined (41, 42), but the relative abundance of Kdo in compositional analysis of the S. meliloti rough LPS (consisting of lipid A core OS molecules lacking O-PS) suggests that there are more than two Kdo residues present (41). The complete core OS structure of R. etli has been determined, and it does contain an outer core Kdo residue that serves as the O-PS ligation site (8), as is the case in K. pneumoniae. However, the genome of R. etli has not been sequenced, so it is not known whether this organism encodes a Wabf homolog. There is good evidence that the core OS structure of R. leguminosarum is identical to that of R. etli (8), although structural analysis as detailed as that performed on R. etli has not been carried out on R. leguminosarum LPS. The LpcB enzyme is involved in the addition of the outer core Kdo residue in R. leguminosarum and has been shown to have Kdo transferase activity using a CMP-Kdo donor, although the structures of the reaction products were not determined (43).

Interestingly, LpcB shows no sequence similarity to either Wabf or the WaaZ gene product of E. coli and Salmonella. BLASTP searches with LpcB shows that it has similarity only to Smb20803, a putative protein encoded by the pSymB plasmid of S. meliloti that has been annotated as a CMP-Kdo synthetase (i.e. KdsB). The remaining proteins showing similarity to Wabf are present in organisms for which there is no known LPS structure, so their function is currently unknown.

Other than WaaA, the enzyme that adds the first two Kdo residues of the inner core (6), Wabf and LpcB (43) are the only verified Kdo transferases. WaaZ is essential for non-stoichiometric addition of a KdoIII residue to KdoII of the inner core of some E. coli and Salmonella species, but its function as a glycosyltransferase has not been examined in vitro (34). These results show that glycosyltransferases with no significant sequence similarity may have similar functions; LpcB adds a Kdo residue to a Gal residue by an α-2,6-linkage (43), and Wabf also adds a Kdo residue to a GlcN residue with the same α-2,6-linkage. It is possible that the lack of sequence similarity may reflect the different acceptor molecules. However, this is likely an oversimplification because WaaZ resembles Wabf (and not LpcB), but forms a Kdo-(2,4)-Kdo linkage. An alternative explanation of the sequence variation in Wabf and LpcB is that they have different evolutionary histories.

The wabH gene product was chosen as the potential GlcN transferase since it shows similarity to other hexose and N-acetylhexosamine transferases (Table II). WabG, WaaE, and ORF10, encoded by the K. pneumoniae waa cluster, also show similarity to glycosyltransferases (data not shown). However, the activities of WabG (10) and WaaE (44) are known. Also, ORF10 has been classified on the CAZY server, which classifies structurally similar carbohydrate-active enzymes as a member of family 2 with inverting glycosyltransferases. It is thought to be involved in the addition of the non-stoichiometric β-GalUA substitutions in the inner core, but has not been

3 Available at afmb.cnrs-mrs.fr/CAZY/.

DISCUSSION

There are several features of the core OS of K. pneumoniae that distinguish it from other members of the Enterobacteriaceae, typified by the well characterized core OS of E. coli and Salmonella. One of these features is the presence of the novel trisaccharide motif Kdo-(2,6)-GlcN-(1,4)-GalUA found in the outer core OS. This trisaccharide can be further substituted by a non-stoichiometric Hep residue added to the Kdo residue (11).
extensively studied. However, now that the identities of the other glycosyltransferases are known, this assignment seems reasonable. Structural analysis of the CWG603 (wabJ wabH) mutant LPS implicated WabH as the GlcN transferase.

The activated sugar precursor for GlcN used for bacterial polysaccharide synthesis is not known, although there has been a report of TDP-GlcN pyrophosphorylase activity in *Pseudomonas aeruginosa* and *E. coli* (45). UDP-GlcN has been identified in mammalian cells, but not in bacteria (35). GlcN makes up the backbone of the lipid A portion of LPS, but the activated precursor involved is UDP-GlcNAc (6), and the lipid A precursor is subsequently deacetylated by LpxC (6). UDP-GlcNAc is formed in two steps from glucosamine 1-phosphate in a reaction catalyzed by the bifunctional enzyme GlmU, with both acetyltransferase and uridylyltransferase activities (46, 47). The overall reaction consists of glucosamine 1-phosphate + acetyl-CoA + UTP → UDP-GlcNAc + CoA + PP, (46, 47). Initial studies on the GlmU enzyme indicated that acetyl transfer preceded uridylyl transfer (47, 48). More recently, it has been shown that GlmU could catalyze the formation of UDP-GlcN from GlcN-1-P and UTP in *vitro*, but at a reduced rate compared with GlcNac-1-P (49). It is not known whether this could occur under normal physiological conditions. An *in vitro* assay indicated that WabH is involved in the transfer of GlcNAc from UDP-GlcNAc to the GalUA residue of the outer core OS of *K. pneumoniae*. There are two possible explanations for these results. 1) WabH has relaxed activity and can incorporate either UDP-GlcNAc or UDP-GlcN. This is unlikely since the sugar nucleotide pool of UDP-GlcNAc in the cell is predicted to be higher than that of UDP-GlcN (if it exists), but no core OS species have been detected that contain GlcNAc instead of GlcN. 2) It is possible that the activated precursor for GlcN is UDP-GlcNAc and that this is followed by a (presently unknown) deacetylase/transfer activity, similar to lipid A biosynthesis. From the evidence currently available, there exists the possibility that WabH is involved in regulating GlcN transfer to the core OS, rather than it having glycosyltransferase activity per se. Although there is no precedent for this, this formal (if unlikely) possibility can be excluded only by *in vitro* studies with purified WabH.

There are four heptosyltransferases (WaaC, WaaF, WabJ, and WaaQ) encoded by the *waa* gene cluster of *K. pneumoniae*. The activities of WaaC and WaaF as HepI and HepII transferases, respectively, involved in inner core biosynthesis have already been well established. By sequence analysis, WabJ appears to be the outer core Hep transferase adding a Hep residue to a glucose residue, and WaaQ appears to be the inner core HepII transferase adding a Hep residue to another Hep residue. The activities of WabJ and WaaQ were confirmed by detection of the LPS structures in the relevant mutants. Complementation of CWG602 (waaL wabL) with wabL (pWQ43) produced a slower migrating species compared with the wild type. This is most likely because of an increase in the amount of non-stoichiometric Hep. Interestingly, when CWG602 (waaL wabL) was complemented with plasmid-encoded wabJ and waaL (pWQ43 and pWQ161, respectively), the lipid A core OS migration returned to that of the wild type, corresponding to wild-type levels of Hep. Indeed, the peaks present in the ESI spectrum of CWG602 (pWQ43(WabJ +)) are identical to those found in that of the wild type (structures 1–4), in addition to a truncated species (structure 7) (Table III). Overexpression of WaaZ, the putative inner core KdoIII transferase of *E. coli* K12, also produces truncated core OS molecules (34). It is not known why overexpression of certain glycosyltransferases leads to core OS truncation. One possible explanation is that an increase in the amount of certain core OS biosynthesis proteins interferes with the protein-protein interactions between the transferases involved in core OS biosynthesis. It is thought that these transferases form a complex on the inner leaflet of the inner membrane and that they act in a coordinated manner to elongate the core OS, although this has not been proven experimentally. Interruption of the protein-protein interactions may prevent the addition of residues by late-acting transferases. Complementation of CWG629 (waaL waaQ) with pWQ48 (WaaQ +) also produced faster migrating lipid A core OS species upon PAGE analysis. These species
were still present when pWQ161 (waaL) was added (Fig. 6, sixth lane), but O-PS ligation was restored, so some full-length lipid A core OS molecules must also be present to provide ligation-competent LPS species. The first complete structure of the core OS region of K. pneumoniae strain R20 (O1/H11002:K20/H11002) identified a novel heptoglycan of three or four D,D-Hep residues linked to O-6 of GlcN (50). A MS study examining the heterogeneity of the LPS from mutants CWG603 (waaL wabJ) and CWG629 (waaL waaQ). A shows the ESI mass spectrum obtained from O-deacylated LPS of CWG603 (waaL wabJ) and the corresponding structures of the main peaks. The main structures of N,O-deacylated LPS that had been treated with hot KOH are shown below. This procedure was used to determine whether HepIV of the outer core or HepIII of the inner core is absent in the mutant. The corresponding MS analysis of CWG629 (waaL waaQ) is shown in B. The α-D-GalUA (GalA) residue is the product of β-elimination of the substituent from C-4 of α,β-GalUA. Non-stoichiometric substitutions are represented by residues J and P. The term acyl in the lipid A structure represents a 3-hydroxytetradecanoyl residue.

**A CWG602 (waaL wabJ)**

**O-deacylated LPS:**

\[
\alpha-Kdo-(2-6)\alpha-GlcN-(1-4)\alpha-GalA-(1-3)\alpha-Hep-(1-3)\alpha-Hep-(1-5)\alpha-Kdo-(2-6)\alpha\text{GalN}-(acyl)-4P-(1-6)\alpha\text{GalN}-(acyl)-1P
\]

| Structure | Calculated mass | J |
|-----------|-----------------|---|
| 1         | 2689.4          | H |
| 2         | 2695.6          | β-GalA |

**Hot KOH treated N,O-deacylated LPS:**

\[
\alpha\text{GalA}-(1-3)\alpha\text{Hep}-(1-3)\alpha\text{Hep}-(1-5)\alpha\text{Kdo}-(2-6)\beta\text{GlcN}-(acyl)-4P-(1-6)\alpha\text{GalN}-(acyl)-1P
\]

| Structure | Calculated mass | J |
|-----------|-----------------|---|
| 9         | 1837.4          | H |
| 10        | 2014.0          | β-GalA |

**B CWG629 (waaL waaQ)**

**O-deacylated LPS:**

\[
\beta\text{Kdo}-(1-4)\beta\text{Kdo}-(2-6)\alpha\text{Kdo}-(1-4)\alpha\text{Kdo}-(1-3)\alpha\text{Kdo}-(1-3)\alpha\text{Kdo}-(1-5)\alpha\text{Kdo}-(2-6)\beta\text{GlcN}-(acyl)-4P-(1-6)\alpha\text{GalN}-(acyl)-1P
\]

| Structure | Calculated mass | J | P |
|-----------|-----------------|---|--|
| 11        | 2497.3          | H | H |
| 12        | 2673.4          | β-GalA | H |
| 13        | 2689.4          | H | α-L-D-Hep |
| 14        | 2865.6          | β-GalA | α-L-D-Hep |

**Hot KOH treated N,O-deacylated LPS:**

\[
\alpha\text{GalA}-(1-3)\alpha\text{Hep}-(1-3)\alpha\text{Hep}-(1-5)\alpha\text{Kdo}-(2-6)\beta\text{GlcN}-(acyl)-4P-(1-6)\alpha\text{GalN}-(acyl)-1P
\]

| Structure | Calculated mass | J | P |
|-----------|-----------------|---|--|
| 15        | 1644.8          | H | H |
| 16        | 1821.4          | β-GalA | H |

Fig. 7. Structural analysis of the LPS from mutants CWG603 (waaL wabJ) and CWG629 (waaL waaQ). A shows the ESI mass spectrum obtained from O-deacylated LPS of CWG603 (waaL wabJ) and the corresponding structures of the main peaks. The main structures of N,O-deacylated LPS that had been treated with hot KOH are shown below. This procedure was used to determine whether HepIV of the outer core or HepIII of the inner core is absent in the mutant. The corresponding MS analysis of CWG629 (waaL waaQ) is shown in B. The α-D-GalUA (GalA) residue is the product of β-elimination of the substituent from C-4 of α,β-GalUA. Non-stoichiometric substitutions are represented by residues J and P. The term acyl in the lipid A structure represents a 3-hydroxytetradecanoyl residue.

The first complete structure of the core OS region of K. pneumoniae strain R20 (O1′:K20′) identified a novel heptoglycan of three or four α,β-Hep residues linked to O-6 of GlcN (50). A MS study examining the heterogeneity of the LPS from
K. pneumoniae strain R20 showed peaks whose mass assignments corresponded to peaks showing the presence of this heptoglycan (51). These first two studies also showed the presence of a KdoIII residue linked to KdoII of the inner core (as in E. coli and Salmonella). The structure of the K. pneumoniae core OS was re-examined in a study of the core OS structures from eight K. pneumoniae serotypes, and different experimental methods were used to prove that KdoIII was indeed linked to O-6 of GlcN in the outer core and not in the inner core (7). In this previous study, only the LPS of serotype O1 contained minor amounts of structures with two Kdo residues and more than four Hep residues. This is the anticipated outcome of the Hep-(1,4)-Kdo-(2,6)-GlcN motif in the outer core. However, these heptoglycan-containing core OS molecules were in such small amounts that they were not isolated as pure fractions; therefore, their structural assignments were based only on data in the literature (50, 51). The genetics and biosynthesis of this heptoglycan are not known. Functions have been assigned to the two heptosyltransferases (WabJ and WaaQ) identified in the waa gene cluster of K. pneumoniae. No additional heptosyltransferases are currently identified elsewhere on the chromosome by BLAST searches of the currently incomplete K. pneumoniae genome sequence with known heptosyltransferases (data not shown). It is possible that the heptoglycan transferases show only limited similarity to known heptosyltransferases or that they are encoded in a region for which good sequence data are currently lacking.

Acknowledgments—We thank Sabine Gronow for providing the plasmid pJB72 and the protocol for purifying KdoB and Dr. Brad Clarke for help in developing the radioactive glycosyltransferase assays.

REFERENCES
1. Podschun, R., and Ullmann, U. (1998) Clin. Microbiol. Rev. 11, 589–603
2. Straus, D. C., Atkinson, D. L., and Garner, C. W. (1985) Infect. Immun. 50, 787–795
3. Straus, D. C. (1987) Infect. Immun. 55, 44–48
4. Domenico, P., Diedrich, D. L., and Straus, D. C. (1985) Can. J. Microbiol. 31, 472–478
5. Holst, O. (1999) in K. pneumoniae Outer Core Biosynthesis. No additional heptosyltransferases are currently identified elsewhere on the chromosome by BLAST searches of the currently incomplete K. pneumoniae genome sequence with known heptosyltransferases (data not shown). It is possible that the heptoglycan transferases show only limited similarity to known heptosyltransferases or that they are encoded in a region for which good sequence data are currently lacking.

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REFERENCES
1. Podschun, R., and Ullmann, U. (1998) Clin. Microbiol. Rev. 11, 589–603
2. Straus, D. C., Atkinson, D. L., and Garner, C. W. (1985) Infect. Immun. 50, 787–795
3. Straus, D. C. (1987) Infect. Immun. 55, 44–48
4. Domenico, P., Diedrich, D. L., and Straus, D. C. (1985) Can. J. Microbiol. 31, 472–478
5. Holst, O. (1999) in K. pneumoniae Outer Core Biosynthesis.

TABLE III
Summary of the major peaks seen by ESI-MS of O-deacylated LPS from Klebsiella mutants and effects of complementing plasmids encoding WabJ and WaaQ

| mle | Structure no. | CWG399 (waaL) | CWG600 (waaL) | CWG603 (waaL, wabH) | CWG602 (pWQ43, WabJ) | CWG602 (pWQ43, WabJ) | CWG629 (pWQ48, WaaQ) |
|-----|---------------|---------------|---------------|---------------------|---------------------|---------------------|---------------------|
| 2208.1 | 7 | + | + |
| 2469.3 | 5 | + | + |
| 2484.2 | 8 | + | + |
| 2497.3 | 9 | + | + |
| 2645.4 | 6 | + | + |
| 2673.4 | 10 | + | + |
| 2699.4 | 11 | + | + |
| 2865.6 | 2 | + | + |
| 2865.6 | 12 | + | + |
| 2881.6 | 3 | + | + |
| 3057.7 | 4 | + | + |

* The structure numbers refer to the structures in Figs. 3, 4, and 7.
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51. Olsthoorn, M. M., Haverkamp, J., and Thomas-Oates, J. E. (1999) J. Mass Spectrom. 34, 622–636
52. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
53. Whitfield, C., Richards, J. C., Perry, M. B., Clarke, B. R., and MacLean, L. L. (1991) J. Bacteriol. 173, 1420–1431
54. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
55. Heinrichs, D. E., Yethon, J. A., Amor, P. A., and Whitfield, C. (1998) J. Biol. Chem. 273, 29497–29505
56. Yethon, J. A., Heinrichs, D. E., Monteiro, M. A., Perry, M. B., and Whitfield, C. (1998) J. Biol. Chem. 273, 26310–26316
57. Wang, L., Briggs, C. E., Rothemund, D., Pratamico, P., Luchansky, J. B., and Reeves, P. R. (2001) Gene (Amst.) 270, 231–236
58. Fry, B. N., Korulik, V., ten Brinke, J. A., Pennings, M. T., Zalm, R., Teunis, B. J., Coloc, P. J., and van der Zeijst, B. A. (1998) Microbiology (Rond.) 144, 2049–2061