Structural Analysis of Oligosaccharides from Lipopolysaccharide (LPS) of Escherichia coli K12 Strain W3100 Reveals a Link between Inner and Outer Core LPS Biosynthesis

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Lipopolysaccharide (LPS) from Escherichia coli K12 W3100 is known to contain several glycoforms, and the basic structure has been investigated previously by methylation analyses (Holst, O. (1989) in Endotoxin in Health and Disease (Brade, H., Opal, S. M., Vogel, S. N., and Morrison, D., eds) pp. 115–154; Marcel Dekker, Inc., New York). In order to reveal dependences of gene activity and LPS structure, we have now determined the composition of de-O-acylated LPS by electrospray ionization-Fourier transform ion cyclotron-mass spectrometry (ESI-FT-MS) and identified 11 different LPS molecules. We have isolated the major glycoforms after de-O- and de-N-acylation and obtained four oligosaccharides that differed in their carbohydrate structure and phosphate substitution. The main oligosaccharide accounted for ~70% of the total and had a molecular mass of 2516 Da according to ESI-FT-MS. The dodecasaccharide structure (glycoform I) as determined by NMR was consistent with MS and compositional analysis. One minor oligosaccharide (5%) of the same carbohydrate structure did not contain the 4-phosphate of the lipid A. Two oligosaccharides contained the same phosphate substitution but differed in their carbohydrate structure, one (5%) which contained an additional β-D-GlcN in 1→7 linkage on a terminal heptose residue (glycoform II) which was N-acetylated in LPS. A minor amount of a molecule lacking the terminal 1-α-D-Hep in the outer core but otherwise identical to the major oligosaccharide (glycoform III) could only be identified by ESI-FT-MS of the de-O-acylated LPS. The other oligosaccharide (20%) contained an α-Kdo-(2→4)-[α-L-Rha-(1→5)]-α-Kdo-(2→4)-α-Kdo branched tetrasaccharide connected to the lipid A (glycoform IV). This novel inner core structure was accompanied by a truncation of the outer core in which the terminal disaccharide 1-α-D-Hep-(1→4)-α-D-Glc was missing. The latter structure was identified for the first time in LPS and revealed that changes in the inner core structure may be accompanied by structural changes in the outer core.

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†The abbreviations used are: LPS, lipopolysaccharide; 3-OH-C14, 3-hydroxytetradecanoic acid; DQF-COSY, double quantum-filtered correlation spectroscopy; ESI-FT-ICR-MS, electrospray ionization-Fourier transform ion cyclotron-mass spectrometry; Hep, L-glycerol-α-D-manno-heptopyranose; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum correlation; HPACEC, high-performance anion exchange chromatography; IRMPD, infrared-multiphoton dissociation; Kdo, 3-deoxy-L-manno-oct-2-ulopyranosonic acid; NOE, nuclear Overhauser enhancement; Rha, rhamnopyranose; TOCSY, total correlation spectroscopy; Tricine, N-(2-hydroxy-1,1-bis(2-hydroxymethyl)ethyl)glycine.
LPS consists of several different molecular species, and early studies indicated the presence of structurally heterogeneous LPS in *E. coli* K12 (13). *E. coli* K12 strains are widely used as hosts in molecular biology and were therefore the first used to elucidate the genetic details of LPS biosynthesis (14–22). Although many details of LPS biosynthesis in *E. coli* K12 are known, there still remain open questions. During the course of this study, Frirdich et al. (23) raised the question whether the structure of the inner core in LPS has an influence on the biosynthesis of the outer core. Overexpression of the gene product of the *waaZ* gene performed in their study led to increased amounts of an /H9251-Kdo-(2\_3\_4)-/H9251-Kdo-(2\_3\_4)-/H9251-Kdo trisaccharide in the inner core that was accompanied by a truncation of the outer core. At this stage, it remained unanswered whether the gene *waaZ* encoded a Kdo transferase that specifically transferred the third Kdo residue onto the common /H9251-(2\_3\_4)-Kdo-disaccharide inner core structure, which is known to be generated by a single bifunctional enzyme (24). It also remained an open question whether the truncation of the outer core was secondary because of the overexpression of the WaaZ gene product. The gene responsible for the transfer of rhamnose to the inner core of *E. coli* K12 has yet to be identified.

We have developed methods to purify the different glycoforms present in LPS (9) and for their conjugation to protein carrier molecules allowing their use in serological studies (25, 26). The advantage of the developed procedures is the fact that they allow the determination of the relative distribution of the various glycoforms and allow the study of their immunoreactivities.

The complete carbohydrate chains of *E. coli* R1 to R4 LPS have been investigated (27, 28); however, such data for *E. coli* K12 LPS are lacking. We have therefore now extended our studies to *E. coli* K12 W3100 LPS and show that it contains two major and two minor glycoforms that are subject to further modification by phosphorylation. Whereas methylation analyses were able to identify the main carbohydrate structure of different parts of the LPS molecule previously and to identify structural modifications (11, 12, 29), the applied methods failed to reveal how far non-stoichiometrically found substitutions were connected to each other. However, as seen for the *E. coli* J-5 mutant, from a biosynthetic point of view this information can be of importance in order to design experiments and understand the regulation of genes and enzymes involved in the biosynthesis of LPS and the factors influencing the function of the outer membrane. We now show that *E. coli* K12 W3100 LPS apart from the known structures contains a so far unknown LPS. The analysis of this structure proves a connection between the biosynthesis of the inner and outer core of LPS and additionally provides a basis for the cloning of the rhamnosyltransferase involved in *E. coli* K12 LPS assembly.

### Table I

| Component \(a\) | mmol/mg | Molar ratio \(b\) |
|----------------|---------|------------------|
| GlcN           | 396     | 2.1              |
| Kdo\(_{\text{AcP}}\) | 218     | 1.2              |
| Kdo\(_{\text{HCl}}\) | 352     | 1.9              |
| P\(_{\text{Ac}}\) | 961     | 4.1              |
| Glc            | 529     | 2.8              |
| Gal            | 188     | 1.0              |
| 1,2-Hep        | 774     | 4.1              |
| C12:0          | 145     | 0.8              |
| C14:0          | 146     | 0.8              |
| 3OH-C14:0      | 595     | 3.2              |

\(a\) Kdo\(_{\text{AcP}}\): Kdo determination after hydrolysis in acetate buffer, pH 4.5; Kdo\(_{\text{HCl}}\): Kdo determination after hydrolysis in 0.1 M HCl; 1,2-Hep, l-glycero-d-manno-heptose.

\(b\) Molar ratios calculated relative to Gal = 1.0.
EXPERIMENTAL PROCEDURES

**Bacteria and Bacterial LPS—**E. coli K12 strain W3100 was cultivated and used for the isolation of LPS by phenol/chloroform/petrol ether extraction as reported (30).

**Analytical Methods—**Neutral sugars, fatty acids, GlcN, Kdo, and bound organic phosphate were determined as described (31).

**Preparation of Deacylated LPS of E. coli K12 W3100—**LPS (1.09 g) was de-O-acylated by mild hydrazinolysis (32) (yield 797 mg) and subjected to alkaline de-N-acylation as described (33). After neutralization by addition of 4 M HCl, desalting by gel filtration on Sephadex G-10 in 10 mM NH₄HCO₃, and lyophilization (yield 366.6 mg), 180 mg of the deacylated oligosaccharide fraction was subjected to high performance anion-exchange chromatography (HPAEC; 9 runs of 20 mg each) using a semi-preparative CarboPak PA100 column (9 × 250 mm) and a DX300 chromatography system (Dionex, Germany). Conditions for semi-preparative and analytical HPAEC were essentially as described.

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**Fig. 2.** Charge deconvoluted ESI-FT-MS spectrum in the negative ion mode of de-O-acylated LPS (A) and of deacylated oligosaccharides (C) from E. coli K12 W3100 LPS. The molecular ions are designated Mₓ to Mₓ, in A, and the mass of M₁, was only poorly resolved from Mₓ, and is therefore shown in the expansion where the region at m/z 1030 of triple charged ions in the not charge-deconvoluted spectrum is shown (B). The corresponding molecular ions of oligosaccharides obtained after de-N-acylation (C) were numbered the same as in A but designated with Mₓ*. The mass numbers given are those of the monoisotopic peaks. Unannotated clusters of signals originated from molecular ions containing varying amounts of Na⁺ and K⁺ adduct ions. For the assignment of the structures see Table II, Fig. 9, and "Results."
previously (9). Briefly, for semipreparative chromatography a gradient of 1–50% B in 60 min with eluents (A) H2O and (B) 1M NaOAc was used. Aliquots of fractions (3 mL) were spotted on silica TLC plates and charred with ethanolic sulfuric acid for detection. Two main fractions (fraction 3, yield 30.2 mg, oligosaccharide (OS) 1; fraction 4, yield 13.92 mg, OS 4) and two minor fractions (fraction 1, yield 4.6 mg, OS 2; fraction 2, yield 6.8 mg, OS 3) were collected, neutralized, and desalted by gel filtration on Sephadex G-10 as described. Analytical HPAEC (Fig. 1) was carried out on a column of CarboPak PA1 (4 × 1100 mm, Dionex) using the eluents (eluent A) H2O and (eluent B) 1M NaOAc and a gradient from 1 to 99% of eluent B in 80 min. The run was monitored by pulsed amperometric detection after post-column addition of NaOH.

**Electrospray Ionization-Fourier Transform Ion Cyclotron-Mass Spectrometry (ESI-FT-ICR MS)**—ESI-FT-ICR MS was performed in the negative and positive ion mode using an APEX II Instrument (Bruker Daltonics, Billerica, MA) equipped with a 7 tesla actively shielded magnet and an Apollo ion source. Mass spectra were acquired using standard experimental sequences as provided by the manufacturer. Samples were dissolved at a concentration of ~10 ng μl⁻¹ in a 50:50:

| Glycoform | Composition | M_calculated | M_observed | Molecule |
|-----------|-------------|--------------|------------|----------|
| II        | GlcNAc,Gal,Glc,Hep,Kdo,GlcNAcyl, P₂ | 3092.09 | 3092.11 | M₃ |
|           | GlcNAc,Gal,Glc,Hep,Kdo,GlcNAcyl, P₂ | 3172.07 | 3172.09 | M₄ |
| I         | Gal,Glc,Hep,Kdo,GlcNAcy, P₂ | 2889.02 | 2889.04 | M₅ |
|           | Gal,Glc,Hep,Kdo,GlcNAcy, P₂ | 2968.99 | 2968.00 | M₆ |
| III       | Gal,Glc,Hep,Kdo,GlcNAcy, P₂ | 2901.02 | 2901.04 | M₇ |
| IV        | Gal,Glc,Hep,Kdo,GlcNAcy, P₂ | 2980.99 | 2981.01 | M₈ |

**Glycoform Composition**

**M_calculated**

| Molecule | M_observed | M_calculated |
|----------|------------|--------------|
| M₃       | 3092.09 | 3092.11 |
| M₄       | 3172.07 | 3172.09 |
| M₅       | 2889.02 | 2889.04 |
| M₆       | 2968.99 | 2968.00 |
| M₇       | 2901.02 | 2901.04 |
| M₈       | 2980.99 | 2981.01 |

**Fig. 3. 600-MHz ¹H NMR spectrum and chemical structure of deacylated OS 1.** In the high field region of the spectrum 2 pairs of signals were observed originating from axial (3α) and equatorial (3e) 3-deoxy protons with chemical shifts characteristic of α-Kdo residues (residues C and D). In addition, 10 signals of anomeric protons were present (labeled as indicated in the structure). Kdo is 3-deoxy-α-D-manno-oct-2-ulospyranosonic acid; L-α-D-Hep is L-glycero-β-D-manno-heptopyranose; Gal is galactopyranose; Glc is glucopyranose; and GlcN is 2-amino-2-deoxyglucopyranose.

The number and identity of hexoses were determined by NMR.

**TABLE II**

Results of ESI-FT-MS of de-O-acylated *E. coli* K12 W3100 LPS

Rha, rhamnose; Gal, galactose; Glc, glucose; GlcNAc, N-acetylgulcosamine; Hep, L-glycero-α-D-manno-heptose; Kdo, 3-deoxy-α-D-manno-oct-2-ulospyranosonic acid; EtN, 2-aminoethanol. GlcNacyl refers to GlcN carrying a 3-OH-C14 in amide linkage (represents the lipid A). The number and identity of hexoses were determined by NMR.

| Glycoform | Composition | M_calculated | M_observed | Molecule |
|-----------|-------------|--------------|------------|----------|
| II        | GlcNAc,Gal,Glc,Hep,Kdo,GlcNAcyl, P₂ | 3092.09 | 3092.11 | M₃ |
|           | GlcNAc,Gal,Glc,Hep,Kdo,GlcNAcyl, P₂ | 3172.07 | 3172.09 | M₄ |
| I         | Gal,Glc,Hep,Kdo,GlcNAcy, P₂ | 2889.02 | 2889.04 | M₅ |
|           | Gal,Glc,Hep,Kdo,GlcNAcy, P₂ | 2968.99 | 2968.00 | M₆ |
| III       | Gal,Glc,Hep,Kdo,GlcNAcy, P₂ | 2901.02 | 2901.04 | M₇ |
| IV        | Gal,Glc,Hep,Kdo,GlcNAcy, P₂ | 2980.99 | 2981.01 | M₈ |

**Glycoform Composition**

| Molecule | M_calculated | M_observed | M_calculated |
|----------|--------------|------------|--------------|
| M₃       | 3092.09 | 3092.11 | 3172.07 | 3172.09 |
| M₄       | 2889.02 | 2889.04 | 2968.99 | 2968.00 |
| M₅       | 2901.02 | 2901.04 | 2980.99 | 2981.01 |

**Structural Analysis of *E. coli* K12 LPS Oligosaccharides**

Previously (9), briefly, for semipreparative chromatography a gradient of 1–50% B in 60 min with eluents (A) H₂O and (B) 1 M NaOAc was used. Aliquots of fractions (3 μl) were spotted on silica TLC plates and charred with ethanolic sulfuric acid for detection. Two main fractions (fraction 3, yield 30.2 mg, oligosaccharide (OS) 1; fraction 4, yield 13.92 mg, OS 4) and two minor fractions (fraction 1, yield 4.6 mg, OS 2; fraction 2, yield 6.8 mg, OS 3) were collected, neutralized, and desalted by gel filtration on Sephadex G-10 as described. Analytical HPAEC (Fig. 1) was carried out on a column of CarboPak PA1 (4 × 250 mm, Dionex) using the eluents (eluent A) H₂O and (eluent B) 1 M NaOAc and a gradient from 1 to 99% of eluent B in 80 min. The run was monitored by pulsed amperometric detection after post-column addition of NaOH.

**NMR Spectroscopy**—NMR spectra were recorded of samples of OS 1 (15 mg), OS 2 (4.6 mg), OS 3 (6.8 mg), and OS 4 (13.9 mg) in 0.5-mL solutions in D₂O using a Bruker DRX 600 Avance spectrometer equipped with a multinuclear probe head with z-gradient. Chemical shift values were determined in reference to acetone 2.225 (1H) and 31.5 ppm (13C). All spectra were recorded at a temperature of 300 K using standard Bruker pulse programs. Sodium deutero-oxide was added to the samples prior to one- and two-dimensional ³¹P NMR spectroscopy to achieve uniform signals as described (34).

**Electrospray Ionization-Fourier Transform Ion Cyclotron-Mass Spectrometry (ESI-FT-ICR MS)**—ESI-FT-ICR MS was performed in the negative and positive ion mode using an APEX II Instrument (Bruker Daltonics, Billerica, MA) equipped with a 7 tesla actively shielded magnet and an Apollo ion source. Mass spectra were acquired using standard experimental sequences as provided by the manufacturer.
Structural Analysis of E. coli K12 LPS Oligosaccharides

RESULTS

Compositional analysis of PCP-extracted LPS from E. coli K12 (Table I) revealed the presence of Rha, Glc, Gal, Hep, Kdo, GlcN, and phosphate in a molar ratio of 0.2:2.8:1.0:4.1:1.9:2.1:5.1. It has been shown previously that all sugars are present in the β-configuration apart from α-Rha and that all sugars are present as pyranoses (4). Fatty acid analysis by gas liquid chromatography also identified the presence of 3-OH-C14:0, tetradecanoic acid, and dodecanoic acid fatty acids indicating that the acylation pattern was the same as reported for other strains of E. coli (35). We have prepared de-O-acylated LPS by mild hydrazinolysis a part of which was further de-N-acylated under strong alkaline conditions (Fig. 1). The mixture of deacylated LPS oligosaccharides was then subjected to HPAEC and four oligosaccharides (OS 1 to OS 4) were obtained. ESI-FT-MS (Fig. 2A) of the de-O-acylated LPS revealed the presence of 11 different LPS structures that had a composition as indicated in Table II. The major molecule had a mass of 2969 Da (M1) which was consistent with a composition of four hexoses, four Hep, two Kdo, two GlcN, four phosphates, and two amide-linked 3-OH-C14:0 fatty acids (glycoform I). After deacylation NMR analysis of this oligosaccharide (OS 1) identified as hexoses one Gal and three Glc residues (see below). An additional molecular ion was observed which contained an additional GlcNAc (glycoform II, 3172 Da; M12), and one molecular species was devoid of one Hep residue (glycoform III, 2777 Da; M10). Another major component was identified that had a mass of 2981 Da, indicating a composition of three hexoses, three Hep, three Kdo, two GlcN, one Rha, four phosphates, and two amide-linked 3-OH-C14 fatty acids (glycoform IV; M11). NMR analysis of the oligosaccharide (OS 4) after deacylation revealed that this molecule contained one Gal and only two Glc as hexoses.

Molecular ions M1, M4, M10, and M11 showed that glycoforms I and II were also present as penta- and trisphosphorylated LPS (Table II). The molecular ion with 3092 Da could have been also due to the presence of an additional 2-aminoethanol substitution in the pentaphosphorylated glycoform I (M11). The detailed investigation of the cluster of triple charged ions in the non-charge deconvoluted ESI-FT-MS spectrum around 1030 Da (Fig. 2B) revealed that both molecules were present (M1 and M11). A triple charged ion of higher intensity was observed with 1029.696 Da which originated from a monoisotopic molecule with a mass of 3091.99 Da (M4) containing 2-aminoethanol. The signal of very low intensity at 1029.696 Da corresponded to a trisphosphorylated glycoform I LPS molecule with a mass of 3092.11 Da (M4). Very small amounts of glycoforms III and IV were identified that contained only three phosphates (2697 Da, M9; 2901 Da, M10). Thus, ESI-FT-MS indicated the presence of four different glycoforms I–IV, which differed in their substitution with GlcNAc, Hep, and Rha. In addition, heterogeneity was observed for the phosphate substitution. Although the majority of all glycoforms contained four phosphates, all of them were also present with three phosphates. Only small amounts of glycoforms I and II contained five phosphates, and a very small amount of a pentaphosphorylated glycoform I LPS contained 2-aminoethanol.

Complete deacylation of LPS and ESI-FT-MS of the mixture of oligosaccharides (Fig. 2C) detected molecular ions with masses of 2436, 2448, 2516, 2528, 2596, and 2677 Da. The separation of the mixture by analytical HPAEC (Fig. 1) revealed the presence of two major (OS 1 and OS 4) and two minor (OS 2 and OS 3) oligosaccharides that were separated by semipreparative HPAEC and further analyzed by ESI-FT-MS and NMR.

Structural Analysis of OS 1 and OS 2—ESI-FT-MS analysis of the mixture of deacylated oligosaccharides (Fig. 2C) and of the isolated OS 1 (not shown) revealed a molecular mass of 2516.67 Da that was in agreement with a chemical composition of four hexoses, four Hep, two GlcN, two Kdo, and four phosphates (M_theoretical = 2516.69 Da). The one-dimensional 1H NMR spectrum (Fig. 3) contained 10 signals of anomic protons of carbohydrates and two pairs of signals of methylene groups belonging to two Kdo residues and identified this oligosaccharide as a dodecasaccharide. Furthermore, 31P NMR confirmed the presence of four phosphates (Fig. 4). The full assignment (Tables III and IV) of NMR signals by 1H,1H-DQF-COSY, TOCSY, and 31C,1H-heteronuclear correlation spectra such as HMQC, HMQC-TOCSY, and HMBC identified two GlcN residues, one in α-configuration (residue A, 3J_H-H_1 = 3 Hz) and
the other in β-configuration (residue B). NOE spectroscopy revealed that these were β-(1→6) connected and according to $^{31}P$,$^1H$-HMQC phosphorylated at positions 1 and 4'. Furthermore, HMBC revealed that one Kdo (residue C) was substituting position 6 of the β-GlcN. Thus, the GlcN-disaccharide represented the lipid A backbone. An NOE between H-6 of the second Kdo and H-3ax (weak) and H-3eq (strong) of Kdo C revealed that these were α-(2→4) connected (36). Three Hep residues (E, F, and H) were identified and NOEs between the anomeric proton of E and protons H-6 and H-7a/b of F in addition to an NOE between the anomeric proton of F and H-3 of E proved that they formed the Hep-(1→7)-Hep-(1→3)-Hep trisaccharide (residues H, F, and E, respectively) commonly found in enterobacterial LPS (4). The structure was confirmed by HMBC correlation signals between the anomeric protons of H and C-7 of F, and of F and C-3 of E, respectively. This trisaccharide substituted the inner Kdo (residue C) in position 5 leading to a NOE between the anomeric proton of E and protons H-5 and -7 of Kdo C. According to NOE data and HMBC three α-Glc residues (K, I, and G) formed the trisaccharide Glc-(1→2)-Glc-(1→3)-Glc which was connected to position 3 of Hep F. These residues thus belonged to the outer core (4). Additionally, the $^3J_{H-1,H-2}$ coupling constant of 4 Hz and the small $^3J_{H-3,H-4}$ (4 Hz) and $^3J_{H-4,H-5}$ (< 3 Hz) coupling constants of residue M identified it as a α-Gal residue that was bound to position 6 of Glc G (HMBC and NOE, low field chemical shift of C-6, and protons H-6a, H-5, and high field shift of H-6b). The fourth Hep (L) was connected to position 6 of Glc K. The configurations at the anemic centers of all residues apart from Kdo were confirmed by a non-decoupled $^1H$,$^1^3^C$-HMQCC spectrum (>170 Hz for α and <168 Hz for β; Fig. 5). The two remaining phosphates were located at position 4 of both Hep
residues E and F (Fig. 4 and Table V) because the $^{31}$P resonances gave correlation signals to the respective protons in HMQC, and the cross-correlation signals of these protons in DQF-COSY showed an additional coupling of $-10$ Hz due to $J_{P,H}$-coupling (9). Thus, OS 1 possessed the chemical structure as depicted in Fig. 3 and represented glycoform I.

The mass spectrometric analysis of OS 2 (not shown) revealed a difference of 80 Da lower mass in comparison to OS 1, indicating the loss of one phosphate (mass = 2436 Da). This was corroborated by the presence of only three phosphorus resonances in $^{31}$P NMR spectroscopy (Fig. 4). The changes of the chemical shifts of protons H-3, -4, and -5 (Table III) and of carbons C-4 and C-5 (Table IV) of the $\beta$-GlCN indicated that the 4'-position was not phosphorylated. This was finally proven by $^1$H,$^3$P-HMQC spectroscopy and IRMPD-ESI-FT-ICR MS. Apart from these chemical shift differences, the same carbohydrate composition and linkages were identified by NMR as in OS 1 which thus were identical in their carbohydrate structure (compare Fig. 3, glycoform I).

**Structural Analysis of OS 3**—The ESI-FT-MS of decylated LPS oligosaccharides (Fig. 2C) and the isolated OS 3 (not shown) revealed a molecular mass 161 Da higher than OS 1 indicating the presence of an additional hexosamine residue. The only amino sugar identified by compositional analysis was GlcN. Accordingly, the $^1$H NMR spectrum (Fig. 6) contained additional resonances (Tables III and IV) in comparison to OS 1 belonging to a GlcN (residue L). The $^3$J$_{H1,H2}$ coupling constant of 8.5 Hz and NOE connectivities between H-1, -3, and -5 revealed that it possessed $\beta$-configuration, and the far downfield shift of C-7 and H-6 of L proved that it was connected to position 7 of Hep L (glycoform II). $^{31}$P NMR identified four phosphates (Fig. 4) at identical substitution sites as in OS 1.
The corresponding molecular ion of the N-acylated sample in ESI-FT-MS had a molecular mass of 3172.09 Da (Fig. 2A) and thus showed that this residue was N-acetylated in LPS. There was no molecular ion present that had an unsubstituted amino group at this position.

**Structural Analysis of OS 4**

The one-dimensional 1H NMR spectrum of OS 4 (Fig. 7) contained three pairs of signals of CH2 groups belonging to three Kdo residues (C, D, and P). In addition, the strong resonance of a methyl group was present at 1.2 ppm, indicating the presence of a 6-deoxy sugar. In the low field region of the spectrum four signals of anomeric protons were identified which had a small 3J_H1,H2-coupling constant of less than 1 Hz and thus had an equatorial proton in position 2.

The assignment of the spin systems revealed that only three of these belonged to Hep residues (E, F, and H) and one belonged to an α-Rha (J_C1,H1 = 171 Hz; E). Further signals of anomeric protons belonged to two α-Glc (G, and I) and one residue each of α-Gal (M), α-GlcN IP (A) and β-GlcN 4P (B). Those residues that were also present in OS 1 formed the same primary structure according to chemical shift analysis, NOESY and HMBC spectra. However, the terminal Glc (K) and Hep (L) that were present in OS 1, OS 2, and OS 3 were missing in this oligosaccharide. The chemical shifts of carbons 4 and 5 of Kdo D (Table IV) showed that these positions were substituted, and because the anomeric proton of the Rha (R) gave a strong NOE to proton 5 of Kdo D they were connected in 1→5-linkage and the additional Kdo (P) was located at position 4 of D. Thus, the side chain Kdo residue D was a branching point in this oligosaccharide and carried, in addition to a third Kdo (P) in position 4, a Rha attached to position 5.

13P NMR (Fig. 4) spectroscopy revealed the presence of four phosphate residues that according to 1H,31P-HMQC were located at the same positions as in OS 1, i.e. the anomeric position of GlcN (A), the 4′ position of the β-GlcN (B), and the 4-positions of the two heptoses E and F.

The ESI-FT-MS spectrum (Fig. 8A) of OS 4 showed a signal of 2529.61 Da, in full agreement with the structure determined by NMR. Furthermore, IRMPD-MS of the parent ion of m/z 2529.61 Da (Fig. 8B) showed fragmentational loss of the terminal residue P (Kdo, 220 Da, mass of 2308.54 Da) and also of residue R (Rha, −142 Da, mass of 2162.48 Da). This unequivocally proved the middle Kdo as attachment site for Rha and Kdo. Further fragmentations gave rise to ions with masses of 1942.42 Da (− Kdo D) and 1442.34 Da (− GlcN2P2, −500 Da, lipid A backbone). The latter fragmentation was also observed directly after the loss of residues P and R which gave rise to an ion of 1662.39 Da. OS 4 thus possessed the chemical structure depicted in Fig. 7 and represented glycoform IV.

**DISCUSSION**

The investigation of deacylated LPS from *E. coli* K12 W3100 by ESI-FT-MS (Fig. 2) indicated the presence of 11 molecular species in this LPS. The presence of four different glycoforms was established, and these differed in their oligosaccharide
structures (Fig. 9). All glycoforms were heterogeneous with respect to their phosphate and 2-aminoethanol substitution. The main oligosaccharide accounting for 70% of the LPS (as determined by HPAEC of deacylated oligosaccharides, Fig. 1) was a tetraphosphorylated dodecasaccharide of the structure depicted in Fig. 9 (glycoform I). A minor component had the same carbohydrate structure but did not contain the 4'-phosphate on the lipid A backbone. This position was only partially substituted with phosphate in all different glycoforms as shown by ESI-FT-MS on the de-O-acylated sample prior to alkali treatment and therefore was not the result of an elimination of 2-aminoethanol monophosphate under the strong alkaline conditions applied for the N-deacylation reaction. One molecular ion indicated the substitution with 2-aminoethanol monophosphate of the main oligosaccharide. This substitution has been identified in several enterobacterial LPS in lipid A and on the 4'-position of the first Hep residue as 2-aminoethanol diphosphate or the 7-position of Kdo as 2-aminoethanol monophosphate (Kdo-PE (4)). A molecular ion of 3092.01 Da (Fig. 2A, M6) was consistent with such a substitution of the main oligosaccharide (Mcalculated 3091.99 Da); however, the same ion could arise from the presence of a molecule containing GlcNAc devoid of phosphate at the 4'-position (Mcalculated 3092.09 Da), and the lack of phosphate at this position was found for all other glycoforms. Investigation of the expanded not charge-deconvoluted ESI-FT-MS spectrum in the region of triple charged ions (Fig. 2B) showed the presence of both LPS species. We have isolated previously Kdo substituted in position 7 with 2-aminoethanol monophosphate or phosphate (37). Thus, the penta-phosphorylated LPS molecules may originate from the substitution with phosphate (Table II, M3 and M9) and 2-aminoethanol monophosphate (M11) at this position. We have tried to identify Kdo phosphate and Kdo-PE by IRMPD-ESI-FT-MS in the de-O-acylated LPS but were unsuccessful due to the complexity of the spectrum in this region.

Another minor component was a tridecasaccharide which in comparison to the main component was additionally substituted with GlcNAc in β-(1→6)-linkage on the terminal 1→6-linked Hep residue in the outer core (glycoform II), and ESI-FT-MS of the de-O-acylated LPS indicated that this molecule was also present with a higher degree of phosphorylation (Table II).

HPAEC led to the isolation of four phosphorylated oligosaccharides, two of which were present in larger amounts. The structural analysis of three of these oligosaccharides (glycoforms I to III), which were now isolated for the first time as complete carbohydrate chains from this LPS, confirmed the results of previous studies based on methylation analysis (4). We have isolated another component of this LPS (glycoform IV), which accounted for the remaining 20–25% and which was
not known to be present in this LPS. The inner core structure consisted of a trisaccharide of Kdo residues connected in α-(1→4)-linkages and was further modified by an additional Rha residue located at the 5-position of the middle Kdo residue. Previous studies have identified in some strains of *E. coli K12* the Rha substitution in the inner core (13), and the α-(1→3)-linkage was shown by methylation analysis of the isolated disaccharide after mild acid hydrolysis of *E. coli K12 W3100* LPS (12). Due to the acidic conditions used in the aforementioned study, the Kdo-trisaccharide structure was destroyed. Therefore, it was assumed that in this LPS the inner core structure may contain either a third Kdo in position 4 or a Rha substitution in position 5 of the side chain Kdo. We now isolated the complete oligosaccharide and unequivocally proved its structure by NMR (Fig. 7), ESI-FT-MS, and MS-MS studies (Fig. 8). The inner core structure of this LPS thus contains a second branched Kdo residue in the side chain. At the same time the terminal Hep-(1→6)-α-D-Glc disaccharide of the outer core is missing. There was no indication that the Rha can be found on oligosaccharides containing only two Kdo, and it appears that the rhamnosyltransferase involved in the biosynthesis of this structure has an absolute requirement for the Kdo trisaccharide as substrate. Furthermore, there was no other glycoform in this LPS that contained three Kdo residues. Why the biosynthesis of the outer core is affected at the same time and does not go to completion cannot be answered at this stage, but it could be speculated that the additional sugars in the inner core have an influence on the conformation of the outer
core sugars precluding an elongation. Additional evidence for this explanation may come from the early observation (13) that the Rha residue is part of the immunodominant epitope in this LPS. Although in other \textit{E. coli} LPS parts of the deep inner core are usually inaccessible for antibodies when they are substituted by further sugars of the inner and outer core, this does not seem to hinder the reactivity with the inner core in this LPS. It can also not be answered at this stage why some \textit{E. coli} \textit{K12} strains modify the inner core carbohydrate structure by Rha or 2-aminoethanol phosphate.

A recent study (23) provided genetic evidence for the correlation of the inner core and the outer core biosynthesis. It was found that the protein WaaZ with yet unknown function from \textit{E. coli} \textit{K12} W3100 led to an increased amount of an \(\alpha\)-Kdo-(2\(\rightarrow\)4)-\(\alpha\)-Kdo-(2\(\rightarrow\)4)-\(\alpha\)-Kdo-trisaccharide in the inner core when overexpressed in \textit{E. coli} F470 which possesses an R1 type core oligosaccharide. Concomitantly, molecules containing three Kdo residues were truncated in the outer core. It remained unclarified whether this effect was secondary due to the overexpression of this enzyme in the R1 background. Inactivation of WaaZ in \textit{E. coli} K12 W3100 led to indistinguishable banding patterns in Tricine SDS-PAGE from the wild-type LPS. Due to expected difficulties in obtaining large enough amounts for structural analysis and difficulties in analyzing complex inner core structures of LPS by NMR, it was not attempted to isolate and analyze the corresponding oligosaccharides from the parent strain. However, the investigation of the \textit{E. coli} K12 W3100 LPS oligosaccharides described in this paper showed that a full structural characterization including NMR analysis was possible. According to our data, the results obtained by Frirdich \textit{et al.} (23) must be interpreted in the sense that the presence of three Kdo residues connected in \(\alpha\)-2\(\rightarrow\)4-linkages in the inner core of \textit{E. coli} LPS are associated with a truncated outer core, and this effect is not due to the overexpression of WaaZ; furthermore, we could show that the substitution of the inner core with Rha occurs only on the Kdo-trisaccharide and exclusively at the middle Kdo. Thus, the inner core structure had an effect on the biosynthesis of the outer core. Cloning experiments aiming at the identification of the gene responsible for rhamnose transfer in this LPS is recommended to be done in a host able to synthesize the inner core Kdo-trisaccharide.

Because much of the structural heterogeneity observed within LPS structures has been identified using derivatized partial structures obtained after hydrolysis and gas-liquid chromatography-mass spectrometry analysis, but also for the sake of simplicity, it has become common practice to depict all structural modifications within the same picture of a basic structure. This may misleadingly imply that all of these

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**Fig. 9. Chemical structures of \textit{E. coli} K12 W3100 de-O-acylated LPS. GlcNacyl refers to GlcN carrying 3-OH-C14 in amide linkage.**

Glycoforms I (70%), II (5%), and IV (20%) were present as tetraphosphorylated LPS in larger amounts in this LPS and could be isolated as deacylated oligosaccharides (OS 1, 3 and 4) and further analyzed by NMR. ESI-FT-MS and NMR revealed that all glycoforms were present in small amounts lacking the phosphate in the 4-position of the \(\beta\)-GlcNacyl residue, of which only the glycoform I could be isolated in larger amounts after deacylation (~5% of LPS, OS 2). Only glycoforms I and II were present in small amounts as pentaphosphorylated LPS. A small amount of 2-aminoethanol was found only in pentaphosphorylated glycoform I LPS. A very minor component (glycoform III) did not contain the outer core heptose.
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modifications occur randomly and in all permutations. However, as can be seen from the results of this and previous studies on the LPS of E. coli strain J-5 (9), one has to keep in mind that this picture may not reflect the real situation because certain combinations of structural modifications may preclude each other. This may lead to false interpretations of experimental data.

The fact that only certain subfractions of the LPS of E. coli J-5 were able to promote folding of PhoE (5) and influence the activity of OmpT (6), it appears that E. coli, and probably other bacteria as well, employs structural modifications of LPS deliberately to support specific functions of the outer membrane. Thus, the observed heterogeneity of LPS may not be seen solely as a statistical distribution of possible modifications according to the fidelities of enzymes involved in LPS biosynthesis but rather as means of controlling function by structure. Determining the specific functions of individual LPS structures will be difficult but rewarding in the sense that a better understanding of the outer membrane assembly will open new doors for therapeutic treatment of infectious diseases.

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