Involvement of waaY, waaQ, and waaP in the Modification of Escherichia coli Lipopolysaccharide and Their Role in the Formation of a Stable Outer Membrane*

(Received for publication, July 1, 1998)

Jeremy A. Yethon‡§, David E. Heinrichs‡§, Mario A. Monteiro¶, Malcolm B. Perry¶, and Chris Whitfield**

From the ‡Department of Microbiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada and the Institute for Biological Sciences, National Research Council, Ottawa, Ontario K1A OR6, Canada

The waaY, waaQ, and waaP genes are located in the central operon of the waa (formerly rfa) locus on the chromosome of Escherichia coli. This locus contains genes whose products are involved in the assembly of the core region of the lipopolysaccharide molecule. In the R1 core prototype strain, E. coli F470, there are nine genes in this operon, and all but waaY, waaQ, and waaP have been assigned function. In this study, the waaY, waaQ, and waaP genes were independently mutated by insertion of a non-polar antibiotic resistance cassette, and the structures of the resulting mutant core oligosaccharides were determined by chemical analyses and phosphorus-nuclear magnetic resonance spectroscopy. All three of these mutations were shown to affect the modification of the heptose region of the core, a region whose structure is critical to outer membrane stability. Mutation of waaY resulted in a core oligosaccharide devoid of phosphate on HepII. Mutation of waaQ resulted in loss of the branch HepIII residue on HepII and impeded the activity of WaaY. Mutation of waaP resulted in loss of phosphoryl substituents on HepI and modified WaaQ and WaaY activity. Only mutation of waaP resulted in hypersensitivity to novobiocin and sodium dodecyl sulfate, a characteristic of deep-rough mutations.

The outer membrane of Gram-negative bacteria is a barrier to many antibiotics and host defense factors (1). The outer leaflet of this membrane is composed almost exclusively of lipopolysaccharide (LPS), a unique glycolipid with structural features essential to outer membrane stability. In Escherichia coli and Salmonella enterica, the LPS molecule is conceptually divided into three distinct regions: 1) a hydrophobic membrane anchor designated lipid A; 2) a short chain of sugar residues with multiple phosphoryl substituents, referred to as the core oligosaccharide (core OS); and 3) a structurally diverse polymer composed of oligosaccharide repeats, termed the O antigen. Lipid A and the core OS are synthesized together as a single unit (lipid A-core, Fig. 1), which serves as an acceptor for preformed O antigen to yield the completed LPS molecule (2, 3).

Five distinct core OS structures have been identified in E. coli (core types K-12, R1, R2, R3, and R4), and two more are known for S. enterica (4, 5). The genes responsible for biosynthesis of the core OS in these bacteria are clustered on the chromosome in the waa (formerly rfa) locus near 81 min on the E. coli K-12 and S. enterica linkage maps. Mutations in many of the glycosyl transferases encoded by this locus result in the production of LPS lacking O antigen (termed rough or R-LPS) since O antigen cannot be ligated to an incomplete lipid A-core acceptor molecule. Strains that produce only R-LPS are more susceptible to complement-mediated serum killing than their wild-type counterparts (reviewed in Ref. 6). Mutations in the waa locus that specifically affect the phosphoryl substitution of the core OS heptose region (Fig. 1) can significantly alter outer membrane permeability, giving rise to a pleiotropic phenotype called deep-rough (7, 8). Characteristics of the deep-rough phenotype include the following: 1) hypersensitivity to detergents and hydrophobic antibiotics; 2) sloughing of LPS from the outer membrane; 3) leakage of periplasmic proteins into the culture medium, and 4) a marked decrease in the protein content of the outer membrane (reviewed in Ref. 9). The phosphoryl substituents in the heptose region are postulated to be so critical to outer membrane stability because their negative charge allows neighboring LPS molecules to be cross-linked by divalent cations (1, 10). Given its crucial role in outer membrane stability, the assembly of the heptose portion of the LPS molecule provides potential targets for novel therapeutic compounds. Development of such therapeutic strategies is limited, however, because the enzymology involved in the heptose region assembly is poorly understood.

Previous studies with S. enterica serovars Minnesota (11, 12) and Typhimurium (7) and with E. coli K-12 (13) have implicated waaP in the phosphorylation of both HepI and HepII (Fig. 1). Mutation of waaP in these organisms was also reported to cause characteristics of the deep-rough phenotype. Furthermore, in E. coli K-12, waaP has been implicated in the addition of HepIII (Fig. 1). Interpretation of these data, however, has been complicated by their reliance on strains with poorly defined or polar mutations. The goal of this study was to identify unequivocally the genes involved in modification of the inner core HepI and HepII residues and to determine the precise contribution of the corresponding gene products to the biosynthesis of the inner core heptose region.

The R1 core is the most prevalent among clinical isolates of
**FIG. 1.** Generalized structure of the lipid A-core portion of lipopolysaccharide common to *E. coli* and *S. enterica*, highlighting the hexose region. Core residues are designated by sugar abbreviations and names as follows: P, phosphate; Kdo, 3-deoxy-d-manno-oct-2-ulonic acid; Hep, 1,3-glycerol-d-manno-heptose; PPEtn, 2-aminoethyl diphasphate. *, structural differences due between the five *E. coli* and two *S. enterica* core types are due to differences between the HexII and HexIII sugars and the nature and positions of side-chain substitutitions of GlcI, HexII, and HexIII.

E. coli (14, 15), and since its structure is known (16) and the genetics of its outer portion have been resolved (see Fig. 2A), studies were performed using the prototype *E. coli* R1 strain, F470. A precise, non-polar insertion mutation was made in the *waaP* gene of *E. coli* F470 to unequivocally establish the role of its gene product in modification of the heptose region. Furthermore, additional functions originally assigned to the *waaP* gene product were shown to be encoded by two genes (*waaY* and *waaQ*) whose functions were previously unknown.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions—** *E. coli* F470 is an R-LPS derivative of an O8:K27 strain (18) and serves as the prototype for R1 core OS studies. Derivatives of F470 used in this study include CWG296 (F470 with a *waaP::aacC1* insertion), CWG2897 (F470 with a *waaQ::aacC1* insertion), and CWG312 (F470 with a *waaY::aacC1* insertion). Bacteria were grown in Luria-Bertani (LB) broth at 37 °C. Growth media were supplemented with ampicillin (100 μg/ml) when necessary. Gentamicin (15 μg/ml), chloramphenicol (30 μg/ml) when necessary.

**In Vitro Mutagenesis and Gene Replacement—** The *waaY*, *waaQ*, and *waaP* genes of the R1 core OS biosynthesis region were independently mutated by insertion of a non-polar gentamicin resistance cassette (the *aacC1* gene from Tn1696). Briefly, each of the genes (with some flanking DNA) was individually PCR amplified, cloned into the pBlueScript II SK(+) phagemid (Stratagene), and sequenced to ensure error-free amplification. The *aacC1* gene from plasmid pUCGM (19) was then inserted into an appropriate site (near the middle) of the coding region of each gene (Fig. 2B). The DNA fragment containing the insertionally inactivated gene was subsequently cloned into the suicide delivery vector pMAK705 (20), and chromosomal gene replacement was performed as described previously (21). Mutations were verified by PCR amplification of the mutated gene from the chromosome and confirmed by sequencing.

**DNA Methods—** Restriction endonuclease digestion and ligation were performed as described by Sambrook et al. (22). Restriction enzymes were purchased from Life Technologies, Inc., New England Biolabs, or Boehringer Mannheim. Plasmids were introduced into *E. coli* strain by electroporation using a Gene Pulser from Bio-Rad. Chromosomal DNA isolation was performed using the Qiagen genomic DNA isolation kit, and plasmid DNA was prepared using Qiaprep Miniprep spin columns (Qiagen Inc., Santa Clarita, CA). When necessary, DNA fragments were isolated from agarose gels using the GeneClean kit from Bio/Can Scientific (Mississauga, Ontario).

**PCR and Sequencing Techniques—** Oligonucleotides were synthesized using a Perkin-Elmer 394 DNA synthesizer, and sequencing was performed using an ABI 377 DNA sequencing apparatus (Perkin-Elmer) in the Guelph Molecular Supercentre at the University of Guelph. PCR amplification was performed using a GeneAmp PCR System 2400 from Perkin-Elmer. The Pwo DNA polymerase enzyme (Boehringer Mannheim) was used as recommended by the manufacturer.

**Cloning and Expression of waaP for Complementation—** Oligonucleotide primers for the amplification of *waaP* from the F470 chromosome were designed to introduce appropriate restriction sites for cloning. The forward primer (5’TGGTGGTCAATAGTGCGGCCTCA-3’) introduced a *BamH*I site (underlined) 3 base pairs downstream of the *waaP* stop codon, and the complementary reverse primer (5’GGGTGGTGCATATGGTTGACCTAAA-3’) introduced an *NdeI* site (underlined) overlapping the *waaP* start codon (bases shown in lowercase indicate mismatches between the primer and chromosomal sequences). The coding region for *waaP* alone was subsequently isolated as a *BamH*I-*NdeI* fragment and cloned behind the arabinose-inducible *P*~*ara*~ promoter of the expression vector pBAD18 (23) to generate plasmid pWQ909. L-Arabinose was used at a final concentration of 0.002% to induce expression of WaaP from plasmid pWQ909.

**Computer Analyses—** Homology searches of nucleotide and amino acid sequences in the National Center for Biotechnology Information database were performed using the BLAST (basic local alignment search tool) or PSI (position-specific iterated)-BLAST server analysis programs (24, 25). Fairwise nucleotide sequence alignments and percent identity scores were obtained using the NALIGN program of the PC/Gene software package (IntelliGenetics, Inc., Mountain View, CA) with an open gap cost of 25 and a unit gap cost of 5. Multiple protein alignments and percentage identity and similarity scores were obtained.
using the PC/Gen program CLUSTALW, with an open gap cost of 10 and a unit gap cost of 10. Routine DNA and protein sequence manipulations were performed using the MacVector and AssemblyLIGN software packages (International Biotechnologies, Inc., New Haven, CT).

**SDS and Novobiocin Sensitivity Testing—**Fold serial dilutions of SDS (200–0.1 mg/ml) and novobiocin (200–1.6 μg/ml) were made in 5-ml volumes of LB. Each series of tubes was point inoculated from an overnight culture of the strain to be tested and then incubated with shaking at 37 °C. Growth was scored as positive if after 8 h the culture was visibly turbid (i.e. OD₆₅₀ > 0.2).

**Preparation of Core OS—**Water-soluble LPS were obtained by the hot phenol/Water extraction of cell (26) and then treated with 2% acetic acid to 100 °C to leave the acid-labile ketosidic linkage between the core OS and lipid A. The water-insoluble lipid A was removed by centrifugation (5000 x g, 30 min), and the supernatant was passed through a column of BioGel P-2 (1 m x 1 cm) with water as eluent. The core-containing fractions were collected and lyophilized.

**Sugar Composition and Methylation Linkage Analyses—**Sugar composition analysis was performed by the alditol acetate method (27). Hydrolysis of glycosidic bonds was achieved with 4x trifluoroacetic acid at 100 °C for 4 h. The samples were then reduced in water with sodium borodeuteride and acetylated with acetic anhydride using residual sodium acetate as the catalyst. Characterization of the alditol acetate derivatives was performed by gas-liquid chromatography (GLC)-mass spectrometry using a Hewlett-Packard chromatograph equipped with a 30-m DB-17 capillary column (210 °C (30 min) to 240 °C at 2 °C/min). Mass spectrometry in the electron impact mode was recorded using a Varian Saturn II mass spectrometer. Linkage data were interpreted based upon the previously published structure of the R1 core (4), as described for analysis of the R1 outer core assembly.2 Methylation linkage analyses were carried out by the procedure of Ciucanu and Kerek (28). The permethylated alditol acetate derivatives were fully characterized by GLC-mass spectrometry in the electron impact mode using a column of DB-17 operated isothermally at 190 °C for 60 min.

**31P NMR Spectroscopy—**31P NMR spectra were recorded with a Bruker DRX 400 MHz instrument at 161.98 MHz with ortho-phosphoric acid as the external reference (0.0 ppm) and with pL = 30 in the proton-decoupling mode. Prior to performing the NMR experiments, the samples were lyophilized three times in H₂O (99.9%). The pH was adjusted with triethylamine when necessary.

**RESULTS**

**Structural Analysis of the Core OS from the R1 Prototype Strain F470—**The established structure of the F470 core OS (Fig. 2A) served as a framework for the interpretation of our results. Sugar composition analysis detected the presence of all the expected core OS sugars (glucose, galactose, and heptose; data not shown). Methylation linkage analysis detected the presence of all the core OS sugars found in F470 (data not shown). Linkage analysis of F470 resulted in a GLC chromatogram that was identical to that from the F470 parent (compare panels A and B in Fig. 3) except for the appearance of a peak (peak 9, Fig. 3B) corresponding to a 3,7-disubstituted heptose derivative (Table I). Based on the F470 structure, this derivative must result from unphosphorylated HepII.31P NMR spectroscopy further corroborated the loss of P from the F470 parent, there was more P than PPEtN, but in CWG312 there was slightly more PPEtN than P (compare panels E and F in Fig. 3). The observed shift in the ratio of P to PPEtN is explained by the loss of P from HepII, leaving only P and PPEtN on HepI. Therefore, the decrease in the appearance of P from HepII in the CWG312 core OS and the biologically significant sequence similarity of WaaY to some kinases, WaaY was concluded to be involved in the phosphorylation of HepII.

**Sequence Analysis and Mutagenesis of waaQ—**In E. coli K-12, R1, R2, and R4 and in S. enterica serovar Typhimurium, waaQ is the first gene of the central operon of the core OS biosynthesis region (Fig. 2B). Pairwise nucleotide alignments of the waaQ genes from these core types showed identities ranging from 68.5 to 98.6%. Multiple sequence alignment of the predicted WaaQ proteins reflected the homology observed at the nucleotide level with a total similarity of 88.1% (64.0% identity). The predicted WaaQ proteins showed limited homology to both WaaC (40.6% total similarity, 10.8% identity) and WaaF (50.1% total similarity, 16.5% identity), the HepI (30) and HepII (31, 32) transferases, respectively (Fig. 2A).

Strain CWG297 was derived by insertion of the aacCI gene into the unique NruI site in the waaQ coding region of F470 (Fig. 2B). The LPS from the resulting mutant migrated similarly to that of the F470 parent in SDS-polyacrylamide gel electrophoresis (data not shown), precluding any significant effect of the mutation on core OS extension. However, the minimum inhibitory concentrations of SDS and of novobiocin for CWG312 were slightly less than for F470 (Table II), suggesting subtle changes in outer membrane properties.

**Structural Analysis of the CWG312 Core OS and Assignment of WaaY Function—**The structure of the deduced CWG312 core OS is shown in Table I. Sugar composition analysis detected the presence of all the core OS sugars found in F470 (data not shown). Linkage analysis of CWG312 resulted in a GLC chromatogram that was identical to that from the F470 parent (compare panels A and B in Fig. 3) except for the appearance of a peak (peak 9, Fig. 3B) corresponding to a 3,7-disubstituted heptose derivative (Table I). Based on the F470 structure, this derivative must result from unphosphorylated HepII.31P NMR spectroscopy further corroborated the loss of P from the CWG312 core OS; in the F470 parent, there was more P than PPEtN, but in CWG312 there was slightly more PPEtN than P (compare panels E and F in Fig. 3). The observed shift in the ratio of P to PPEtN is explained by the loss of P from HepII, leaving only P and PPEtN on HepI. Therefore, the decrease in the appearance of P from HepII in the CWG312 core OS and the biologically significant sequence similarity of WaaY to some kinases, WaaY was concluded to be involved in the phosphorylation of HepII.

**Sequence Analysis and Mutagenesis of waaY—**Sequence analysis of the CWG312 core OS indicated that WaaY is the first gene of the central operon of the core OS biosynthesis region (Fig. 2B). Pairwise nucleotide alignments of the waaY genes from these core types showed identities ranging from 68.5 to 98.6%. Multiple sequence alignment of the predicted WaaY proteins reflected the homology observed at the nucleotide level with a total similarity of 88.1% (64.0% identity). The predicted WaaY proteins showed limited homology to both WaaC (40.6% total similarity, 10.8% identity) and WaaF (50.1% total similarity, 16.5% identity), the HepI (30) and HepII (31, 32) transferases, respectively (Fig. 2A).
Structural Analysis of the CWG297 Core OS and Assignment of WaaQ Function—Sugar composition analysis detected the presence of all the core OS sugars found in F470 (data not shown). The structure of the CWG297 core OS shown in Table I was deduced from methylation linkage analysis and 31P NMR spectroscopy as follows. First, methylation linkage analysis of the CWG297 core OS showed all of the outer core derivatives in the expected ratios (Table I), but the terminal heptose derivative (resulting from HepIII) was completely absent (note the absence of peak 6 in Fig. 3C). The disappearance of the HepIII side branch was also confirmed by FAB-MS (data not shown). Given the similarity of WaaQ to WaaC and WaaF (the HepI and HepII transferases, respectively; Fig. 2A) and the complete absence of HepIII in the CWG297 core OS, it was concluded that waaQ encodes the transferase for the branch HepIII residue. Interestingly, the appearance of a 3-linked heptose derivative in the linkage analysis of CWG297 (peak 8, Fig. 3C) also indicated the loss of a phosphoryl substituent from either HepI or HepII (both are 3-linked after the loss of HepIII) due to the waaQ mutation. To resolve which heptose residue was lacking, its phosphoryl substituent, the 31P NMR spectrum of the CWG297 core OS, was compared with the spectra from F470 and CWG312 (waaY). Given the similar P to PPEtN ratios for CWG297 and CWG312 (compare panels F and G in Fig. 3), it was concluded that the same phosphoryl substituent (P on HepII) was absent in both strains. If the waaQ mutation had affected phosphoryl substitution of HepII, the ratio of P relative to PPEtN would have increased rather than decreased. The observed loss of P from HepII in CWG297 did not contradict the assignment of WaaQ as the HepIII transferase since WaaY had already been assigned as the enzyme responsible for the phosphorylation of HepII (see above). Rather, the transfer of HepIII by WaaQ seems to be a prerequisite to the phosphorylation of HepII catalyzed by WaaY.

Sequence Analysis and Mutagenesis of waaP—In E. coli K-12, R1, R2, and R4 and in S. enterica serovar Typhimurium, waaP occurs as the third gene of the central operon of the core OS biosynthesis region (Fig. 2B). Pairwise nucleotide alignments indicated that the waaP gene from these core types was highly conserved (71.8–98.1% identity). Similarity at the protein level was even higher, with multiple sequence alignment of the predicted WaaP proteins showing a total similarity of 93.3% and an identity of 75.9%. In BLASTP searches of the available data bases, WaaP showed no significant similarity to...
Structures of the core OS from F470 and derivatives as deduced from linkage analyses

The peaks in the GLC chromatograms from the linkage analyses (Fig. 3, A–D) were correlated to the substituted sugar residues from which they were derived. Approximate molar ratios were calculated from the integrated peak areas. The published structure of the F470 core OS (Fig. 2A) was used as a framework for the interpretation of results as discussed in the text.

| Strain       | Peak Linkage | Approximate molar ratio | Core OS structure          |
|--------------|--------------|-------------------------|---------------------------|
| F470 (parent)| Glc- (1→1)  | 1.0                     | HepIII                    |
|              | Gal- (1→1)  | 1.0                     |                           |
|              | →3)-Glc- (1→1) | 0.7                   |                           |
|              | →2)-Gal- (1→1) | 1.3                 | GalI-1→2-GlcII-1→3-GlcI-1→3-HepII-1→3-HepI-1→ |
|              | Hep- (1→1)  | 0.6                     |                           |
|              | →2,3)-Glc- (1→1) | 1.0                |                           |

| Strain       | Peak Linkage | Approximate molar ratio | Core OS structure          |
|--------------|--------------|-------------------------|---------------------------|
| CWG312 (waaY)| Glc- (1→1)  | 0.9                     | HepIII                    |
|              | Gal- (1→1)  | 0.8                     |                           |
|              | →3)-Glc- (1→1) | 1.1                 |                           |
|              | →2)-Gal- (1→1) | 1.0                 | GalI-1→2-GlcII-1→3-GlcI-1→3-HepII-1→3-HepI-1→ |
|              | Hep- (1→1)  | 0.7                     |                           |
|              | →2,3)-Glc- (1→1) | 1.1                |                           |

| Strain       | Peak Linkage | Approximate molar ratio | Core OS structure          |
|--------------|--------------|-------------------------|---------------------------|
| CWG297 (waaQ)| Glc- (1→1)  | 0.9                     | HepIII                    |
|              | Gal- (1→1)  | 1.0                     |                           |
|              | →3)-Glc- (1→1) | 1.1                 |                           |
|              | →2)-Gal- (1→1) | 1.0                 |                           |
|              | →2,3)-Glc- (1→1) | 1.1                |                           |

| Strain       | Peak Linkage | Approximate molar ratio | Core OS structure          |
|--------------|--------------|-------------------------|---------------------------|
| CWG296 (waaP)| Glc- (1→1)  | 0.5                     | HepIII                    |
|              | Gal- (1→1)  | 0.9                     |                           |
|              | →2)-Glc- (1→1) | 0.7                 |                           |
|              | →3)-Glc- (1→1) | 1.0                 |                           |
|              | →2)-Gal- (1→1) | 1.1                 |                           |
|              | →2,3)-Glc- (1→1) | 0.6                | GalI-1→2-GlcII-1→3-GlcI-1→3-HepII-1→3-HepI-1→ |

* In CWG296, the β-Glc residue is only present in about 50% of the core OS.

### Minimum inhibitory concentration (MIC) of SDS and novobiocin for F470 and derivatives

| Strain       | MIC SDS (mg/ml) | MIC Novobiocin (µg/ml) |
|--------------|-----------------|------------------------|
| F470 (parent)| >200            | 200                    |
| CWG312 (waaY)| 200             | 100                    |
| CWG297 (waaQ)| 200             | 100                    |
| CWG296 (waaP)| 0.1             | 6.3                    |
| CWG296 (waaP) + pWQ909 | 200            | 100                    |

indicating that mutation of waaP resulted in the loss of all phosphoryl substituents from HepI and HepII. The GLC chromatograms from the CWG296 and F470 methylation linkage analyses were then compared to determine any other effects of the waaP mutation on the core OS structure. Of note, the terminal heptose derivative from HepIII was almost completely absent in the CWG296 core OS (note the absence of peak 6 in Fig. 3D). This lack of terminal heptose was also observed by FAB-MS (data not shown) and implicated the waaP mutation in the loss of HepIII in addition to the above-mentioned deficiency in phosphoryl substituents. The absence of phosphoryl substituents and of HepIII was further corroborated by the appearance of a large peak in the CWG296 GLC chromatogram (peak 8, Fig. 3D) corresponding to a 3-substituted heptose derivative from HepI and HepII. The calculated molar ratio of this derivative (Table I) was somewhat lower than expected, but the observed signal was clearly twice that from CWG297 (compare peak 8 in Fig. 3, C and D; see also Table I), as expected based on their predicted structures (the prolonged retention time of the 3-substituted heptose derivative on the GLC column may account for this slight discrepancy in molar ratios). Finally, the amounts of terminal glucose (from the β-Glc side branch) and of 2,3-disubstituted glucose (from GlcII) were noticeably decreased in CWG296 (compare peaks 1 and 7 in Fig. 3, A and D; see also Table I). The decrease in 2,3-disubstituted glucose was offset by the appearance of an approximately equal amount of 2-substituted glucose (note the appearance of peak 3, Fig. 3D), reflecting GlcII lacking the β-Glc substitution. In summary, the mutation of waaP resulted in a core OS, indicating that mutation of waaP resulted in the loss of all phosphoryl substituents from HepI and HepII. The GLC chromatograms from the CWG296 and F470 methylation linkage analyses were then compared to determine any other effects of the waaP mutation on the core OS structure. Of note, the terminal heptose derivative from HepIII was almost completely absent in the CWG296 core OS (note the absence of peak 6 in Fig. 3D). This lack of terminal heptose was also observed by FAB-MS (data not shown) and implicated the waaP mutation in the loss of HepIII in addition to the above-mentioned deficiency in phosphoryl substituents. The absence of phosphoryl substituents and of HepIII was further corroborated by the appearance of a large peak in the CWG296 GLC chromatogram (peak 8, Fig. 3D) corresponding to a 3-substituted heptose derivative from HepI and HepII. The calculated molar ratio of this derivative (Table I) was somewhat lower than expected, but the observed signal was clearly twice that from CWG297 (compare peak 8 in Fig. 3, C and D; see also Table I), as expected based on their predicted structures (the prolonged retention time of the 3-substituted heptose derivative on the GLC column may account for this slight discrepancy in molar ratios). Finally, the amounts of terminal glucose (from the β-Glc side branch) and of 2,3-disubstituted glucose (from GlcII) were noticeably decreased in CWG296 (compare peaks 1 and 7 in Fig. 3, A and D; see also Table I). The decrease in 2,3-disubstituted glucose was offset by the appearance of an approximately equal amount of 2-substituted glucose (note the appearance of peak 3, Fig. 3D), reflecting GlcII lacking the β-Glc substitution. In summary, the mutation of waaP resulted in a core OS,
which was devoid of all phosphoryl substituents and the branch HepIII residue and which seemed to contain a lower percentage of β-Glc substituted GlcII. As WaaY and WaaQ had already been assigned in this study as the enzymes responsible for the phosphorylation of HepII and the transfer of HepIII, respectively (see above), and because WaaV had been previously assigned as the β-Glc transferase (see Fig. 2A), it was concluded that the enzyme encoded by waaP was responsible for the phosphoryl substitution of HepI. This conclusion is supported by the sequence similarity of WaaP to known kinases, first reported in this study. The loss of other substituents and sugar residues from the CWG296 core OS does not contradict this assignment but suggests that the activity of WaaP is a prerequisite to the efficient functioning of other enzymes.

**DISCUSSION**

The data presented here identify WaaY as the enzyme that phosphorylates HepII, WaaQ as the transferase for the branch HepIII residue and WaaP as the enzyme responsible for the addition of either P or PEtN to HepI. With these assignments, a function has now been ascribed to every gene in the waaQ operon of the core OS biosynthesis region in the *E. coli* R1 prototype strain, F470 (see Fig. 2A). Moreover, the deduced structures of the core OS from the waaY, waaQ, and waaP mutants are consistent with a sequence of events in the decoration of the heptose region of the core OS where 1) WaaP adds a phosphoryl substituent to HepI, 2) WaaQ adds HepIII to HepII, and 3) WaaY adds P to HepII.

The conclusion that the activity of WaaP is a prerequisite to the functioning of both WaaQ and WaaY is based on the fact that the core OS of CWG296 (waaP) does not have HepIII or a P substituent on HepII (Table I), even though functional copies of the waaQ and waaY genes are present on the chromosome. Likewise, the activity of WaaQ is concluded to be required prior to the functioning of WaaY because the core OS of CWG297 (waaQ) does not have the P substituent on HepII (Table I), even though the waaY gene in this mutant is fully functional. These results can be explained by a fastidious substrate requirement in each sequential reaction, such that WaaQ can only effect the transfer of HepIII to a lipid A-core acceptor with P or PEtN arising from subsequent addition of P on HepI, and WaaY can only phosphorylate HepII if the lipid A-core acceptor has both HepIII and a phosphoryl substituent on HepI. Alternatively, these data could reflect a requirement for specific protein-protein interactions to provide a functional multi-enzyme complex.

Re-evaluation of previous structural studies in *E. coli* K-12 (13), in light of the data presented here, further confirms the assignment of WaaY and WaaP function. Parker et al. (13) created a polar mutation in *E. coli* K-12 that eliminated the function of all genes downstream of waaQ operon and then determined the core OS structures (by tandem mass spectrometry) of the resulting mutant and of the mutant complemented with both waaQ and waaP. As expected, the strain without a functional copy of the waaP gene had no phosphoryl substituents or branch HepIII residue in the heptose region. Complementation of the mutant with waaQ and waaP, however, resulted in a core OS with both HepIII and P or PEtN on HepI, but no P on HepII, again as expected given the absence of a functional waaY gene on the chromosome (due to the polarity of the mutation). Correct and complete interpretation of these results was not possible prior to the unequivocal gene assignments reported here.

The data presented here also provide an explanation for the multiple functions attributed to waaP in the literature. For example, the suggested involvement of WaaP in the transfer of HepIII in *E. coli* K-12 (13) has now been resolved. WaaQ is the HepIII transferase but requires the prior functioning of WaaP.
Role of waaY, waaQ, and waaP in E. coli LPS Assembly

11. Drige, W., Ruschmann, E., Lüderitz, O., and Westphal, O. (1968) Eur. J. Biochem. 4, 134–138
12. Mühldorf, P., Risoe, H. J., Lüderitz, O., and Westphal, O. (1968) Eur. J. Biochem. 4, 139–145
13. Parker, C. T., Kloer, A. W., Schnaitman, C. A., Stein, M. A., Gottesman, S., and Gibson, B. W. (1992) J. Bacteriol. 174, 2525–2538
14. Appelmelk, B. J., An, Y.-Q., Hekker, T. A. M., Thijss, L. G., MacLaren, D. M., and de Graaf, J. (1994) Microbiology 140, 1119–1124
15. Gibbs, A. P., Barclay, G. R., Poxton, I. R., and di Padova, F. (1992) J. Infect. Dis. 166, 1051–1057
16. Jansson, P.-E., Lindberg, A. A., Lindberg, B., and Wallin, R. (1981) Eur. J. Biochem. 115, 571–577
17. Hasin, M., and Kennedy, E. P. (1982) J. Biol. Chem. 257, 12475–12477
18. Schmidt, G., Jann, B., and Jann, K. (1969) Eur. J. Biochem. 10, 501–510
19. Schweizer, H. P. (1993) BioTechniques 15, 831–833
20. Hamilton, C. M., Aldea, M., Washburn, B. K., Babtizke, P., and Kushner, S. R. (1989) J. Bacteriol. 171, 4617–4622
21. Amor, P. A., and Whitfield, C. (1997) Mol. Microbiol. 26, 145–161
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
23. Guzman, L.-M., Belin, D., Carson, M. J., and Beckwith, J. (1995) J. Bacteriol. 177, 4121–4130
24. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
25. 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
26. Westphal, O., and Jann, K. (1965) Methods Carbohydr. Chem. 5, 83–91
27. Sawardeker, J. H., Stoneker, J. H., and Jeanes, A. (1967) Anal. Chem. 39, 1602–1604
28. Ciucanu, I., and Kerek, F. (1984) Carbohydr. Res. 131, 209–217
29. Helander, I. M., Kilpeläinen, I., and Vaara, M. (1981) Mol. Microbiol. 11, 481–487
30. Kadmos, J. L., and Raetz, C. R. H. (1998) J. Biol. Chem. 273, 2799–2807
31. Sirisena, D. M., MacLachlan, P. R., Liu, S.-L., Hessel, A., and Sanderson, K. E. (1994) J. Bacteriol. 176, 2379–2385
32. Braebetz, W., Müller-Loennies, S., Holst, O., and Brade, H. (1997) Eur. J. Biochem. 247, 716–724
33. Parker, C. T., Pradel, E., and Schnaitman, C. A. (1992) J. Bacteriol. 174, 930–934
34. Süsskind, M., Müller-Loennies, S., Nimmich, W., Brade, H., and Holst, O. (1995) Carbohydr. Res. 269, C1–C7
35. Severn, W. B., Kelly, R. F., Richards, J. C., and Whitfield, C. (1996) J. Bacteriol. 178, 1731–1741