Core Oligosaccharides of *Plesiomonas shigelloides* O54:H2 (Strain CNCTC 113/92)

STRUCTURAL AND SEROLOGICAL ANALYSIS OF THE LIPOPOLYSACCHARIDE CORE REGION, THE O-ANTIGEN BIOLOGICAL REPEATING UNIT, AND THE LINKAGE BETWEEN THEM*

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The structure of the core oligosaccharide moiety of the lipopolysaccharide (LPS) of *Plesiomonas shigelloides* O54 (strain CNCTC 113/92) has been investigated by 1H and 13C NMR, fast atom bombardment mass spectrometry (MS)/MS, matrix-assisted laser-desorption/ionization time-of-flight MS, monosaccharide and methylation analysis, and immunological methods. It was concluded that the main core oligosaccharide of this strain is composed of a decasaccharide with the following structure:

$$\begin{align*}
\beta-\alpha-D-Galp & \quad \beta-\alpha-D-Glc \quad \beta-\alpha-D-Galp \\
(1\rightarrow4) & \quad (1\rightarrow3) & \quad (1\rightarrow5) \quad \text{Kdo}
\end{align*}$$

![Structure 1](image)

in which \(\beta-\alpha-D-\text{Hepp}\) is \(L\)-glycerol-\(\alpha-D\)-manno-heptopyranose. The nonasaccharide variant of the core oligosaccharide (−10%), devoid of \(\beta-D-Glc\) substituting the \(\alpha-D-Glc\) at C-6, was also identified. The core oligosaccharide substituted at C-4 of the outer core \(\beta-D-Glc\) residue with the single O-polysaccharide repeating unit was also isolated yielding a hexadecasaccharide structure. The determination of the monosaccharides involved in the linkage between the O-specific polysaccharide part and the core, as well as the presence of \(\beta-\alpha-D-\text{Hepp}-(1\rightarrow\text{ instead of }3)\)-\(\beta-D-\text{Hepp}-(1\rightarrow\) in the repeating unit, revealed the structure of the biological repeating unit of the O-antigen. The core oligosaccharides are not substituted by phosphate residues and represent novel core type of bacterial LPS that is characteristic for the *Plesiomonas shigelloides* serotype O54. Serological screening of 69 different O-serotypes of *P. shigelloides* suggests that epitopes similar to the core oligosaccharide of serotype O54 (strain CNCTC 113/92) might also be present in the core region of the serotypes O24 (strain CNCTC 92/89), O37 (strain CNCTC 39/89) and O96 (strain CNCTC 5133) LPS.

*Plesiomonas shigelloides* is a Gram-negative, flagellated, rod-shaped bacterium. This ubiquitous and facultatively anaerobic organism has been isolated from such sources as fresh-water, surface water, and many wild and domestic animals. The infections correlate strongly with the surface water contamination and are particularly common in tropical and subtropical habitats (1).

Human infections with *P. shigelloides* are mostly related to drinking untreated water, eating uncooked shellfish (2, 3), and visiting countries with low sanitary standards (4, 5). Recent studies implicated *P. shigelloides* as an opportunistic pathogen in immunocompromised hosts (6) and especially neonates (6–10). However, it has also been associated with diarrheal illness (11) and other diseases in normal hosts. *P. shigelloides* has been isolated from an assortment of clinical specimens, including cerebrospinal fluid, wounds, and respiratory tract. It causes gastrointestinal and localized infections originating from infected wounds, which can disseminate to other parts of the body (12). The cases of meningitis and bacteremia (10) caused by *P. shigelloides* are of special interest due to their seriousness.

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**P. shigelloides** has been traditionally classified as a member of the *Vibrionaceae* family based on phenotypic characteristics such as polar flagella, oxidase production, and fermentation properties (1). However, phylogenetic analysis and assessment of the genus *Plesiomonas* deduced from small rRNA sequences indicate a closer relationship with members of *Enterobacteriaceae* (13).

**FIG. 1.** MALDI-TOF mass spectra of the core oligosaccharides of the *P. shigelloides* O54. A, OSIVB; B, OSIVA; and C, OSIII. The MALDI-TOF MS spectra were obtained in the positive reflection mode with 2,5-dihydroxybenzoic acid as matrix. Prior to analysis, the samples were washed twice with 50% methanol directly on the target. *m/z* values represent monoisotopic masses.
The serotyping scheme of P. shigelloides was proposed by Aldova, Shimada, and Sakazaki (14–19). Some O-antigens have shown cross-reactivity with antisera directed against lipopolysaccharides (LPS) of Shigella sonnei, Shigella dysenteriae 1, 7 and 8, Shigella boydi 2, 9, and 13 and Shigella flexneri 6 (15, 20). Two P. shigelloides strains were found to share the structure with O-antigens of S. flexneri and S. dysenteriae (20, 21). The unique structures of the O-specific polysaccharides and core oligosaccharides remain unknown, except those of O-specific polysaccharides from strains 22074, 12254 (21, 22) and CNCTC 113/92 (22). The O-specific polysaccharide of strain CNCTC 113/92 LPS (serotype O54) is composed of a hexasaccharide repeating unit with the following structure:

\[
\beta-\text{D}-\text{Gal} \rightarrow (1\rightarrow 3) \beta-\text{D}-\text{GlcNAc} \rightarrow \beta-\text{D}-\text{Hepp} \rightarrow (1\rightarrow 3) \beta-\text{D}-\text{Hepp} \rightarrow (1\rightarrow 3) \beta-\text{D}-\text{Hepp} \rightarrow (1\rightarrow 3) \beta-\text{D}-\text{Glcp} \rightarrow \beta-\text{D}-\text{Gal} \rightarrow (1\rightarrow 3)
\]

The core oligosaccharide is important for biological and physical properties of the overall lipopolysaccharide and plays a significant role in interactions with the host. Thus we now report on structural and immunochemical studies of the core oligosaccharides isolated from P. shigelloides strain CNCTC 113/92 LPS.

EXPERIMENTAL PROCEDURES

Bacteria—Plesiomonas shigelloides strain CNCTC 113/92, classified as serovar O54:H2 according to Aldova’s antigenic scheme (14–17, 19) and 68 different P. shigelloides O-serotypes (O1, O2, O4–O6, O9, O11–O13, O15, O17, O19, O21, O22, O24–O28, O33–O46, O48, O50, O51, O56, O58, O62, O64–O68, O70–O72, O74–O77, O81–O86, O91–O98), i.e. a group representative for all currently known serotypes, were obtained from the Institute of Hygiene and Epidemiology, Prague, Czech Republic. The bacteria were grown and harvested as described previously (22, 23).

Lipopolysaccharide and Core Oligosaccharides—LPS was extracted from bacterial cells by the hot phenol/water method (24) and purified as reported earlier (23). The yield of LPS was 2% of the dry bacterial mass. LPS (200 mg) was degraded by treatment with 1.5% acetic acid containing 2% SDS at 100 °C for 15 min. The reaction mixture was freeze-dried, the SDS removed by extraction with 96% ethanol, and the residue dried, the SDS removed by extraction with 96% ethanol, and the residue freeze-dried.

Core Oligosaccharides from Plesiomonas shigelloides

The core oligosaccharides were further fractionated by chromatography on Bio-Gel P-10, where the core oligosaccharides were separated from the core polysaccharides (LPS) of P. shigelloides. The core oligosaccharides were further fractionated by chromatography on Bio-Gel P-10, where the core oligosaccharides were separated from the core polysaccharides (LPS) of P. shigelloides. The core oligosaccharides were further fractionated by chromatography on Bio-Gel P-10, where the core oligosaccharides were separated from the core polysaccharides (LPS) of P. shigelloides. The core oligosaccharides were further fractionated by chromatography on Bio-Gel P-10, where the core oligosaccharides were separated from the core polysaccharides (LPS) of P. shigelloides.

N-Methylation of Oligosaccharides—The core oligosaccharides were methylated according to the method of Hakomori (33). The methyl ester groups of the latter methylated oligosaccharides were reduced with Superdeuteroxide (LiB(C6H5)4,2H) as described by Bhat et al. (34). The methylated sugars were analyzed as partially methylated alditol acetates by GC-MS as described previously (23). GC-MS was carried out with a Hewlett-Packard 5971A system using an HP-1 fused-silica capillary column (0.2 mm × 12 m) and a temperature program 150 °C to 270 °C at 8 °C min⁻¹. Amino acid analysis was carried out as described (35, 36). The core oligosaccharide (1 mg) was hydrolyzed with 6 N hydrochloric acid at 100 °C for 24 h and concentrated to dryness. Subsequently, n-butanol (0.5 ml) and acetyl chloride (50 µl) were added, and the reaction was carried out at 120 °C for 20 min, followed by the evaporation to dryness. Heptafluorobutyric anhydride (100 µl) was added, and the mixture was heated for 5 min at 150 °C. The N-heptafluorobutyryl n-butyl ester derivative of amino acid was analyzed by GC-MS on the same system as described above, but a temperature program 100 °C to 270 °C at 5 °C min⁻¹.

N-Acetylation—Oligosaccharide OIII (5 mg) was dissolved in saturated NaHCO3 (2 ml) at 0 °C and treated with acetic anhydride (3 × 100 µl, with 10-min intervals). Reaction mixture was stored for additional 30 min at 0 °C, the product purified on a column (1.6 × 100 cm) of Bio-Gel P-2 and the N-acetylated OSIII oligosaccharide examined by NMR spectroscopy and MALDI-TOF MS.

Mass Spectrometry—MALDI MS of the investigated oligosaccharides, in positive or negative mode, was run on a Bruker Reflex III time-of-flight instrument. Conjugates of core oligosaccharides with BSA were analyzed using a Kratos Compact-SEQ instrument. 2,5-Dihydroxybenzoic acid and sinapinic acid were used as matrices for analyses of oligosaccharides and glycoconjugates, respectively.

NMR Spectrometry—NMR spectra of the oligosaccharides were obtained for 4H2O solutions and D2O solutions containing 10% of D2O, at 35 °C on Bruker DRX 400 and DRX 600 spectrometers. All spectra were obtained using acetone (δ3, 2.225, δ3, 31.05) as internal reference. The core oligosaccharide fractions were repeatedly exchanged with D2O with intermediate lyophilization. The data were acquired and processed using standard Bruker software. The processed spectra were assigned with the help of the SPARKY program (37). The signals were assigned by one- and two-dimensional experiments (COSY, clean-TOSCY, NOESY, ROESY, HMQC-DEPT, and HSQC with and without carbon decoupling). In the clean-TOSCY experiments the mixing times...
used were 30, 60, and 100 ms. The delay time in the HMBC was 60 ms and the mixing times in the NOESY and ROESY experiments were 200 ms.

Preparation of Oligosaccharide Conjugates with BSA—The core oligosaccharide (OSIVA) was isolated and purified as described above. The conjugation was carried out as described previously (39). Briefly, core oligosaccharide OSIVA (2.5 mg) solution in H2O (100 μl) was mixed with an equal volume of BSA (1 mg) solution in H2O. Dimethylformamide was added to a final concentration of 2%, and the mixture was freeze-dried. Dry preparation was heated at 110 °C for 30 min, dissolved in PBS (1 ml), and dialyzed against PBS (3 × 1 liter). The products were analyzed by MALDI-TOF MS, and their antigenic properties were determined in the immuno blotting test, using polyclonal anti-P. shigelloides CNCTC 113/92 antibodies.

Immunization Procedures and Serological Methods—Rabbits were immunized with the P. shigelloides core oligosaccharide-BSA conjugate, suspended in a complete Freund adjuvant, and polyclonal antibodies against the conjugates were obtained by the procedures previously described (39). Enzyme-linked immunosorbent assay (ELISA), using LPS as solid-phase antigen, was performed by a modification (40) of the method described by Voller et al. (41). Immunoblotting was done as previously described (23). A goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad) was used as the second antibody and p-nitrophenyl phosphate and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium were applied as detection systems for ELISA and immunoblotting, respectively.

RESULTS

Isolation and Chemical Analysis of Core Oligosaccharides—The LPS of P. shigelloides CNCTC 113/92 was isolated by conventional methods and analyzed by SDS-PAGE, showing fractions consisting of core oligosaccharide substituted with different numbers of oligosaccharide repeating units as well as unsubstituted core oligosaccharides. The O-specific polysaccharide and core oligosaccharides were liberated by mild acidic hydrolysis of the LPS and isolated by gel filtration on Bio-Gel P-10. In addition to the polysaccharide fraction, which was analyzed previously (22), two fractions with lower molecular mass components were obtained, i.e. OSIII (yield, 16.5% of LPS) and OSIV (yield, 6% of LPS). The fraction OSIV was further separated on Bio-Gel P-2 giving the two main oligosaccharides OSIVA (yield, 4.5% of LPS) and OSIVB (yield, 0.5% of LPS). Because the initial NMR investigation indicated the presence of uronic acid, Kdo, and one non-acetylated glucosamine residue in the oligosaccharides, all subsequent sugar and methylation analyses were done on N-acetylated and carboxylreduced oligosaccharides to detect these residues. Composition analysis of the carboxyl-reduced and N-acetylated oligosaccharide OSIVB together with determination of the absolute configuration revealed the presence of L-He, D-Glc, D-Gal, and D-GlcN (relative proportions of 2.7:1.8:2.9:0.8) in the carboxyl-reduced OSIVB oligosaccharide. Methylation analysis was performed on this carboxyl-reduced and N-acetylated OSIVB but also on only N-acetylated OSIVB. The methyl esters of the latter methylated material were reduced with Superdeuteride generating two deuterium on C-6 of the former uronic acid. These analyses showed the presence of 2,3,7-trisubstituted L-He, 3,4-di substituted L-Hepp, terminal L-Hepp, 4-substituted D-GlcN, terminal D-Glc, 4-substituted D-Galp, and D-GlcN, and 6-substituted Kdo (relative proportions 0.8:1:1:1.0:0.7:0.9:1.8:0.6:0.7) in the original core oligosaccharide OSIVB.

In oligosaccharide OSIVA the ratio of terminal D-GlcP was twice as high as in OSIVB and 4,6-disubstituted D-GlcN was identified instead of 4-substituted D-GlcP. All other components and ratios were found to be the same as in OSIVB. The substitution positions and the ring forms were supported by NMR data (see below).

The MALDI-TOF mass spectra of the oligosaccharides (Fig. 1, A and B) showed main ions at m/z 1660.55 [M + Na]+, 1638.53 [M + H]+, and 1642.53 [M-H2O+Na]+ for OSIVB and m/z 1822.76 [M + Na]+, 1844.75 [M-H+2Na]+, and 1804.74 [M-H2ONa]+ for OSIVA. This suggests a nonasaccharide in OSIVB and a decasaccharide in OSIVA differing only in one hexose unit (162.21Da difference). The nine sugars, two Gal, one Glc, three Hep, one GalA, one GlcN, and one Kdo, give together a monoisotopic mass of 1637.52 and an average mass of 1638.41. The mass spectrum of the isolated OSIII (Fig. 1C) component showed main ions at m/z 2865.94 [M + H]+, 2887.91 [M + Na]+, and 2847.93 [M-H2O+H]+. The mass difference of 1065.15 between OSIII and OSIVA can be explained by one repeating unit of the O-specific polysaccharide substituting the core. The mass of OSIII thus supports a hexadecasaccharide structure with one repeating unit linked to the core oligosaccharide.

NMR Analysis of the Core Oligosaccharide OSIVB and OSIVA—The 1H (Fig. 2A) and HSQC-DEPT (Fig. 3) NMR spectra of the core oligosaccharide OSIVB contained main signals for eight anemic protons and carbons, and in addition a Kdo spin system confirming a nonasaccharide (the sugar residues are indicated by capital letters as shown in the structure below, and these letters refer to the corresponding sugars through the entire text, tables, and figures). The 1H (Fig. 2B) and HSQC-DEPT NMR spectra of the core oligosaccharide OSIVA contained main signals for nine anemic protons and carbons and a Kdo spin system, thus confirming a decasaccharide structure. Because all the 1H NMR spectra were complex and contained overlapping signals, the major signals and spin systems were assigned by COSY, TOCSY with different mixing times, and HSQC experiments. By comparing the chemical shifts with previously published NMR data for respective monosaccharides (42–44) and considering the 3JH,H values for the coupling between ring protons, estimated from the cross-peaks in the two-dimensional spectra, the sugars could be identified and their anomeric configuration determined.

Starting with the signal for the anemic proton, H-1, the COSY spectrum identified the H-2 signal and the TOCSY spectra with different mixing times the H-3 to H-7 signals. The H-7 signals of heptose residues were identified in the TOCSY experiments starting with the assigned H-3 and H-4 signals. The HSQC-TOCSY experiments were used for unambiguous assignment of overlapping signals. From the assigned 1H signals and the one bond C–H connectivities, the carbon signals were assigned in the gradient-enhanced HSQC-DEPT spectrum (Fig. 3), and the linkage positions were determined from the high chemical shifts of the signals from the substituted carbons. The CH2 carbon signals were readily identified in the HSQC-DEPT experiment from negative cross-peaks. An unequivocal identification of the H-7, C-7 as a negative cross-peak in the HSQC-DEPT experiment was further confirmed in the HSQC-TOCSY experiment. By these procedures all the spin systems comprising 1H and 13C resonances were determined (Table 1).

Residue G with the H-1/C-1 signals at δ 5.44/101.5 ppm and non-resolved JH1,H2 = 2 Hz was recognized as the 4-substituted o-D-GalpA residue based on the characteristic five proton spin system, the high chemical shifts of the H-5 (δ 4.52), H-4 (δ 4.43), H-3 (δ 4.12), and C-4 (δ 79.9) signals, the large vicinal couplings between H-2 and H-3 and small vicinal coupling between H-3, H-4, and H-5. Residue D with the H-1/C-1 signals at δ 5.23/100.3 ppm, JH1,H2 = 2 Hz was assigned as the 2,3,7-substituted l-glycero-α-D-manno-Hepp residue from the 1H and 13C chemical shifts, small vicinal couplings between H-1, H-2, and H-3, and the relatively high chemical shifts of the C-2 (δ 79.2), C-3 (δ 77.4), and C-7 (δ 70.9) signals. Residue H with the H-1/C-1 signals at δ 5.17/96.8 ppm, JH1,H2 = 3.6 Hz was assigned as the 4-substituted α-D-GlcP residue based on the
low chemical shifts of the C-2 signal (δ 55.2), the relative high chemical shift of the C-4 signal (δ 78.9) and the large vicinal couplings between all ring protons. Residue B with the H-1/C-1 signals at δ 5.09/101.3 ppm, J_{H1,1H} < 2 Hz was recognized as the 3,4-disubstituted l-glycero-d-manno-hepp residue on the basis of the small vicinal couplings between H-1, H-2, and H-3...
and the relatively high chemical shifts of the C-3 (δ 75.8) and C-4 (δ 74.8) signals. Residue F with the H-1/C-1 signals at δ 4.91/101.4 ppm, J_H1,H2 < 2 Hz was recognized as the terminal 1-glycero-α-d-manno-Hepp residue due to the small vicinal couplings between H-1, H-2, and H-3 and similar chemical shifts as those of the monosaccharide 1-α-d-Hepp. Residue E with the

| Residue | Oligosaccharide | Chemical shift | ppm  |
|---------|----------------|----------------|------|
| A→5)-Kdo | * * * | 1.85 | 2.16 |
| B→3,4)-l-glycero-α-d-manno-Hepp(1→ | * * * | 5.15 | 4.05 |
| C  β-D-Galp(1→ | * * * | 4.44 | 3.54 |
| D→2,3,7)-l-glycero-α-d-manno-Hepp(1→ | * * * | 5.25 | 4.13 |
| E  β-D-Glcpl(1→ | * * * | 4.55 | 3.23 |
| F→4)-α-d-GalpA(1→ | * * * | 5.45 | 3.89 |
| H→4)-α-d-GlcplN(1→ | * * | 5.12 | 3.32 |
| H→4)-α-d-GlcplN(1→ | * * | 5.18 | 3.33 |
| I  β-D-Glcpl(1→ | * | 4.49 | 3.28 |
| K  β-D-Galp(1→ | * * | 4.48 | 3.25 |
| L→3)-β-D-GlcplNAc(1→ | * | 4.62 | 3.85 |
| M→4)-α-L-Rhap(1→ | * | 4.85 | 3.77 |
| N→3)-2-O-Ac-6d-β-D-manno-Hepp(1→ | * | 5.02 | 3.61 |
| O→3)-d-glycero-β-D-manno-Hepp(1→ | * | 4.76 | 3.98 |
| P→4)-α-L-Rhap(1→ | * | 4.95 | 3.98 |
| Q  β-D-Galp(1→ | * | 5.30 | 4.13 |

| Residue | Atom | Connectivities to | Inter-residue atom* residue |
|---------|------|----------------|-----------------------------|
| G→4)-α-d-GalpA(1→ | H-1/C=1 | 5.44 | 77.6 |
| D→2,3,7)-l-glycero-α-d-manno-Hepp(1→ | H-1/C=1 | 5.23 | 75.8 |
| H→4)-α-d-GlcplN(1→ | H-1/C=1 | 5.17 | 79.9 |
| B→3,4)-l-glycero-α-d-manno-Hepp(1→ | H-1/C=1 | 5.10 | 75.0 |
| F→4)-α-L-Rhap(1→ | H-1/C=1 | 4.92 | 70.9 |
| E  β-D-Glcpl(1→ | H-1/C=1 | 4.53 | 79.2 |
| K  β-D-Galp(1→ | H-1/C=1 | 4.43 | 78.9 |
| C  β-D-Galp(1→ | H-1/C=1 | 4.42 | 74.8 |
| I  β-D-Glcpl(1→ | H-1/C=1 | 4.49 | 68.2 |

*Assignment of resonance can be interchanged.

* Corresponding residues present in a different environment.

TABLE I

1H and 13C NMR chemical shifts of the P. shigelloides O54 (strain CNCTC 113/92) core oligosaccharides

Spectra were obtained for 2H2O solutions at 35 °C. Acetone (δ 2.225, δ 3.105) was used as internal reference. The presence of a residue in the respective oligosaccharide is marked with an asterisk. The chemical shifts are given as averaged values for the residues in the same environment.

| Residue | Oligosaccharide | Chemical shift | ppm  |
|---------|----------------|----------------|------|
| A→5)-Kdo | * * * | 1.85 | 2.16 |
| B→3,4)-l-glycero-α-d-manno-Hepp(1→ | * * * | 5.15 | 4.05 |
| C  β-D-Galp(1→ | * * * | 4.44 | 3.54 |
| D→2,3,7)-l-glycero-α-d-manno-Hepp(1→ | * * * | 5.25 | 4.13 |
| E  β-D-Glcpl(1→ | * * * | 4.55 | 3.23 |
| F→4)-α-d-GalpA(1→ | * * * | 5.45 | 3.89 |
| H→4)-α-d-GlcplN(1→ | * * | 5.12 | 3.32 |
| H→4)-α-d-GlcplN(1→ | * * | 5.18 | 3.33 |
| I  β-D-Glcpl(1→ | * | 4.49 | 3.28 |
| K  β-D-Galp(1→ | * * | 4.48 | 3.25 |
| L→3)-β-D-GlcplNAc(1→ | * | 4.62 | 3.85 |
| M→4)-α-L-Rhap(1→ | * | 4.85 | 3.77 |
| N→3)-2-O-Ac-6d-β-D-manno-Hepp(1→ | * | 5.02 | 3.61 |
| O→3)-d-glycero-β-D-manno-Hepp(1→ | * | 4.76 | 3.98 |
| P→4)-α-L-Rhap(1→ | * | 4.95 | 3.98 |
| Q  β-D-Galp(1→ | * | 5.30 | 4.13 |

*Assignment of resonance can be interchanged.

* Corresponding residues present in a different environment.

TABLE II

Selected inter-residue 1H,13C connectivities from the anomeric atoms of the core oligosaccharides OSIVA and OSIVB of P. shigelloides O54 (strain CNCTC 113/92)

The chemical shifts are given as averaged values for the residues in the same environment.
The H-1/C-1 signals at δ 4.53/103.9 ppm, JH-1,H-2 7.8 Hz was recognized as terminal β-D-Glp from the similarity of the 1H and 13C chemical shifts with those of β-D-Glc and the large vicinal couplings between all protons in the sugar ring. Residue K with the H-1/C-1 signals at δ 4.43/104.1 ppm, JH-1,H-2 7.8 Hz as well as residue C with the H-1/C-1 signals at δ 4.42/104.3 ppm, JH-1,H-1 7.8 Hz were assigned as terminal β-D-Galp residues due to the large coupling between H-1, H-2, and H-3 and the small vicinal coupling between H-3, H-4, and H-5, and chemical shifts similar to those of β-D-Galp. Residue A was identified as a 5-substituted Kdo on the basis of characteristic deoxy proton signals, found at δ 1.86 ppm (H-3ax) and δ 2.16 ppm (H-3eq), and a high chemical shift of the C-5 signal (δ 75.0 ppm). In OSIVA an additional terminal β-D-Glp (residue I), δ 4.49/103.9 ppm, JH-1,H-2 7.8 Hz, was found and residue H with the H-1/C-1 signals at δ 5.16/97.0 ppm, JH-1,H-2 3.6 Hz was found to be additionally substituted at C-6 due to characteristic downfield shift of the C-6 signal (δ 68.2 ppm). The 1JH-1,H-1 values obtained from an HMQC experiment, confirmed the C-2-propanosyl configuration for residues B (173 Hz), D (175 Hz), F (173 Hz), G (173 Hz), and H (174 Hz) and β-pyranosyl configuration for residues C, E, and K (163 Hz for all these residues). The results are in agreement with data from the sugar and methylation analyses.

In some of the batches of core oligosaccharides a glycine was identified, by the presence of an additional carbonyl signal at δ 169.2 ppm, and a negative CH2 signal (Hδ δ 3.96 ppm, Cδ δ 41.6 ppm) in the HSQC-DEPT spectrum. The presence of glycine in some of the preparations of the core oligosaccharides was confirmed by amino acid analysis and mass spectrometry. However, only MS data (data not shown) suggested that the glycine was linked to the isolated core oligosaccharides.

Each disaccharide element in the core oligosaccharides was identified by HMBC (Fig. 3, Table II) and ROESY (Fig. 4, Table III) experiments that showed inter-residue connectivities between adjacent sugar residues and thus provided the sequence of monosaccharides in the oligosaccharides (Fig. 6). For OSIVA inter-residue NOEs were found between H-1 of K and H-4 of H, H-1 of H and H-4 of G, H-1 of G and H-3 of D, H-1 of D and H-3 of B, H-1 of B and H-5 of A, H-1 of C and H-4 of B, H-1 of E and

**TABLE III**

Selected inter-residue NOE connectivities from the anomeric atoms of the core oligosaccharides OSIVA and OSIVB of *P. shigelloides* O54 (strain CNCTC 113/92)

| Residue | Atom H1 | δH1 | Connectivities to δH1 | Inter-residue atom/residue |
|---------|---------|-----|----------------------|---------------------------|
| G       |         | 5.44| 4.08                 | H-3 of D                  |
| D       |         | 5.23| 4.17                 | H-3 of B                  |
| H       |         | 5.17| 4.43                 | H-4 of G                  |
| B       |         | 5.10| 4.11                 | H-5 of A                  |
| F       |         | 4.92| 3.56, 3.74           | H-7, H-7' of D            |
| E       |         | 4.53| 4.11                 | H-2 of D                  |
| K       |         | 4.43| 4.11, 3.89           | H-4 of H                  |
| C       |         | 4.42| 4.17                 | H-4 of B                  |
| I       |         | 4.49| 3.97                 | H-6 of H                  |

* Residues present only in the core oligosaccharide OSIVA.
H-2 of D, and H-1 of F and H-7,7′ of D. In OSIVA connectivities between H-1 of the additional glucose (residue I) and H-6 of H were established in NOESY experiment.

The HMBC spectra showed cross-peaks between the anomeric proton and the carbon at the linkage position and between the anomeric carbon and the proton at the linkage position (Table II), which confirmed the structure of the core nona- and decasaccharide in the LPS of P. shigelloides strain CNCTC 113/92 (Fig. 6).

**NMR Analysis of the Core Oligosaccharide OSIII**—The 1H (Fig. 2C) and HSQC-DEPT (Fig. 5) NMR spectra, recorded for isolated OSIII, contained signals that derived from the core oligosaccharide as well as from the O-polysaccharide and supported the structure of a hexadecasaccharide as the main component. Signals from each monosaccharide were assigned according to the described procedures for OSIVA and OSIVB, taking into account NMR data concerning the O-polysaccharide (22) (Table I). Two significant differences were found with regard to the respective units: (i) 3,4)-β-D-GlcNAc-(1→ (residue L) of the repeating unit was found to be linked to C-4 of β-D-GlcN (residue I, δ 4.53/103.2 ppm) and (ii) —3)-β-D-Hepp-(1→ (residue O) was found instead of —3,4)-β-D-Hepp-(1→. These structural elements were confirmed by both HMBC and NOE connectivities (Tables IV and V) and further corroborated

**Fig. 5.** HSQC-DEPT spectrum of the OSIII oligosaccharide of P. shigelloides O54. The spectrum was obtained for 2H2O solutions at 600 MHz and 35 °C. The cross-peaks are labeled as explained in the legend to Fig. 2.

**Table IV**

| Residue          | Atom H-1 | Connectivities to δH | Inter-residue atom/residue |
|------------------|----------|----------------------|----------------------------|
| G                | —4)-α-D-GalpA-(1→ | 5.45 | 4.14 | H-3 of D |
| Q                | β-D-Gal-(1→ | 5.30 | 3.61 | H-4 of P |
| D                | —2,3,7)-L-glycero-α-D-manno-Hepp-(1→ | 5.28 | 4.20 | H-3 of B |
| H                | —4,6)-α-D-GlcN-(1→ | 5.19 | 4.44 | H-4 of G |
| B                | —3,4)-L-glycero-α-D-manno-Hepp-(1→ | 5.12 | 4.15 | H-5 of A |
| N                | —3)-2-OAc-6d-β-D-manno-Hepp-(1→ | 5.01 | 3.63 | H-4 of M |
| P                | —4)-α-L-Rhap-(1→ | 4.95 | 3.68 | H-3 of O |
| F                | L-glycero-α-D-manno-Hepp-(1→ | 4.94 | 3.65, 3.76 | H-7,7′ of D |
| M                | —4)-α-L-Rhap-(1→ | 4.84 | 3.62 | H-3 of L |
| O                | —3)-β-D-gluco-β-D-manno-Hepp-(1→ | 4.75 | 4.07 | H-3 of N |
| L                | —3)-β-D-GlcNAc-(1→ | 4.61 | 3.58 | H-4 of I |
| E                | β-D-Glc-(1→ | 4.57 | 4.17 | H-2 of D |
| K                | β-D-Galp-(1→ | 4.52 | 3.89 | H-4 of H |
| C                | β-D-Galp-(1→ | 4.47 | 4.20 | H-4 of B |
| I                | —4)-β-D-Glc-(1→ | 4.52 | 4.17 | H6 of H |
by the results of the methylation analysis of OSIII. The linkage between the O-specific polysaccharide part, i.e. \(\rightarrow 3\)-\(\beta\)-d-GlcpNAc-(1\(\rightarrow\) (residue L) and the core structure, together with the presence of \(\rightarrow 3\)-\(\beta\)-d-Hepp-(1\(\rightarrow\) (residue O) instead of \(\rightarrow 3,4\)-\(\beta\)-d-Hepp-(1\(\rightarrow\) previously found in the repeating unit, showed the structure of the biological repeating unit of the O-antigen. Thus the combined results suggest the following hexadecasaccharide structure of the core oligosaccharide substituted by one repeating unit of the O-specific polysaccharide of the P. shigelloides strain 113/92 (Fig. 6).

Serological Studies—Most of the O-specific polysaccharides are still unknown, and no data are available on the number of different core structures within the Plesiomonas genus. Because the investigated oligosaccharide OSIVA represents the
first complete structure of a Plesiomonas shigelloides core, anti-OSIVA-BSA polyclonal antibodies were used to scan by immunoblotting all the currently available P. shigelloides strains comprising 69 different O-serotypes for the presence of epitopes similar to those found in the P. shigelloides O54 (strain CNCTC 113/92).

The OSIVA oligosaccharide, obtained by mild acidic hydrolysis and separation by size exclusion chromatography, was linked covalently to BSA (38) and the reaction product was checked by MALDI-TOF MS. The MALDI-TOF mass spectrum of the OSIVA conjugated to BSA showed main ion at m/z 68,300. The reference spectrum of BSA gave the main ion at m/z 66,510. The mass difference between BSA and the BSA conjugate suggests that mainly one oligosaccharide molecule was conjugated to one BSA molecule.

Rabbits were immunized with the glycoconjugate and sera with polyclonal anti-conjugate antibodies were obtained. The reactivity of anti-conjugate antibodies with homologous LPS was tested in ELISA assay and in immunoblotting. The end point titer in ELISA (A_{405 nm} 0.2 at dilution 1/3200) and a distinct reaction of anti-conjugate antibodies with the fast migrating fraction of LPS visualized in immunoblotting showed that the conjugate was a good immunogen in rabbits. Whole-cell lysates of P. shigelloides strains representing 69 O-serotypes (O1, O2, O4–O6, O9, O11–O13, O15, O17, O19, O21, O22, O24–O28, O33–O46, O48, O50, O51, O54, O56, O58, O59, O62, O64–O68, O70–O72, O74–O77, O81–O86, O91–O98) were subjected to proteinase K digestion in the lysing buffer, followed by the SDS-PAGE and immunoblotting analysis. The LPS separated by SDS-PAGE were stained using the method of Tsai and Frasch (28) and the reactivity of anti-conjugate antibodies with LPS isolated from various P. shigelloides strains was compared.

The staining patterns for the cross-reacting strains obtained from proteinase K-digested lysates were compared with that of the isolated and purified LPS. The antibodies against P. shigelloides strain 113/92 OSIVA oligosaccharide reacted distinctly with fast migrating LPS fractions, representing LPS with unsubstituted core oligosaccharides, of the homologous strain. A strong cross-reaction was also observed with the fast migrating LPS fractions of serotypes O37 (strain 39/89), O24 (strain 92/89), and O96 (strain 5133) (Fig. 7). However, the blotting test also showed weak reaction of the anti-OSIVA antibodies with high molecular weight LPS fractions of the P. shigelloides O67 (strain 137/92), in which the core is substituted with the O-specific polysaccharide.

**DISCUSSION**

We present here the first complete structure of a Plesiomonas shigelloides core oligosaccharide, the structure of the biological repeating unit of the O-antigen, and the linkage between them. The opinions differ on the classification of the genus Plesiomonas, because it has some characteristics in common with both Enterobacteriaceae and Vibrionaceae families. A comparison of the 5 S rRNA sequences of a number of Enterobacteriaceae and Vibrionaceae shows that P. shigelloides is more related to Proteus mirabilis and Proteus vulgaris than to any other member of Vibrionaceae tested (1). In the core oligosaccharides of Enterobacteriaceae and related families two regions are distinguished: the outer core, composed mostly of hexoses, and the inner core, built of LPS-specific components, i.e. heptose and Kdo (45). We found that the investigated core oligosaccharide shares some structural elements with the core types found in the Enterobacteriaceae family, whereas similarities of the P. shigelloides O54 core to other known core oligosaccharides within the Vibrionaceae family are scarce.

**FIG. 7. Reactivity of polyclonal antibodies against P. shigelloides O54 core oligosaccharide with LPS and proteinase K-digested whole cell lysates in immunoblotting.** Whole cell lysates and purified LPS were separated by SDS-PAGE using a 15% separating gel and visualized by the silver staining method (A) or transferred onto nitrocellulose (B). Polyclonal antibodies against OSIVA were 200-fold diluted. Only the cross-reacting P. shigelloides serotypes, i.e. O24 (CNCTC 92/89), O37 (CNCTC 39/89), O54 (CNCTC 113/92), and O96 (CNCTC 5133) are shown. Asterisks, the lanes containing whole cell lysates (20 μl/lane) digested with proteinase K are designated with s cle, and the lanes containing the isolated and purified lipopolysaccharides are marked LPS (7.5 μg/lane).

The core oligosaccharide of P. shigelloides contains the structural element :

\[
\begin{align*}
1-\alpha-\text{D-Hep} & \\
\uparrow & \\
1 & \\
\rightarrow 3-1-\alpha-\text{D-Hep} & -(1 \rightarrow 3)-1-\alpha-\text{D-Hep} & -(1 \rightarrow 5)-\text{Kdo}
\end{align*}
\]

**STRUCTURE 3**

present in the majority of characterized enterobacterial and non-enterobacterial core structures. The inner core region of enterobacteria is usually substituted by charged groups such as phosphate, pyrophosphate, 2-aminoethylphosphate, or 2-aminoethylpyrophosphate, but the core oligosaccharides of P. shigelloides O54 lack these charged groups.

The characteristic feature of the family Vibrionaceae is the presence of one phosphorylated Kdo residue in the representative strains of all genera of Vibrionaceae except Plesiomonas (46). In the analyses of the de-O-N-acetylated preparations of Plesiomonas LPS, we found that the LPS of P. shigelloides...
possesses two Kdo residues in the inner core. We also found that none of the sugar residues within the core oligosaccharide of the P. shigelloides LPS was substituted by phosphate groups.

The isolation of the OSIII oligosaccharide, i.e. the complete core oligosaccharide substituted with one repeating unit, not only showed the structure of the biological repeating unit of the O-antigen but also allowed for the identification of the linkage between the O-specific polysaccharide core and the region. The \( \beta \)-D-GlcNAc(1→(residue L) found at the reducing end of the O-specific polysaccharide repeating unit is linked to O-4 (C-4 at \( \delta \) 80.3 ppm) of \( \beta \)-D-Glc(1→(residue I) of the core oligosaccharide OSIVA. The anomic configuration of that linkage is retained as in the O-polysaccharide (\( \beta \)-configuration).

The herein reported core oligosaccharide structure is the first one described for the LPS of the genus Plesiomonas. However, we have found that, despite the fact that Plesiomonas is classified within the Vibrionaceae family, the core region of the P. shigelloides O54 (strain CNCTC 113/92) LPS possesses some characteristic structural features of the enterobacterial core oligosaccharides, and it differs substantially from those described for other genera among Vibrionaceae (47–49).

The results obtained from serological screening of 69 P. shigelloides O-serotypes suggest that structural elements of the core oligosaccharide of serotype O54, recognized by the polyclonal antibodies directed against the core oligosaccharide conjugate, are only shared by the strains of the serotypes O24 (strain CNCTC 92/89), O37 (strain CNCTC 39/89), and O96 (strain CNCTC 5133). The presence of many non-reactive core oligosaccharides suggests the lack of a uniform core structure among the screened P. shigelloides O-serotypes. Such observations were reported previously for bacteria belonging to other genera, e.g. Escherichia, Proteus, Citrobacter, and Salmonella, possessing diverse core types within a species (45, 50).

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