# Complete Lipopolysaccharide of *Plesiomonas shigelloides* O74:H5 (Strain CNCTC 144/92). 1. Structural Analysis of the Highly Hydrophobic Lipopolysaccharide, Including the O-Antigen, Its Biological Repeating Unit, the Core Oligosaccharide, and the Linkage between Them[†,‡]

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ABSTRACT: The lipopolysaccharide of *Plesiomonas shigelloides* serotype O74:H5 (strain CNCTC 144/92) was obtained with the hot phenol/water method, but unlike most of the S-type enterobacterial lipopolysaccharides, the O-antigens were preferentially extracted into the phenol phase. The poly- and oligosaccharides released by mild acidic hydrolysis of the lipopolysaccharide from both phenol and water phases were separated and investigated by $^{1}$H and $^{13}$C NMR spectroscopy, MALDI-TOF mass spectrometry, and sugar and methylation analysis. The O-specific polysaccharide and oligosaccharides consisting of the core, the core with one repeating unit, and the core with two repeating units were isolated. It was concluded that the O-specific polysaccharide is composed of a trisaccharide repeating unit with the [→2)-β-D-Qui*p*3NAcyl-(1→3)-α-L-Rha*p*2OAc-(1→3)-α-D-Fuc*p*NAc-(1→] structure, in which D-Qui3NAcyl is 3-amino-3,6-dideoxy-D-glucose acylated with 3-hydroxy-2,3-dimethyl-5-oxopyrrolidine-2-carboxylic acid. The major oligosaccharide consisted of a single repeating unit and a core oligosaccharide. This undecasaccharide contains information about the biological repeating unit and the type and position of the linkage between the O-specific chain and core. The presence of a terminal β-D-Qui*p*3NAcyl-(1→ residue and the →3)-β-D-Fuc*p*NAc-(1→4)-α-D-Gal*p*A element showed the structure of the biological repeating unit of the O-antigen and the substitution position to the core. The →3)-β-D-Fuc*p*NAc-(1→ residue has the anomeric configuration inverted compared to the same residue in the repeating unit. The core oligosaccharide was composed of a nonphosphorylated octasaccharide, which represents a novel core type of *P. shigelloides* LPS characteristic of serotype O74. The similarity between the isolated O-specific polysaccharide and that found on intact bacterial cells and lipopolysaccharide was confirmed by HR-MAS NMR experiments.

*Plesiomonas shigelloides* is a Gram-negative, flagellated, rod-shaped bacterium. This ubiquitous and facultatively anaerobic organism has been isolated from such sources as freshwater, surface water, and many wild and domestic animals. The observed infections correlate strongly with surface water contamination. They are common in tropical and subtropical habitats (*1*), but the presence of *P. shigelloides* in the surface waters of the Nordic countries has also been reported (*2*).

Human infections with *P. shigelloides* are mostly related to drinking untreated water, eating uncooked shellfish (*3*, *4*), and visiting countries with low sanitary standards (*5*, *6*). Recent studies implicated *P. shigelloides* as an opportunistic pathogen in immunocompromised hosts (*7*) and especially neonates (*8*−*11*). However, it has also been associated with diarrheal illness (*12*) 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 (*13*). The cases of meningitis and bacteremia (*11*) caused by *P. shigelloides* are of special interest due to their seriousness.

The lipopolysaccharide (LPS)[1] consists of O-specific polysaccharide, core oligosaccharide, and lipid A. All these components are important for the biological and physical properties of the LPS and could be involved in the

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pathogen−host interactions. To date, only the structures of the O-specific polysaccharides from strains 22074, 12254 (*14*), and CNCTC 113/92 (*15*) and the structure of the core oligosaccharide of strain CNCTC 113/92 (*16*) are known. To improve our knowledge of the structures of *Plesiomonas* LPS, we now report structural studies of the complete LPS isolated from *P. shigelloides* O74:H5 (strain CNCTC 144/92). This strain is a clinical isolate collected from a patient in Bohemia. LPS preparations did not exhibit serological cross reactivity with antibodies directed against *P. shigelloides* O54:H2 (strain CNCTC 113/92) core oligosaccharide (*16*), indicating that the core oligosaccharides of the two strains are different. The structural studies of the complete LPS presented here concern the O-specific polysaccharide, the core oligosaccharide, and the linkage between them. The lipid A part has been described in ref *51*.

The similarity between the isolated O-specific polysaccharide and that on bacterial cells in situ was shown by high-resolution magic angle spinning (HR-MAS) NMR spectroscopy.

## EXPERIMENTAL PROCEDURES

*Bacteria. P. shigelloides* strain CNCTC 144/92, classified as serovar O74:H5 according to Aldova's antigenic scheme (*17*−*21*), was obtained from the Institute of Hygiene and Epidemiology (Prague, Czech Republic). The bacteria were grown and harvested as described previously (*15*, *22*). Bacteria for the HR-MAS HSQC NMR analysis were grown in a liquid medium as described above, containing D-[U-$^{13}$C]glucose (99% $^{13}$C, Cambridge Isotope Laboratories, Woburn, MA), and the culture volume was scaled down to 5 mL.

*Lipopolysaccharide and Core Oligosaccharides.* LPS was extracted from bacterial cells by the hot phenol/water method (*23*) and purified as previously reported (*22*). As the yield of LPS from the water phase was low (0.6−1.1% of dry bacterial mass), the phenol phase was also collected and dialyzed extensively against deionized water. LPS was isolated by ultracentrifugation, and the yield was ∼2%. LPS (200 mg) from both phases were separately 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 suspended in water and centrifuged. The supernatants were fractionated by gel permeation chromatography, performed on a column (1.6 cm × 100 cm) of Bio-Gel P-10 equilibrated with 0.05 M pyridine/acetic acid buffer at pH 5.6. Eluates were monitored with a Knauer differential refractometer, and all fractions were first freeze-dried and then checked by $^1$H NMR spectroscopy and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). The fractionation yielded two main fractions containing O-specific polysaccharide ($PS_{H_2O}$I, 30 mg; $PS_{PhOH}$I, 58 mg) and oligosaccharide ($OS_{H_2O}$III, 32 mg; $OS_{PhOH}$III, 27 mg) and minor fractions containing shorter O-specific polysaccharide chains linked to the core (PSII) and unsubstituted core oligosaccharide (OSIV).

*Analytical Methods.* The LPS was analyzed by SDS−PAGE according to the method of Laemmli (*24*) with modifications as described previously (*25*), and the LPS bands were visualized by the silver staining method (*26*). Monosaccharides were analyzed as their alditol acetates by GC−MS (*22*, *27*). The absolute configurations of the sugars were determined as described by Gerwig et al. (*28*, *29*) using (−)-2-butanol for the formation of 2-butyl glycosides and 2-butyl esters for uronic acids. The trimethylsilylated butyl glycosides were then identified by comparison with authentic samples [produced from respective monosaccharide and (−)-2-butanol] via GC−MS. Carboxyl reduction of the core oligosaccharide fractions was carried out according to the method of Taylor et al. (*30*) as previously described (*22*). Methylations were performed on both native and carboxyl-reduced oligosaccharides according to the method of Hakomori (*31*). The methylated sugars were analyzed as partially methylated alditol acetates by GC−MS as previously described (*22*). 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 gradient from 150 to 270 °C at 8 °C/min. Amino acid analysis was carried out on an LKB ALPHA PLUS amino acid analyzer after hydrolysis of the oligosaccharides with 6 M hydrochloric acid at 110 °C for 24 h.

*O-Deacetylation of Polysaccharide.* Polysaccharide (1 mg) was treated with 1 mL of aqueous 12.5% $NH_3$ for 16 h at 22 °C followed by dilution with water and lyophilization. The product was analyzed by $^1$H NMR spectroscopy.

*Mass Spectrometry.* Positive mode MALDI MS of the investigated fractions was carried out on a Bruker Reflex III time-of-flight instrument. 2,5-Dihydroxybenzoic acid was used as a matrix for analysis of oligosaccharides.

*NMR Spectroscopy.* All NMR spectra were recorded on Bruker DRX 400 and DRX 600 spectrometers. NMR spectra of polysaccharide and oligosaccharide fractions were obtained for $^2H_2O$ solutions at 30 °C using acetone ($\delta_H$ 2.225, $\delta_C$ 31.05) as an internal reference. The polysaccharide and core oligosaccharides were first repeatedly exchanged with $^2H_2O$ (99%) with intermediate lyophilization. The data were acquired and processed using standard Bruker software. The processed spectra were assigned with the help of SPARKY (*32*). The signals were assigned by one- and two-dimensional experiments (COSY, clean-TOCSY, NOESY, ROESY, HMBC, HSQC-DEPT, and HSQC with and without carbon decoupling). In the clean-TOCSY experiments, the mixing times of 30, 60, and 100 ms were used. The delay time in the HMBC was 60 ms and the mixing time in the NOESY and ROESY experiments 200 ms. $^1$H NMR spectra of the polysaccharide were also acquired at −10 °C for $H_2O$ solutions containing 15% acetone-$d_6$, using the residual acetone-$d_5$ ($\delta$ 2.204) as an internal reference.

NMR spectra of bacteria and LPS suspensions in $^2H_2O$ were obtained using the high-resolution magic angle spinning (HR-MAS) technique on a Bruker DRX 600 spectrometer. HR-MAS NMR experiments were carried out at a spin rate

[1] Abbreviations: LPS, lipopolysaccharide; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; GC, gas chromatography; COSY, correlated spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; ROESY, rotating frame nuclear Overhauser effect spectroscopy; HMBC, heteronuclear multiple-bond correlation; HMQC, heteronuclear multiple-quantum coherence; HSQC, heteronuclear single-quantum coherence; DEPT, distortionless enhancement by polarization transfer; L,D-Hep, L-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; D-[U-$^{13}$C]glucose, uniformly $^{13}$C-substituted D-glucose.

of 5 kHz at 23 °C (the measured temperature of the pressurized air used for sample spinning) using a Bruker 4 mm HR-MAS probe and a $ZrO_2$ rotor as previously described (*33*). Acetone in $^2H_2O$ placed in the rotor was used as a reference ($\delta_H$ 2.225, $\delta_C$ 31.05) for HR-MAS NMR in a separate experiment prior to the actual run. The HSQC spectra obtained for the $^{13}C$-labeled bacteria were processed using exponential multiplication in the $F_2$ dimenssion and a line broadening parameter adjusted to 10 Hz, to focus on the large O-antigen molecules.

## RESULTS

*Isolation of O-Specific Polysaccharide and Core Oligosaccharides.* The LPS of *P. shigelloides* CNCTC 144/92 was isolated by the hot phenol/water method. As the yield of LPS obtained from the water phase ($LPS_{H_2O}$) was low, the phenol phase ($LPS_{PhOH}$) was also investigated, resulting in the isolation of another batch of LPS material. Both $LPS_{H_2O}$ and $LPS_{PhOH}$ were analyzed by SDS−PAGE (Figure 1), showing fractions consisting of core oligosaccharide substituted with different numbers of oligosaccharide repeating units as well as unsubstituted core oligosaccharides. $LPS_{PhOH}$ exhibited a higher degree of polymerization within its O-specific polysaccharide than $LPS_{H_2O}$ did.

The O-specific polysaccharide and different oligosaccharide components were released by mild acidic hydrolysis of the LPS and isolated by gel filtration on Bio-Gel P-10. Four main fractions were obtained: PSI (yield, 15% of $LPS_{H_2O}$ and 28% of $LPS_{PhOH}$), PSII (yield, 1−5% of $LPS_{H_2O}$ and $LPS_{PhOH}$), OSIII (yield, 16% of $LPS_{H_2O}$ and 11% of $LPS_{PhOH}$), and OSIV (yield, ∼3% of $LPS_{H_2O}$ and ∼5% of $LPS_{PhOH}$). The fractions were analyzed by NMR spectroscopy and MALDI-TOF MS, which showed that PSI consisted of the O-specific polysaccharide, PSII of two O-repeats and the core, OSIII of the core and one repeating unit, and OSIV of the core oligosaccharide.

*Structure Analysis of the O-Specific Polysaccharide.* Monosaccharide analysis of PSI together with determination of the absolute configuration and methylation analysis indicated the presence of equimolar amounts of 3-substituted L-Rha*p*, 3-substituted D-Fuc*p*N, and 2-substituted Qui*p*3N in the O-specific polysaccharide.

The NMR spectra (Figure 1) of PSI isolated from $LPS_{PhOH}$ and $LPS_{H_2O}$ indicated a partly O-acetylated trisaccharide repeating unit, stoichiometrically N-acylated with a substituent (**R**), containing a methylene and two methyl groups. As all the $^1H$ NMR spectra were complex, the major signals and spin systems were assigned by several two-dimensional experiments (Table S1 of the Supporting Information). The sugar residues are denoted with uppercase letters through the manuscript. In the main spin systems of $PSI_{PhOH}$ and $PSI_{H_2O}$, residue **I** with the H-1/C-1 signals at $\delta$ 5.54/96.2 ($J_{H1,H2}$ < 2 Hz) was assigned as a 3-substituted α-D-Fuc*p*NAc residue on the basis of the signal of an exocyclic $CH_3$ group ($\delta$ 1.27/16.2), the small chemical shifts of the C-2 signal ($\delta$ 48.7), the large chemical shift of the C-3 signal ($\delta$ 77.7), and the small vicinal couplings among H-3, H-4, and H-5. Residue **K** with the H-1/C-1 signals at $\delta$ 4.92/99.8 ($J_{H1,H2}$ < 2 Hz) was assigned as the 3-substituted α-L-Rha*p* residue on the basis of the signal of an exocyclic $CH_3$ group ($\delta$ 1.23/17.6), the large chemical shift of the C-3 signal ($\delta$ 78.9), and the small vicinal couplings among H-1, H-2, and H-3.

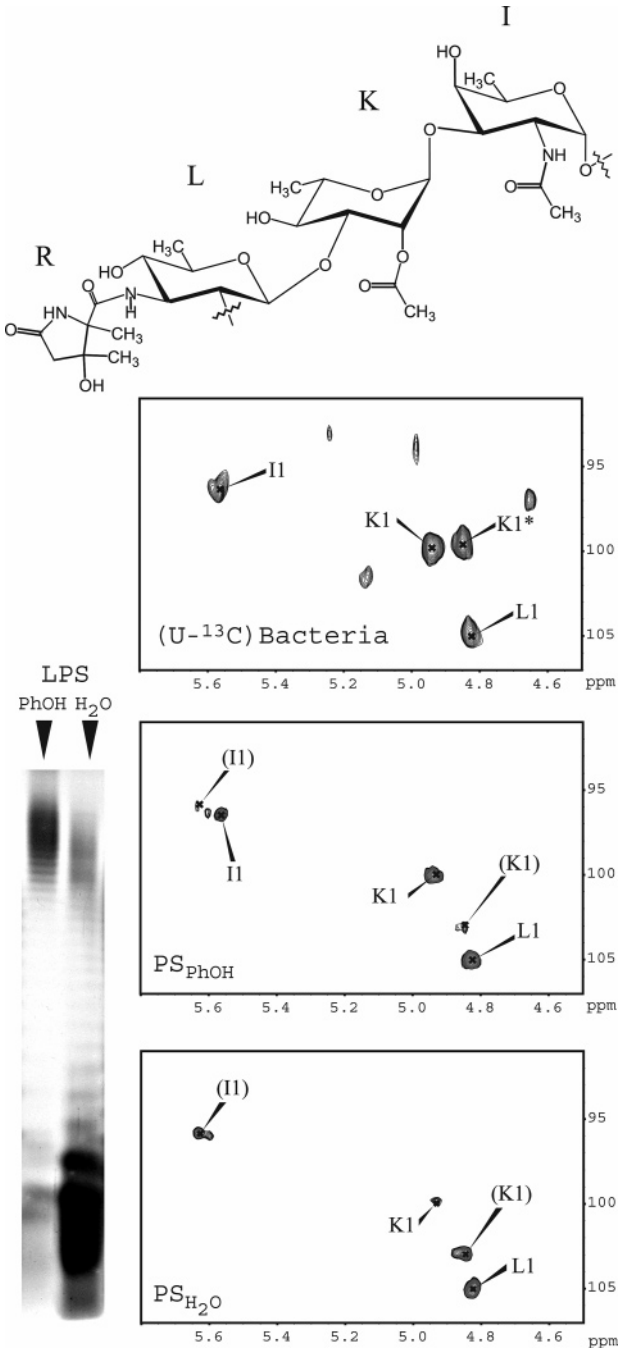



FIGURE 1: Anomeric region of the HSQC NMR spectra of the O-antigen of *P. shigelloides* O74:H5 in situ and the O-specific polysaccharides obtained from LPS from phenol and water phases and SDS−PAGE analysis of *P. shigelloides* O74:H5 lipopolysaccharide. The HSQC spectrum of intact bacteria was acquired for $^2H_2O$ suspensions of biosynthetically U-$^{13}C$-enriched bacteria with the HR-MAS technique on a Bruker DRX 600 spectrometer (5 kHz spin rate, at 23 °C). The spectra of the isolated polysaccharides were recorded for $^2H_2O$ solutions at 600 MHz and 30 °C. The uppercase letters in the anomeric region refer to designations of carbohydrate residues. The $^1H$ and $^{13}C$ resonances of the O-specific polysaccharide isolated from the phenol phase were identified in the HR-MAS HSQC NMR spectrum of intact bacteria. The uppercase letters refer to the carbohydrate residues. The chemical shift variant for the H-1 resonance of residue **K** in the microenvironment of the bacterial cell envelope is marked with an asterisk. The resonances of the PS population (minor) devoid of the O-acetyl groups are in parentheses. LPS isolated from the phenol phase and water phase, using the water/phenol method, was purified and analyzed by SDS−PAGE (5 μg/lane), using a 15% separating gel, and visualized by the silver staining method.

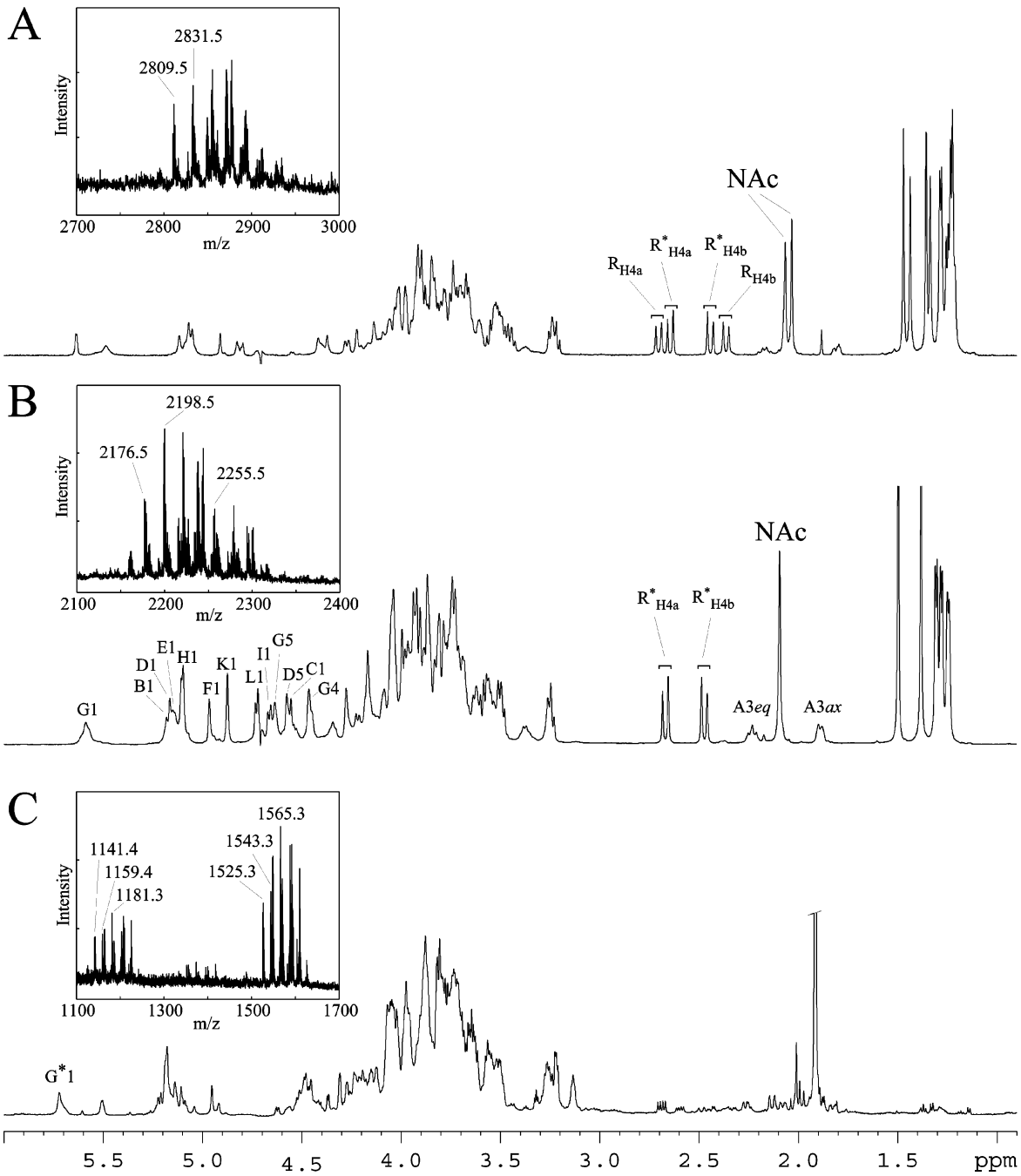



FIGURE 2: $^1$H NMR and MALDI-TOF mass spectra of the core oligosaccharides of *P. shigelloides* O74:H5. (A) PSII, core oligosaccharide substituted with two O-repeats, $[M + Na]^+$ 2809.5, $[M - H + 2Na]^+$ 2831.5; (B) OSIII, core oligosaccharide substituted with a single O-repeat, $[M + Na]^+$ 2176.5, $[M - H + 2Na]^+$ 2198.5, $[M + Gly - H + 2Na]^+$ 2255.5; and (C) OSIV, core oligosaccharide, $[M - H_2O + Na]^+$ 1525.3, $[M + Na]^+$ 1543.3, $[M - H + 2Na]^+$ 1565.3. Ions at $[M - H_2O + Na]^+$ 1141.4, $[M + Na]^+$ 1159.4, and $[M - H + 2Na]^+$ 1181.3 represent the OSIV population devoid two heptose residues. The $^1$H NMR spectra were obtained for $^2H_2O$ solutions at 600 MHz and 30 °C. The insets show the corresponding MALDI-TOF mass spectra obtained in the positive reflectron mode with 2,5-dihydroxybenzoic acid as the matrix. *m*/*z* values represent monoisotopic masses. The uppercase letters in the anomeric regions of the $^1$H NMR spectra refer to carbohydrate residues as shown in the structure, and the Arabic numerals refer to protons in the respective residue. The H-4a,4b resonances of the $CH_2$ group of **R** are designated with an asterisk when present in a single repeating unit.

Residue **L** with the H-1/C-1 signals at $\delta$ 4.81/105.0 ($J_{H1,H2} \sim 8$ Hz) was assigned as the 2-substituted $\beta$-D-Qui*p*3NHR residue on the basis of the signal of an exocyclic $CH_3$ group ($\delta$ 1.22/17.7), small chemical shifts of the C-3 signal ($\delta$ 57.1), the relatively large chemical shift of the C-2 signal ($\delta$ 74.2), and the large vicinal couplings between all ring protons. Each disaccharide element in the polysaccharide was identified by NOESY and HMBC experiments, providing the sequence of the monosaccharides. The inter-residue NOEs were found between H-1 of $\beta$-D-Qui*p*3NHR and H-3 ($\delta$ 4.04) of 2-*O*-Ac-$\alpha$-L-Rha*p*, H-1 of 2-*O*-Ac-$\alpha$-L-Rha*p* and H-3 ($\delta$ 3.70) of $\alpha$-D-Fuc*p*NAc, and H-1 of $\alpha$-D-Fuc*p*NAc and H-2 ($\delta$ 3.56) of $\beta$-D-Qui*p*3NHR. The HMBC spectra exhibited cross-peaks between H-1/C-1 of $\beta$-D-Qui*p*3NHR and H-3/C-3 ($\delta$ 4.04/78.9) of 2-*O*-Ac-$\alpha$-L-Rha*p*, H-1/C-1 of 2-*O*-Ac-$\alpha$-L-Rha*p* and H-3/C-3 ($\delta$ 3.70/77.7) of $\alpha$-D-Fuc*p*NAc, and H-1/C-1 of $\alpha$-D-Fuc*p*NAc and H-2/C-2 ($\delta$ 3.56/74.2) of $\beta$-D-Qui*p*3NHR. The spin system of the N-acyl substituent (**R**), containing a $CH_2$ group ($\delta$ 2.42, 2.71/45.5, $J_{H,H} \sim 17$ Hz) and two $CH_3$ groups ($\delta$ 1.46/18.6 and 1.35/23.1), was corroborated by TOCSY,

HSQC-DEPT, HMBC, and NOESY spectra (Table S1). The large coupling between protons of the $CH_2$ resonance together with the multiple bond correlations observed in the HMBC spectra supported the cyclic structure of the *N*-acyl group being 3-hydroxy-2,3-dimethyl-5-oxopyrrolidine-2-carboxylic acid (3-hydroxy-2,3-dimethyl-5-oxoproline). The location of this group was deduced from the inter-residue correlation between C-1 ($\delta$ 175.3) of **R** and H-3 ($\delta$ 3.98) of $\beta$-D-Qui*p*3NHR observed in the HMBC spectrum. Table S1 contains the complete $^1$H and $^{13}$C assignments of both O-acetylated and non-O-acetylated $PS_{PhOH}$ and $PS_{H_2O}$ resonances. The obtained data showed that the repeating unit of the O-specific polysaccharide has the →2)-$\beta$-D-Qui*p*3NHR-(1→3)-2-*O*-Ac-$\alpha$-L-Rha*p*-(1→3)-$\alpha$-D-Fuc*p*NAc-(1→ structure. The absolute configuration of Qui*p*3NHR was derived from the $^{13}$C glycosylation shift analysis on the basis of a small $\beta$-effect (−0.3 ppm) on C-2 of L-Rha*p* upon substitution as previously described for the $\beta$-D-Qui*p*3NHR-(1→3)-$\alpha$-L-Rha*p* disaccharide (*34*, *35*). The repeating unit of the O-deacetylated polysaccharide has the same structure as the O-specific polysaccharide of *Pseudomonas fluorescens* IMV 2366 (*35*). NMR spectra of O-deacetylated polysaccharides were compared with spectra from the native polysaccharide. The chemical shift differences demonstrated an O-acetyl group at O-2 of the Rha residue, i.e., 2-*O*-Ac-Rha, as the chemical shifts of the H-2 and C-2 signals were shifted upfield after O-deacetylation by 1.09 and 1.5 ppm, respectively. The chemical shifts of signals for the adjacent atoms such as H-1/C-1 and H-3/C-3 were also affected as expected for O-acetylation (*36*).

Signals from O-acetyl groups were only present in the NMR spectra of PSI and PS populations with fewer O-repeats. The degree of O-acetylation was determined by integration of the anomeric signals of 2-*O*-Ac-$\alpha$-L-Rha*p* in the $^1$H NMR spectra of $PSI_{H_2O}$ and $PSI_{PhOH}$, relative to the resolved resonance of a methylene proton at 2.67 ppm of the N-acyl (integral value of 1, corresponding to a single proton) present in the →2)-$\beta$-D-Qui*p*3NHR−(1→. The ratios of the integral values have shown that in the polysaccharide from the water phase 56% of the repeating units were O-acetylated whereas in that from the phenol phase 84% were O-acetylated.

The O-acetylation was never observed in the core oligosaccharide substituted with one (OSIII) and two repeating units (PSII) (Figure 2).

The similarity of the isolated O-specific polysaccharides and the O-antigens observed directly on the bacterial cells was investigated by high-resolution magic angle spinning (HR-MAS) NMR spectroscopy combined with $^{13}$C labeling (Figure 1 and Figure S1). The U-$^{13}$C biosynthetically enriched bacteria (~5 mg) were obtained from the small-scale culture, using D-[U-$^{13}$C]glucose, and analyzed in the HR-MAS HSQC NMR experiment.

The detailed assignments of the spin systems originating from O-acetylated and non-O-acetylated populations of the isolated O-specific polysaccharides were compared to these of the O-antigens observed directly on bacteria (Table S1). The chemical shift values of the O-specific polysaccharide components in situ were in agreement with those for the isolated $PS_{PhOH}$ and not those of the non-O-acetylated PS, demonstrating that the O-specific polysaccharide is present on bacteria predominantly in the O-acetylated form (Figure 1 and Figure S1). The increased O-acetylation-related heterogeneity of the isolated $PS_{PhOH}$, compared to this observed directly on bacteria, could result from the mild acidic hydrolysis, used to release the heteropolysaccharide from the lipid A part, as the procedure can lead to a possible loss of O-acetyl groups. The presence of chemical shift variants for the H-1 resonance of residue **K**, and H-2 and H-3 resonances of residue **I**, suggests that these residues might have different spatial conformations in the microenvironment of the bacterial cell envelope. Additional signals found in the HR-MAS HSQC NMR spectrum originated mainly from the lipid components of the cell envelope.

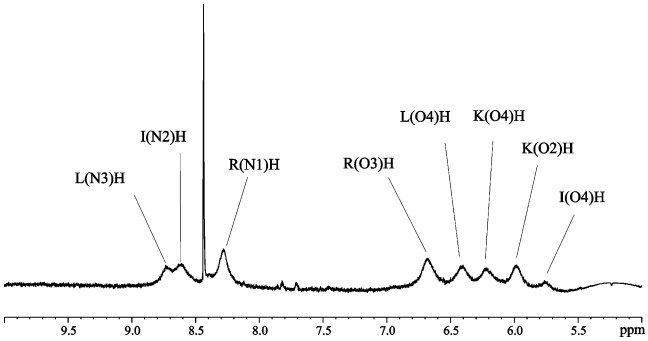



FIGURE 3: $^1$H NMR spectrum of exchangeable protons of amide and hydroxyl groups of the O-specific polysaccharide of *P. shigelloides* O74. The PSI of *P. shigelloides* O74 was O-deacetylated prior to analysis to simplify the assignment. The spectrum was obtained for a $H_2O$ solution, containing 15% acetone-$d_6$ at −10 °C. The proton assignments were corroborated by 2D NOESY experiments. The peak labels designate exchangeable protons of amide, **R** (N1)H, **I** (N2)H, and **L** (N3)H, and hydroxyl, **I** (O4)H, **K** (O2)H, **K** (O4)H, **L** (O4)H, and **R** (O3)H, groups of the respective residues.

*Assignment of Exchangeable Protons of Amide and Hydroxyl Groups*. To complete the assignment of the O-specific polysaccharide resonances, the exchangeable protons of the amide and hydroxy groups were investigated. $^1$H NMR spectra of the O-deacetylated polysaccharide were recorded. The sample in a solution of 85% $H_2O$ and 15% acetone-$d_6$ was run at a low temperature (−10 °C) to decrease the exchange rate and thus allow the observation of exchangeable protons (*37*, *38*).

The $^1$H NMR spectrum (Figure 3) exhibited signals for OH and NH protons, and these were assigned by different two-dimensional (2D) experiments. The three NH signals at 8.61, 8.73, and 8.29 ppm were assigned as (N2)H of residue **I**, (N3)H of residue **L**, and (N1)H of residue **R**, respectively. These assignments were supported by the intraresidue NOE between (N2)H of **I** and the $CH_3$ group of the N-acetyl group, between (N3)H of **L** and H-2 and H-4 of **L**, and between (N1)H of **R** and the $CH_3$ group of **R**. Only five signals were found for the OH protons from the repeating unit of the de-O-acetylated O-specific polysaccharide at $\delta$ 6.69 [(O3)H of **R**], 6.39 [(O4)H of **L**], 6.20 [(O4)H of **K**], 6.00 [(O2)H of **K**], and 5.76 [(O4)H of **I**]. Additional inter-residue NOEs observed between (N3)H of residue **L** ($\delta$ 8.73) and (N1)H of residue **R** ($\delta$ 8.29) confirmed the linkage position of this N-acyl group.

*Structure Analysis of Core Oligosaccharides OSIII and OSIV*. An initial NMR investigation indicated the presence of uronic acid and Kdo residues in oligosaccharides OSIII and OSIV; thus, all subsequent sugar and methylation

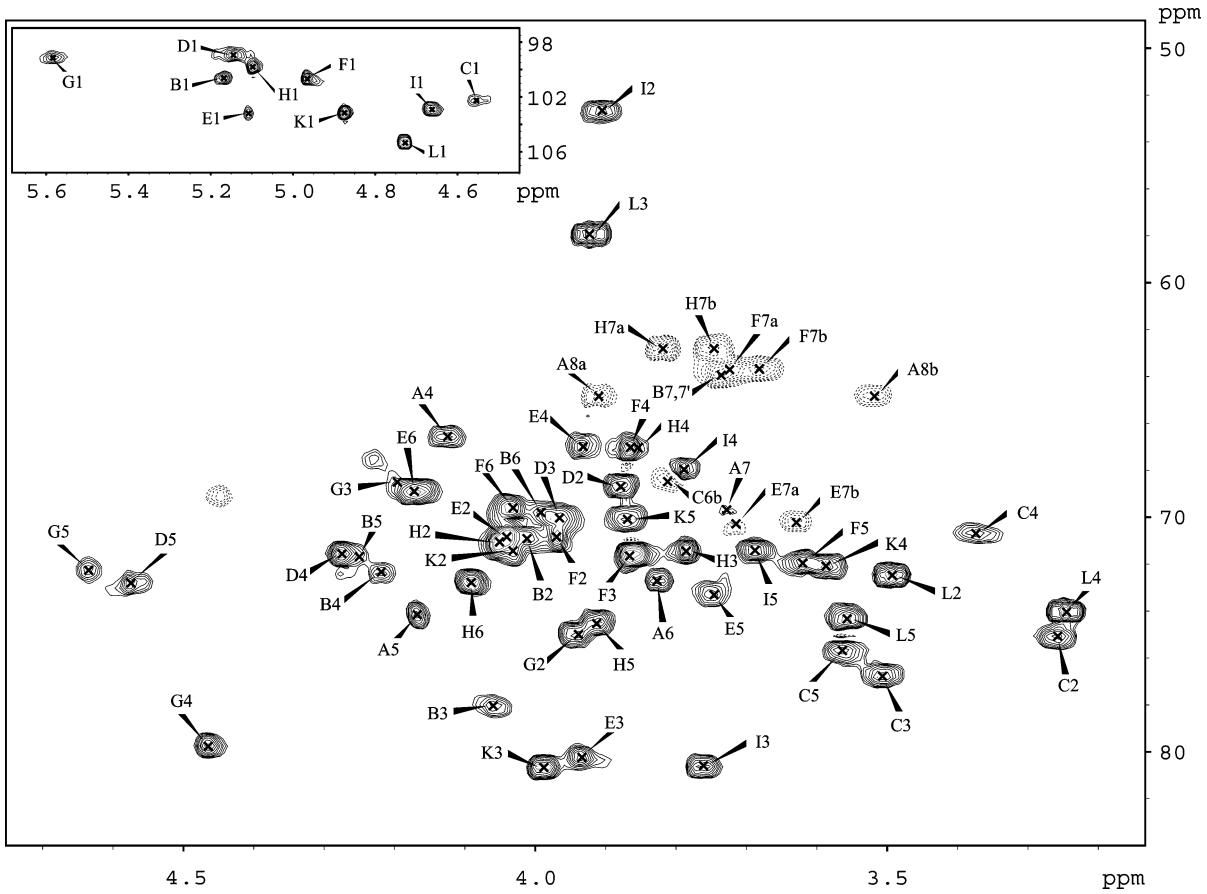



FIGURE 4: HSQC-DEPT spectrum of the OSIII oligosaccharide of *P. shigelloides* O74. The spectrum was obtained for $^2H_2O$ solutions at 600 MHz and 30 °C. The uppercase letters refer to carbohydrate residues as shown in Figure 6.

analyses were carried out on carboxyl-reduced material so that these residues could be detected. Composition analysis of carboxyl-reduced oligosaccharide OSIII together with determination of the absolute configuration revealed the presence of L,D-Hep, D,D-Hep, D-Glc, D-Gal, L-Rha, D-FucN, and D-Qui3N. Methylation analysis performed on carboxyl-reduced OSIII showed the presence of 3,7-disubstituted L,D-Hep*p*, 3,4-disubstituted L,D-Hep*p*, terminal L,D-Hep*p*, terminal D,D-Hep*p*, 2,4-disubstituted D-Gal*p*, terminal D-Gal*p*, 6-substituted D-Glc*p*, 3-substituted L-Rha*p*, 3-substituted D-Fuc*p*N, terminal D-Qui*p*3N, and 5-substituted Kdo. Core oligosaccharide OSIV was devoid of L-Rha*p*, D-Fuc*p*N, and D-Qui*p*3N but included 2-substituted D-Gal*p* instead of 2,4-disubstituted D-Gal*p*.

The MALDI-TOF mass spectra of the oligosaccharide OSIV fractions (Figure 2C) showed clusters of ions separated by 22 Da with the monosodiated monoisotopic molecules ($[M + Na]^+$) at *m*/*z* 1525.3 and 1543.3. These ions correspond to four Hep molecules, one Glc, two GalA molecules, and one Kdo, which give together a monoisotopic mass of 1520.4, and if Kdo is in the anhydro form a monoisotopic mass of 1502.4, suggesting an octasaccharide in OSIV. The mass spectrum of OSIII (Figure 2B) with a cluster at *m*/*z* 2176.5 ($[M + Na]^+$) supports an undecasaccharide structure with one repeating unit (633 Da) linked to the core oligosaccharide.

The $^1H$ (Figure 2B) and HSQC-DEPT (Figure 4) NMR spectra, recorded for main core oligosaccharide OSIII, contained signals for 10 anomeric protons and carbons, and a Kdo spin system. These signals derived from the core oligosaccharide as well as from the O-specific polysaccharide and supported an undecasaccharide as the main component. As 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, HSQC-DEPT, and HSQC-TOCSY. By comparing the chemical shifts with previously published NMR data for respective monosaccharides (*39*−*41*) and considering the $^3J_{H,H}$ values for the coupling between ring protons, estimated from the cross-peaks in the two-dimensional spectra, we could identify the sugars and determine their anomeric configuration. All the spin systems comprising $^1H$ and $^{13}C$ resonances (Table 1) were determined by applying the previously described procedures (*16*). The D-*glycero*-α-D-*manno*-heptose was differentiated from the L-*glycero*-α-D-*manno*-heptose on the basis of the chemical shifts of their C-6 resonances. The C-6 signal of L,D-Hep*p* appears in the HSQC spectrum at ∼70 ppm, whereas the C-6 signal of D,D-Hep*p* resonates at ∼72−73 ppm (*41*).

Residue **A** was identified as a 5-substituted Kdo on the basis of characteristic deoxy proton signals of H-$3_{ax}$ ($\delta$ 1.89) and H-$3_{eq}$ ($\delta$ 2.23) and a large chemical shift of the C-5 signal ($\delta$ 74.1).

Residue **B** with the H-1/C-1 signals at $\delta$ 5.17/100.7 ($J_{H-1,H-2} < 2$ Hz) was recognized as a 3,4-disubstituted L-*glycero*-α-D-*manno*-Hep*p* residue on the basis of the small vicinal couplings among H-1, H-2, and H-3 and the relatively large chemical shift of the C-3 ($\delta$ 78.1) and C-4 ($\delta$ 72.5) signals.

Table 1: $^{1}H$ and $^{13}C$ NMR Chemical Shifts of the *P. shigelloides* O74:H5 (strain CNCTC 144/92) Core Oligosaccharides[a]

| | | fraction | | chemical shift (ppm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | residue | OSIII | OSIV | H-1<br>C-1 | H-2(H-$3_{ax}$)<br>C-2 | H-3(H-$3_{eq}$)<br>C-3 | H-4(H-4a,4b)<br>C-4 | H-5<br>C-5 | H-6a,6b<br>C-6<br>$CH_3$-1 | H-7a,7b<br>C-7<br>$CH_3$-2 | H-8a, 8b<br>C-8<br>$CH_3CO$ |
| **A** | →5)-Kdo | yes | yes | | 1.89 | 2.23 | 4.12 | 4.17 | 3.82 | 3.73 | 3.52, 3.91 |
| | | | | – | 96.7 | 34.7 | 66.7 | 74.1 | 72.7 | 69.9 | 64.9 |
| **B** | →3,4)-L-α-D-Hep*p*-(1→ | yes | yes | 5.17 | 4.03 | 4.07 | 4.23 (4.34) | 4.25 | 3.99 | 3.74 | |
| | | | | 100.7 | 71.4 | 78.1 | 72.4 (72.5) | 72.1 | 69.9 | 63.9 | |
| **C** | →6)-β-D-Glc*p*-(1→ | yes | yes | 4.56 | 3.25 | 3.51 | 3.37 | 3.56 | 3.81 | | |
| | | | | 102.3 | 75.2 | 76.8 | 70.7 | 75.7 | 68.5 | | |
| **D** | α-D-Gal*p*A-(1→ | yes | yes | 5.15 | 3.88 | 3.96 | 4.28 | 4.57 | | | |
| | | | | 99.0 | 68.7 | 70.1 | 71.6 | 72.8 | 175.7 | | |
| **E** | →3,7)-L-α-D-Hep*p*-(1→ | yes | yes | 5.11 | 4.04 | 3.93 | 3.93 | 3.75 | 4.17 | 3.66, 3.72 | |
| | | | | 103.2 | 70.9 | 80.2 | 67.0 | 73.4 | 68.9 | 70.4 | |
| **F** | l-α-D-Hep*p*-(1→ | yes | yes | 4.97 | 3.97 | 3.86 | 3.86 | 3.62 | 4.03 | 3.68, 3.73 | |
| | | | | 100.7 | 70.9 | 71.7 | 67.0 | 72.1 | 69.6 | 63.7 | |
| **G** | →2,4)-α-D-Gal*p*A-(1→ | yes | | 5.59 | 3.94 | 4.16 | 4.47 | 4.63 | | | |
| | | | | 99.1 | 75.0 | 69.0 | 79.8 | 72.4 | 173.9 | | |
| **G**[b] | →2)-α-D-Gal*p*A-(1→ | | yes | 5.89 | 4.16 | 4.20 | 4.42 | 4.61 | | | |
| | | | | 99.2 | 72.5 | 68.3 | 72.0 | 74.9 | 175.9 | | |
| **H** | D-α-D-Hep*p*-(1→ | yes | yes | 5.10 | 4.04 | 3.81 | 3.86 | 3.91 | 4.09 | 3.75, 3.82 | |
| | | | | 99.9 | 70.3 | 71.6 | 67.1 | 74.5 | 72.8 | 62.8 | |
| **I*** | →3)-β-D-FucNAc*p*-(1→ | yes | | 4.67 | 3.91 | 3.76 | 3.78 | 3.69 | 1.25 | | 2.09 |
| | | | | 102.9 | 52.7 | 80.7 | 68.0 | 71.5 | 16.5 | | 23.5, 175.9 |
| **K*** | →3)-α-L-Rha*p*-(1→ | yes | | 4.88 | 4.05 | 3.99 | 3.58 | 3.87 | 1.28 | | |
| | | | | 103.2 | 71.1 | 80.7 | 72.1 | 70.1 | 17.7 | | |
| **L*** | β-D-Qui3NR*p*-(1→ | yes | | 4.73 | 3.49 | 3.92 | 3.24 | 3.56 | 1.31 | | |
| | | | | 105.4 | 72.5 | 57.9 | 74.0 | 74.4 | 18.0 | | |
| **R*** | 3-hydroxy-2,3-dimethyl-5-oxoproline | yes | | – | – | – | 2.47, 2.67 | – | 1.49 | 1.38 | |
| | | | | 175.6 | 71.8 | 78.4 | 45.7 | 179.4 | 18.7 | 23.5 | |

[a] Spectra were obtained for $^{2}H_2O$ solutions at 30 °C. Acetone ($\delta_H$ 2.225, $\delta_C$ 31.05) was used as internal reference. The chemical shifts are given as averaged values for the residues in the same environment. The chemical shifts of the O-repeat residues marked with an asterisk were different from those described for the O-specific polysaccharide fraction. For a comparison, see Figure 6 and Table S2 of the Supporting Information. [b] The residue present only in OSIV.

Residue **C** with the H-1/C-1 signals at $\delta$ 4.56/102.3 ($J_{H-1,H-2} \sim 8$ Hz) was recognized as 6-substituted β-D-Glc*p* on basis of the large vicinal couplings between all ring protons and the characteristic downfield shift of the C-6 signal ($\delta$ 68.5).

Residue **D** with the H-1/C-1 signals at $\delta$ 5.15/99.0 ($J_{H-1,H-2} < 2$ Hz) was assigned as the terminal α-D-Gal*p*A residue on the basis of the characteristic five-proton spin system, the large chemical shifts of the H-4 ($\delta$ 4.28), H-5 ($\delta$ 4.57), and C-6 ($\delta$ 175.7) signals, and the small vicinal couplings among H-3, H-4, and H-5.

Residue **E** with the H-1/C-1 signals at $\delta$ 5.11/103.2 ($J_{H-1,H-2} < 2$ Hz) was recognized as the 3,7-disubstituted L-*glycero*-α-D-*manno*-Hep*p* residue from the $^{1}H$ and $^{13}C$ chemical shifts, the small vicinal couplings among H-1, H-2, and H-3, and the relatively large chemical shifts of the C-3 ($\delta$ 80.2) and C-7 ($\delta$ 70.4) signals.

Residue **F** with the H-1/C-1 signals at $\delta$ 4.97/100.7 ($J_{H-1,H-2} < 2$ Hz) was recognized as the terminal L-*glycero*-α-D-*manno*-Hep*p* residue from the $^{1}H$ and $^{13}C$ chemical shifts, the small vicinal couplings among H-1, H-2, and H-3, and the C-6 signal at $\delta$ 69.6 as in the monosaccharide L-α-D-Hep (*41*).

Residue **G** with the H-1/C-1 signals at $\delta$ 5.59/99.1 ($J_{H-1,H-2} < 2$ Hz) was assigned as the 2,4-disubstituted α-D-Gal*p*A residue on the basis of the characteristic five-proton spin system, the large chemical shifts of H-5 ($\delta$ 4.63), H-4 ($\delta$ 4.47), and H-3 ($\delta$ 4.16), the large $^{13}C$ chemical shift of the C-2 ($\delta$ 75.0), C-4 ($\delta$ 79.8), and C-6 ($\delta$ 173.9) signals, large vicinal couplings between H-2 and H-3, and small vicinal couplings among H-3, H-4, and H-5.

Residue **H** with the H-1/C-1 signals at $\delta$ 5.10/99.9 ($J_{H-1,H-2} < 2$ Hz) was recognized as the terminal D-*glycero*-α-D-*manno*-Hep*p* residue due to the small vicinal couplings among H-1, H-2, and H-3 and the characteristic chemical shift of the C-6 signal at $\delta$ 72.8 as in the monosaccharide D-α-D-Hep (*41*).

Residue **I** with the H-1/C-1 signals at $\delta$ 4.67/102.9 ($J_{H-1,H-2} \sim 8$ Hz) was assigned as the 3-substituted β-D-Fuc*p*NAc residue on the basis of the characteristic signal of the exocyclic $CH_3$ group ($\delta_H$ 1.25, $\delta_C$ 16.5), the small chemical shift of the C-2 ($\delta$ 52.7) signal, the large chemical shift of the C-3 ($\delta$ 80.7) signal, and small vicinal couplings among H-3, H-4, and H-5.

Residue **K** with the H-1/C-1 signals at $\delta$ 4.88/103.2 ($J_{H-1,H-2} < 2$ Hz) was assigned as the 3-substituted α-L-Rha*p* residue on the basis of the characteristic signal of the exocyclic $CH_3$ group ($\delta_H$ 1.28, $\delta_C$ 17.7), the large $^{13}C$ chemical shift of the C-3 signal ($\delta$ 80.7), and small vicinal couplings among H-1, H-2, and H-3.

Residue **L** with the H-1/C-1 signals at $\delta$ 4.73/105.4 ($J_{H-1,H-2} \sim 8$ Hz) was assigned as the terminal β-D-Qui*p*3NR residue on the basis of the characteristic signal of the exocyclic $CH_3$ group ($\delta_H$ 1.31, $\delta_C$ 18.0), the small $^{13}C$ chemical shift of the C-3 ($\delta$ 57.9) signal, and large vicinal couplings between all ring protons.

The $^{1}J_{C-1,H-1}$ values, obtained from a HSQC experiment run without carbon decoupling, confirmed the α-pyranosyl

Table 2: Selected Inter-Residue NOE and $^3J_{H,C}$ Connectivities from the Anomeric Atoms of Core Oligosaccharide OSIII of *P. shigelloides* O74 (strain CNCTC 144/92)[a]

| | | atom $\delta_H/\delta_C$ H-1/C-1 | connectivity to $\delta_C$ | connectivity to $\delta_H$ | inter-residue atom/residue |
|---|---|---|---|---|---|
| **B** | →3,4)-L-α-D-Hep*p*-(1→ | 5.17<br>100.7 | | 4.17 | H-5 of **A** |
| **C** | →6)-*β*-D-Glc*p*-(1→ | 4.56<br>102.3 | 72.26 | 4.27 | C-4, H-4 of **B** |
| **D** | α-D-Gal*p*A-(1→ | 5.15<br>99.0 | | 3.82* | H-6 of **C** |
| **E** | →3,7)-L-α-D-Hep*p*-(1→ | 5.11<br>103.2 | 78.0 | 4.08* | C-3, H-3 of **B** |
| **F** | L-α-D-Hep*p*-(1→ | 4.97<br>100.7 | 70.6 | 3.64, 3.72 | C-7, H-7,7′ of **E** |
| **G** | →2,4)-α-D-Gal*p*A-(1→ | 5.59<br>99.1 | | 3.93* | H-3 of **E** |
| **H** | D-α-D-Hep*p*-(1→ | 5.10<br>99.9 | | 3.94<br>5.59* | H-2 of **G**<br>H-1 of **G** |
| **I** | →3)-*β*-D-Fuc*p*NAc-(1→ | 4.67<br>102.9 | 79.8 | 4.47 | C-4, H-4 of **G** |
| **K** | →3)-α-L-Rha*p*-(1→ | 4.88<br>103.2 | 80.7 | 3.77 | C-3, H-3 of **I** |
| **L** | *β*-D-Qui*p*3NR-(1→ | 4.73<br>105.4 | 80.7 | 4.00 | C-3, H-3 of **K** |
| **R** | 3-hydroxy-2,3-dimethyl-5-oxoproline | —<br>175.6 | | 3.92 | H-3 of **L** |

[a] The chemical shifts are given as averaged values for the residues in the same environment. The values marked with asterisks represent NOE connectivities only.

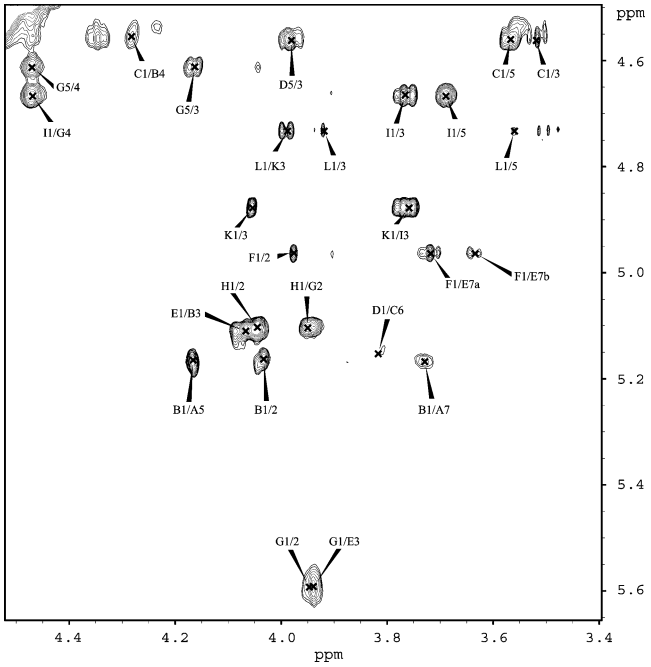



FIGURE 5: Part of the NOESY spectrum of the OSIII core oligosaccharide of *P. shigelloides* O74. The spectrum was obtained for a $^2H_2O$ solution at 600 MHz and 30 °C. The NOE connectivities were recorded with a mixing time of 200 ms. The cross-peaks are labeled as shown in Figure 6.

configuration for residues **K** (171 Hz), **G** (179 Hz), **H** (174 Hz), **F** (174 Hz), **B** (176 Hz), **E** (174 Hz), and **D** (178 Hz) and the *β*-pyranosyl configuration for residues **I** (164 Hz), **L** (163 Hz), and **C** (161 Hz).

The inter-residue connectivities between adjacent sugar residues were observed by NOESY (Figure 5 and Table 2) and HMBC (Table 2) experiments. The results identified the disaccharide elements in the core oligosaccharides and thus provided the sequence of monosaccharides in the oligosaccharide (Figure 6). For OSIII, inter-residue NOEs were found between H-1 of **L** and H-3 of **K**, H-1 of **K** and H-3 of **I**, H-1 of **I** and H-4 of **G**, H-1 of **G** and H-3 of **E**, H-1 of **E** and H-3 of **B**, H-1 of **B** and H-5 of **A**, H-1 of **H** and H-2 of **G**, H-1 of **F** and H-7,7′ of **E**, H-1 of **D** and H-6 of **C**, and H-1 of **C** and H-4 of **B**.

The HMBC spectra exhibited 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 2), which confirmed the sequence of sugar residues in the core unadecasaccharide. The results are in agreement with data from the sugar and methylation analyses. Thus, the combined results suggest the following structure of the core octasaccharide substituted with one repeating unit of the O-specific polysaccharide of *P. shigelloides* strain 144/92 (Figure 6).

The linkage between the O-specific polysaccharide, i.e., →3)-*β*-D-Fuc*p*NAc-(1→ (residue **I**), and the core structure, together with the presence of *β*-D-Qui*p*3NR-(1→ (residue **L**) instead of →2)-*β*-D-Qui*p*3NR-(1→ found in the O-repeats, demonstrated the structure of the biological repeating unit of the O-antigen.

It is worth noting that the H-4a,4b chemical shift values of the $CH_2$ group of residue **R** ($\delta_H$ 2.47, 2.67) in a single repeating unit differed from those described for the O-specific polysaccharide ($\delta_H$ 2.42, 2.73). The ratio between the characteristic outer (**$R_{H4a}$**, **$R_{H4b}$**) and inner (**$R^*_{H4a}$**, **$R^*_{H4b}$**) doublets (Figure 2) correlated with the degree of polymerization of the analyzed fractions.

In core oligosaccharide OSIII, glycine was identified by the presence of an additional carbonyl resonance at $\delta$ 169.0 in the HMBC spectrum and a negative $CH_2$ cross-peak ($H_\alpha$ $\delta$ 3.99, $C_\alpha$ $\delta$ 41.2) in the HSQC-DEPT spectrum. The presence of glycine in core oligosaccharide OSIII was further corroborated by amino acid analysis and mass spectrometry.

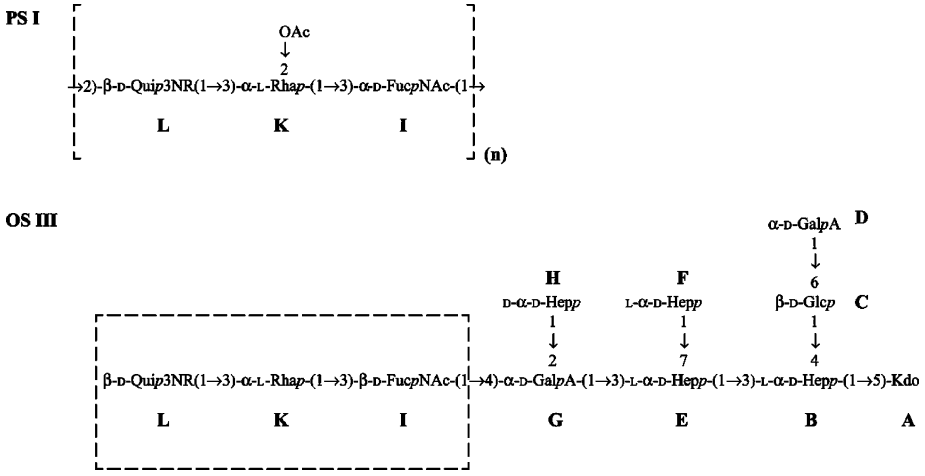



FIGURE 6: Structures of the OSIII oligosaccharide and the polysaccharide O-repeat (top) of *P. shigelloides* O74. The uppercase letters refer to carbohydrate residues. Residue **L** constitutes a nonreducing end of the biological repeating unit of the O-antigen (framed with a dashed line). Residues **A**−**H** constitute core oligosaccharide OSIV. In OSIII, residue **I** has the anomeric configuration reversed, compared with the O-repeat in the PS.

The mass difference of 57 between the $[M - H + 2Na]^+$ ions at *m*/*z* 2198.5 and 2255.5 observed in the MALDI-TOF spectrum revealed the glycinylated form of core oligosaccharide OSIII. However, a connectivity between the glycine and the oligosaccharide is only implicit as neither NOESY nor HMBC experiments showed any supporting correlation.

The $^1$H and HSQC-DEPT spectra, recorded for oligosaccharide OSIV, contained only signals that originated from the core oligosaccharide. Signals of the monosaccharides were assigned according to the procedures described for OSIII (Table 1). The main difference was the presence of →2)-α-D-Gal*p*A-(1→ (residue **G**$^b$, $\delta$ 5.89/99.2) instead of →2,4)-α-D-Gal*p*A-(1→, supporting the idea that C-4 of residue **G** is the linkage point between the core oligosaccharide and the O-specific polysaccharide.

In addition, a minor spin system identified as terminal α-D-Gal*p*A ($\delta$ 5.57/101.9) alongside that of →2)-α-D-Gal*p*A-(1→ and additional signals at $\delta$ 3.77 and 3.87/62.1 attributed to H-7a and H-7b/C-7 of the →3)-L-α-D-Hep*p*-(1→ variant of residue **E** were found. These results were in agreement with the MALDI-TOF analysis of core oligosaccharide OSIV showing additionally a minor cluster of the monosodiated monoisotopic molecule $[M + Na]^+$ at *m*/*z* 1141.4 (Kdo in the anhydro form) and 1159.4. These ions could be explained by the presence of an additional core oligosaccharide population devoid of residues **H** (D-α-D-Hep*p*) and **F** (L-α-D-Hep*p*). The structure of OSIV was further corroborated by the results of the MALDI-TOF MS and methylation analysis.

## DISCUSSION

We present here the complete structure of *P. shigelloides* lipopolysaccharide possessing a new core oligosaccharide, the structure of the biological repeating unit of the O-antigen, and the linkage between them. The detailed analysis of lipid A and its linkage to the core oligosaccharide are described in ref *51*.

The lipopolysaccharide of *P. shigelloides* serotype O74 was obtained with the hot phenol/water method; however, rather unexpectedly, the yield of the LPS obtained from the water phase ($LPS_{H_2O}$) was low, and a substantially larger amount of LPS was recovered form the phenol phase. The SDS−PAGE (Figure 1) analysis of both $LPS_{H_2O}$ and $LPS_{PhOH}$ revealed that $LPS_{PhOH}$ contained the O-specific chains with the higher number of O-repeats, showing a pattern typical for S-LPS.

The hot phenol/water extraction has been used extensively, during the LPS isolation from various Gram-negative bacteria with the majority of the S-type lipopolysaccharides recovered from the water phase. Typically it is the R-type LPS, which is retained in the phenol phase due to a higher ratio of fatty acids to carbohydrate. The lipid A preparations obtained from both $LPS_{H_2O}$ and $LPS_{PhOH}$ were analyzed by MALDI-TOF MS and exhibited corresponding ions with different intensity profiles, suggesting the same structure of lipid A, varying in the heterogeneity as described in ref *51*.

However, it was reported that S-type lipopolysaccharides from some *Acinetobacter*, *Azorhizobium*, and *Yersinia* spp. were primarily isolated as a phenol-soluble fraction (*42*−*45*). Unlike most of the S-type enterobacterial lipopolysaccharides, the O-antigens of *P. shigelloides* O74 were preferentially extracted into the phenol phase, suggesting unusual physicochemical properties of this LPS.

The NMR analysis of PSI isolated from $LPS_{PhOH}$ and $LPS_{H_2O}$ indicated that the O-specific polysaccharides share the same structure of the repeating units, possessing only a few free hydroxyl groups. The O-repeats contain one deoxy sugar (Rha), two dideoxy amino sugars, such as N-acetylated 2-amino-2,6-dideoxygalactose (Fuc*p*NAc) and 3-amino-3,6-dideoxyglucose (Qui*p*3N) N-acylated with a 3-hydroxy-2,3-dimethyl-5-oxoproline. The O-repeats differed only in a degree of O-acetylation at O-2 of the Rha*p* residue: 84% of the repeating units from $LPS_{PhOH}$ were O-acetylated, whereas 56% were O-acetylated in the O-repeats from $LPS_{H_2O}$. The deoxy and amino sugars, the N-acyl, scarce hydroxyl groups, and the O-acetyls present in both $LPS_{PhOH}$ and $LPS_{H_2O}$ all contribute to the hydrophobicity of the LPS of *P. shigelloides* O74.

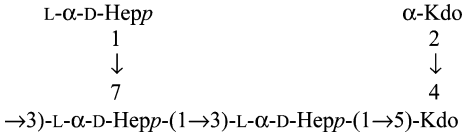
The high-resolution magic angle spinning (HR-MAS) NMR allows observation of the O-antigen carbohydrate structures directly on the surface of the bacterial cells. Moreover, the changes in the HR-MAS NMR spectra provide

immediate distinction of structures differing in the O-acetylation or other chemical modification. A HR-MAS HSQC NMR experiment combined with biosynthetic U-$^{13}$C enrichment of bacteria in a small-scale culture, using D-[U-$^{13}$C]glucose, was used to confirm the assignments of the O-specific polysaccharide components in situ. Here we have demonstrated that among the native O-antigens on the surface of *P. shigelloides* O74 the O-acetylated O-repeats prevail as the chemical shift values of the O-specific polysaccharide components in situ were in agreement with those for the isolated $PS_{PhOH}$ and not those of the O-deacetylated PS (Figure 1, Table S1, and Figure S1).

As shown by HR-MAS NMR, the more hydrophobic character of the phenol soluble LPS could be explained by a higher degree of the O-acetylation of the native O-repeats, possibly influencing the overall conformation of the O-antigens and leading to its preference for a nonaqueous surrounding.

The core oligosaccharides of Enterobacteriaceae and related families are built of the outer core, composed mainly of hexoses, and the inner core, containing the LPS-specific components, i.e., heptose and Kdo. The inner core region of enterobacteria is usually substituted with charged groups such as phosphate, pyrophosphate, 2-aminoethyl phosphate, and 2-aminoethyl pyrophosphate. The core oligosaccharides isolated from LPS of *P. shigelloides*, belonging to separate O-serotypes, differ in their structures and cross-reactivity (*16*). The core oligosaccharide of *P. shigelloides* O74, similar to that of *P. shigelloides* O54, lacks these charged groups, and unlike in the core oligosaccharides of Enterobacteriaceae, the outer and inner core parts cannot be clearly distinguished.

The core oligosaccharide of *P. shigelloides* O74 contains the same structural element




present in the majority of characterized enterobacterial and non-enterobacterial core structures. This inner core structural element is further substituted with the *β*-D-Gal*p*A-(1→6)-*β*-D-Glc*p*-(1→ disaccharide (strain O74) compared to terminal *β*-D-Glc*p* and *β*-D-Gal*p* residues in the O54 strain. The outer core region of the core oligosaccharide of *P. shigelloides* O74 is limited only to the D-α-D-Hep*p*-(1→2)-α-D-Gal*p*A-(1→ disaccharide.

The preliminary structural data obtained for four serologically nonrelated core types (data not published) suggest that none of these oligosaccharides is substituted with phosphate groups.

In the core oligosaccharides devoid of phosphate residues, the only negative charges are provided by carboxyl groups of Kdo and galacturonic acid, sufficiently stabilizing the outer membrane of *P. shigelloides*. We have determined in our preliminary studies that *P. shigelloides* strains (O54, O68, and O74) with known core structures do not release, into the liquid medium, substantial amounts of the toxic LPS complex from the outer membrane during growth. The susceptibility of these bacteria to antimicrobial agents is comparable with that of other enteropathogens such as *Salmonella* and *Shigella* possessing LPS with the typical phosphorylated core oligosaccharides (*46*−*48*).

Although *P. shigelloides* is a rare pathogen in humans, individual cases are usually serious and difficult to treat with a mortality rate exceeding 60% for septicaemia (*7*, *49*). The infections are most common but not limited to the tropical countries, and the bacteria have been isolated more often in countries with moderate or cold climate, e.g., in Sweden (*2*), in Finland (*48*), and even in a lake above the Polar Circle (*50*).

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 the identification of the terminal residue of the O-specific side chains and the linkage to the core. The →3)-*β*-D-Fuc*p*NAc-(1→ residue (residue **I***) found at the reducing end of the O-specific polysaccharide repeating unit is linked to O-4 of the →2)-α-D-Gal*p*A-(1→ residue (residue **G**) of core oligosaccharide OSIV. The anomeric configuration of that linkage is altered (*β*-configuration), compared to that of the O-specific polysaccharide (α-configuration).

The core oligosaccharide described herein represents a new structure described for the LPS of the genus *Plesiomonas*. The results obtained further support the previously described observation, suggesting the lack of a uniform core structure among the *P. shigelloides* O-serotypes.

## SUPPORTING INFORMATION AVAILABLE

The HSQC spectra of the O-antigen of *P. shigelloides* O74 on intact U-$^{13}$C-enriched bacteria (U-$^{13}$C-bacteria), obtained using HR-MAS NMR, and that of the O-specific polysaccharide isolated from the phenol phase ($PS_{PhOH}$) are compared in Figure S1. Table S1 presents the complete assignment of $^{1}$H and $^{13}$C NMR chemical shifts of the O-antigen in situ and the O-specific polysaccharides obtained from phenol and water phases of LPS. This material is available free of charge via the Internet at http://pubs.acs.org.

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