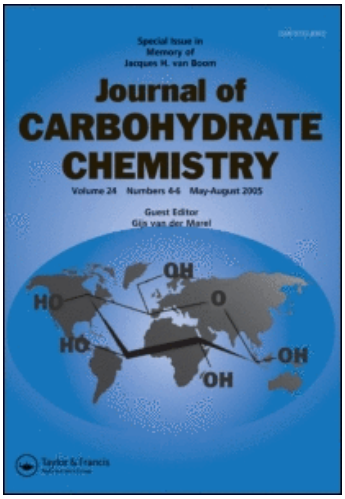

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Anna N. Kondakova[ab]; Evgeny Vinogradov[c]; Buko Lindner[b]; Nina A. Kocharova[a]; Antoni Rozalski[d]; Yuriy A. Knirel[a]

[a] N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia [b] Research Center Borstel, Leibniz Center for Medicine and Biosciences, bors, Germany [c] National Research Council, Institute for Biological Sciences, Ottawa, Canada [d] University of Lodz, Institute of Microbiology and Immunology, Lodz, Poland

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Anna N. Kondakova

N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia and Research Center Borstel, Leibniz Center for Medicine and Biosciences, Borstel, Germany

Evgeny Vinogradov

Institute for Biological Sciences, National Research Council, Ottawa, Canada

Buko Lindner

Research Center Borstel, Leibniz Center for Medicine and Biosciences, Borstel, Germany

Nina A. Kocharova

N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia

Antoni Rozalski

Institute of Microbiology and Immunology, University of Lodz, Lodz, Poland

Yuriy A. Knirel

N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia

Received March 29, 2006; accepted June 1, 2006.
Address correspondence to Anna N. Kondakova, N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospekt 47, 119991, Moscow GSP-1, Russia. E-mail: annakond@gmail.com

Enterobacteria of the genus *Providencia* are opportunistic pathogens causing diarrhea in travelers and children. Lipopolysaccharide (LPS) is the major surface antigen of *Providencia* and the major target of the immune response. O-polysaccharide structures have been elucidated in several *Providencia* O-serogroups, but little is known about the LPS core structure. We isolated core oligosaccharides from the R-type LPS of a number of *Providencia* O-serogroups and studied them by high-resolution mass spectrometry, including capillary skimmer dissociation technique; three selected oligosaccharides were analyzed also by NMR spectroscopy. The conserved inner core and variable outer core regions were distinguished and the full core oligosaccharide structures established in *Providencia* O8, O35, and O49. *Providencia* LPSs were found to share some structural features with *Proteus* LPSs.

Keywords *Providencia*, Core oligosaccharide, Lipopolysaccharide structure, Electrospray ionization mass spectrometry

INTRODUCTION

The genera *Proteus*, *Providencia*, and *Morganella* are a unique group of Gram-negative bacteria in the family Enterobacteriaceae called *Proteae*. Due to the ability to oxidatively deaminate a wide range of amino acids, these bacteria play an important role in degradation processes that occur in the natural environment. Under favorable conditions, they cause several types of infections, mainly urinary tract infections, wound infections, and enteric diseases. Bacteria of the genus *Providencia* are associated with diarrhea in travelers and children but can be found also in nondiarrheic stool specimens.[1] The genus is subdivided into five species: *P. alcalifaciens*, *P. heimbachae*, *P. rettgerii*, *P. rustigianii*, and *P. stuartii*, three of which—*P. alcalifaciens*, *P. rustigianii*, and *P. stuartii*—are serotyped based on the lipopolysaccharide (LPS, endotoxin) O-antigen and flagellar H-antigen structures.[2] Serological relationships are observed between different *Providencia* strains as well as between strains of *Providencia* and *Proteus*, *Morganella*, *Escherichia coli*, *Salmonella*, and *Shigella*.

The LPS is the major component of the outer membrane of the cell envelope of Gram-negative bacteria, which forms its outer leaflet linked to the phospholipid inner leaflet by hydrophobic interactions. The LPS is considered as a virulence factor of pathogenic bacteria, including *Providencia*.[1] The S-type LPS has an O-polysaccharide (O-antigen), which is built up of oligosaccharide repeats (O-units). A number of LPS molecules are devoid of any O-polysaccharide (R-type LPS) and in others the O-antigen is represented by one nonpolymerized O-unit (SR-type LPS). The O-antigen determines the serological O-specificity of bacterial strains. The lipid moiety (lipid A) is the endotoxic principle of the LPS, which is linked to the O-antigen via an intervening core oligosaccharide. In enteric and some other bacteria, the core oligosaccharide may be divided into two entities: the inner region, which is attached to lipid A, and the outer

region, which serves as the attachment site for the O-polysaccharide. Within the genus or family, the structure of the inner core oligosaccharide tends to be well conserved, and the fact that the core oligosaccharides from the distantly related bacteria share structural features in the inner core is a reflection of the importance of the core for the outer membrane integrity. The outer core shows more structural diversity, as it is more exposed to the selective pressures of host responses, bacteriophages, and environmental stresses.[3] It often carries determinants of the immunospecificity additional to the O-antigen (e.g., like in *Proteus* bacteria).[4] A number of O-polysaccharide structures of *Providencia* LPS have been elucidated (refs.[5,6] and refs. cited therein), but little is known about the core oligosaccharide structure.[7]

Recently,[8,9] high-resolution mass spectrometry was successfully employed for structure elucidation of the core oligosaccharides of the *Proteus* LPS. In this study, we applied electrospray ionization ion cyclotrone resonance Fourier transform mass spectrometry (ESI ICR FT MS), including positive ion capillary skimmer dissociation (CSD) fragmentation, for screening of the core oligosaccharides released by mild acid hydrolysis of the LPS from 29 *Providencia* strains representing different O-serogroups. The full structures were determined by NMR spectroscopy for selected oligosaccharides from the O-antigen-lacking R-type LPS of *Providencia* O8, O35, and O49.

MATERIALS AND METHODS

Bacterial Strains, Growth, and Isolation of LPS

Providencia reference strains came from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest) and were cultivated under aerobic conditions in tryptic soy broth supplemented with 1% glucose or 0.6% yeast extract. The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with distilled water, and lyophilized.

LPS was isolated from bacterial cells of each strain in a yield of ~5% by phenol-water extraction[10] and purified by treatment with aqueous 50% CCl_3CO_2H at 4°C as described;[11] the supernatant was dialyzed against distilled water and lyophilized.

Mild Acid Degradation of LPS

The LPS from each strain (100 mg) was hydrolyzed with 6 mL of aqueous 2% HOAc at 100°C for 2 to 3 h and a lipid precipitate was removed by centrifugation at 13,000 × *g* for 20 min. The carbohydrate portion was fractionated by gel-permeation chromatography on a column (56 × 2.6 cm) of Sephadex G-50 (S) (Amersham Biosciences, Uppsala, Sweden) in 0.05 M pyridinium acetate

buffer pH 4.5 at the flow rate of 0.5 mL · min^{-1} with monitoring by a differential refractometer (Knauer, Berlin, Germany) to give a high-molecular-mass O-polysaccharide, one or two oligosaccharide fractions, and low-molecular-mass compounds. For MS studies, the oligosaccharides were used without further purification. For NMR spectroscopic studies, the oligosaccharides from the LPS of *Providencia* O8, O35, and O49 were purified by anion-exchange chromatography on a 5 mL HiTrap Q column (Amersham Biosciences) in a gradient of $0 \rightarrow 1$ M NaCl over 1 h at a flow rate 3 mL · min^{-1} using pulsed amperometric detection (Dionex) for monitoring. Compounds were desalted by gel filtration on a column (50×1.6 cm) of Sephadex G-15 (Amersham Biosciences) as described above.

Alkaline Degradation of LPS

LPS from *Providencia* O8, O35, and O49 (20 mg each) was heated at 100°C for 4 h in 4 M KOH, cooled, and neutralized with 2 M HCl. The precipitate was removed by centrifugation and the supernatant desalted by gel filtration on Sephadex G-50 in 0.05 M pyridinium acetate buffer pH 4.5.

Chemical Analyses

For monosaccharide analysis, a sample of each oligosaccharide (1 mg) was hydrolyzed with 2 M CF_3CO_2H (120°C, 2 h), dried under a stream of nitrogen, and reduced with $NaBH_4$. After evaporation with glacial HOAc and MeOH (2×1 mL), the sample was dried, acetylated with Ac_2O (0.5 mL, 100°C, 20 min), dried, and analyzed by GLC-MS on an HP Ultra 1 column (25 m × 0.3 mm) using a Varian Saturn 2000 ion-trap instrument (Palo Alto, USA) and a temperature gradient of 180°C (3 min) to 290°C at 10°C min^{-1}. In addition, oligosaccharide samples (1 mg) were dephosphorylated by the treatment with aq 48% HF (0.5 mL) at 4°C for 16 h and, after removal of the reagent by lyophilization using a NaOH cartridge to absorb HF, were studied by GLC as described above.

NMR Spectroscopy

Samples were dried twice from D_2O prior to the measurements. ^1H and ^{13}C NMR spectra were recorded using a Varian UNITY/Inova 500 spectrometer (Palo Alto, CA, USA) for D_2O solutions at 25°C with acetone as internal standard (δ_H 2.225 and δ_C 31.5 ppm) using standard pulse sequences COSY, TOCSY (mixing time 120 ms), NOESY (mixing time 300 ms), $^1H^{13}C$ HSQC and HSQC-TOCSY (mixing time 80 ms), $^1H^{31}P$ HMQC ($^1H^{31}P$ coupling

constant value was set to 11 Hz), and HMQC-TOCSY (mixing time 80 ms). Spectra were assigned with the help of the computer program PRONTO.[12]

Mass Spectrometry

ESI ICR FT MS of oligosaccharides derived by mild acid degradation of LPS was performed in the negative ion mode using an APEX II Instrument (Bruker Daltonics, Billerica, MA, USA) equipped with a 7 Tesla magnet and an Apollo ion source. Mass spectra were acquired using standard experimental sequences as provided by the manufacturer. Mass accuracy has been checked through the external calibration. For negative ion experiments, samples ($\sim$10 ng $\cdot$ μL^{-1}) were dissolved in a 50:50:0.001 (v/v/v) mixture of 2-propanol, water, and triethylamine. For the positive ion experiments, a 30:10:0.4 (v/v/v) mixture of water, acetonitrile, and HOAc was used. Samples were sprayed at a flow rate of 2 μL $\cdot$ min^{-1}. Capillary entrance voltage was set to 3.8 kV, and drying gas temperature to 150°C. The spectra, which showed several charge states for each component, were charge deconvoluted using software provided by the manufacturer (Bruker XMASS 6.0.0), and mass numbers given refer to the monoisotopic molecular masses. In negative ion MS, the capillary exit voltage was set to -100 V and in some cases increased to -200 V for getting a better signal intensity. Positive ion CSD was induced by increasing the capillary exit voltage from -100 V to -150 or -200 V to ensure the optimal conditions for fragment ion formation.

ESI MS of core-lipid A backbone oligosaccharide obtained by alkaline degradation of LPS was performed using a Q-Star Quadropole/time-of-flight instrument (Applied Biosystems/Sciex, Concord, ON). Samples were injected using a Crystal Model 310 CE capillary electrophoresis instrument (ATI Unicam, Boston, MA, USA) coupled to a Q-Star via a microionspray interface. A sheath solution (isopropanol-methanol, 2:1) was delivered at a flow rate of 1 μL/min to a low dead volume tee. The separation were obtained on about 90 cm length bare fused-silica capillary using 10 mM ammonium acetate/ ammonium hydroxide in deionized water pH 9.0, containing 5% methanol. A voltage of 25 kV was typically applied at the injection. Mass spectra were acquired with dwell times of 2.0 S per scan in positive ion detection mode. Fragment ions formed by collision activation of selected precursor ions with nitrogen in the RF-only quadrupole collision cell were recorded by a time-of-flight mass analyzer. Collision energies were typically 120 eV (laboratory frame of reference).

RESULTS

The LPS of 32 strains representing different *Providencia* O-serogroups were hydrolyzed under mild acidic conditions to cleave the acid-labile glycosidic

linkage of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residues, including the linkage between the core and lipid A. The products were fractionated by gel-permeation chromatography on Sephadex G-50 to give one or two oligosaccharide fractions from each LPS, amounting to a total of 49 oligosaccharide samples. Eleven of them, including all fractions from *Providencia* O2, O51, and O52, were found to represent the cyclic dodecasaccharide enterobacterial common antigen (molecular mass peak at m/z 2429.9)[13–15] and were not studied further. Nine samples were found to originate from the SR-type containing the core with one O-unit attached (A fractions); their structural studies will be reported elsewhere. The remaining 29 samples, representing the core oligosaccharides derived from R-type LPS (B fractions) or a mixture of A and B fractions, were studied in this work.

Mass Spectrometric and GLC Studies of Core Oligosaccharides

Negative ion ESI ICR FT mass spectra of the fraction B oligosaccharides (Fig. 1) demonstrated their structural heterogeneity. In most cases, the

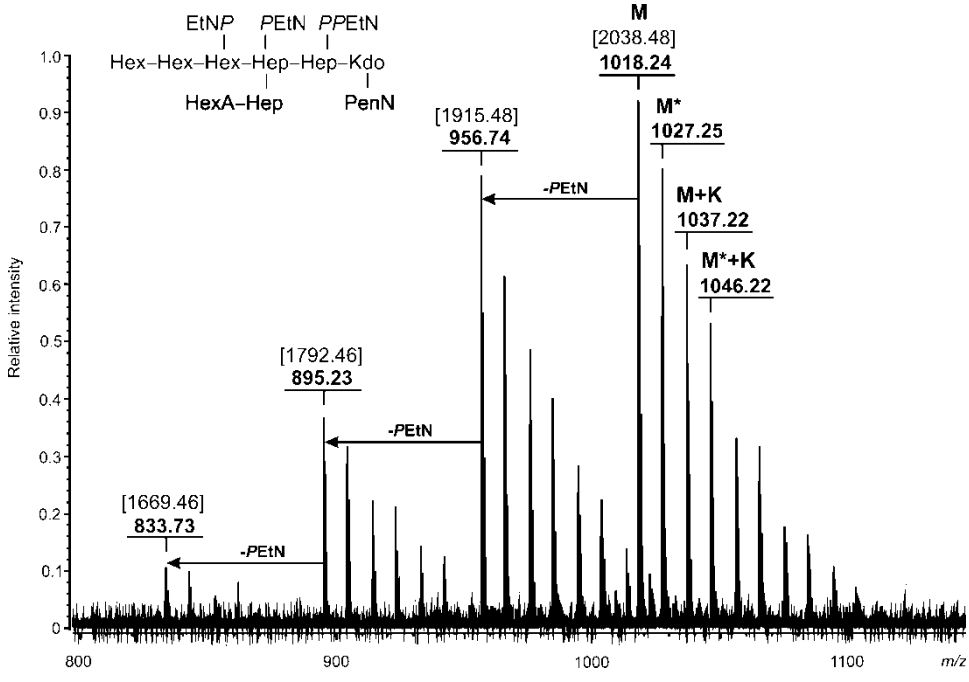

Figure 1: Part of a negative ion ESI FT-ICR mass spectrum of fraction B oligosaccharide from *P. alcalifaciens* O35. M and M* indicate peaks of doubly charged ions for oligosaccharides with anhydro-Kdo and Kdo at the reducing end, respectively. The corresponding molecular masses in Da are shown in square brackets.

spectra contained one abundant mass peak corresponding to the major core glycoform accompanied by peaks of Na- or K-adduct ions and those for compounds with Kdo in an anhydro form.[16] Most of the spectra contained molecular mass peaks in the region of m/z 1900–2600 (m/z 800–1300 as doubly charged anions in the spectra), which could be assigned to core oligosaccharides derived from the R-type LPS lacking the O-polysaccharide chain. This mass region is rather typical of the enterobacterial LPS full core, which includes a common inner core tetrasaccharide made of one Kdo and three L-*glycero*-D-*manno*-heptose (Hep) residues (Hep_3Kdo_1, m/z 796.25) bearing various substituents, including an outer core oligosaccharide and nonsugar residues.

Based on the high-resolution MS molecular mass determination, composition of core oligosaccharides in 27 samples was inferred neglecting the monosaccharide configurations (Table 1). In addition to Kdo and Hep, they contain residues of hexose (Hex), *N*-acetylhexosamine (HexNAc), hexuronic acid (HexA), pentosamine (PenN), phosphate, and 2-aminoethyl phosphate (*P*EtN). All oligosaccharides were found to contain a common $Hex_1HexA_1Hep_3Kdo_1PenN_1P_1PEtN_2$ fragment with Kdo occurring predominantly in an anhydro form (m/z 1591.37). Except for *Providencia* O8, additional residues of Hex (2 to 5), HexNAc (0 to 2), and *P*EtN (0 to 2) are also present. As judged by mass differences observed in the spectra, the most common cause of structural heterogeneity is the absence of phosphate ($\Delta m/z$ 79.97) and/or *P*EtN ($\Delta m/z$ 123.01), whereas the absence of Hex ($\Delta m/z$ 162.05), HexNAc ($\Delta m/z$ 203.08), and/or HexA ($\Delta m/z$ 176.03) is less common (Table 1). These data indicate the occurrence of incomplete core glycoforms, whereas the lack of phosphate and *P*EtN groups could result from both intrinsic heterogeneity and partial loss by cleavage of diphosphate groups during mild acid degradation of LPS.

Positive ion capillary skimmer dissociation (CSD) ESI FTICR MS of *Providencia* core oligosaccharides (Fig. 2) showed abundant fragmentation. The major pathway consisted of step-by-step loss of monosaccharide residues starting from the nonreducing end of the molecule, giving rise to a number of Y-fragments. A similar fragmentation pathway has been observed earlier in our MS studies of the *Proteus* core oligosaccharide structure.[8,9] Since CSD fragmentation is nonselective, in general, structural information could be inferred from these spectra only tentatively; the fragmentation and the presence of two different glycoforms were distinguished by comparison of normal and CSD mass spectra. However, the presence in the CSD spectra of the majority of the oligosaccharides mass peaks of the Y-fragment at m/z 1468.37 and a fragment thereof at m/z 1306.31 derived by loss of Hex confirmed the common $Hex_1HexA_1Hep_3Kdo_1PenN_1P_1PEtN_1$ fragment (calculated molecular mass 1468.36 Da) inferred by negative ion ESI FTICR MS (Table 1).

Components beyond the common fragment could be assigned to the outer core region, whose composition was inferred from the CSD fragmentation

Table 1: Composition and heterogeneity of the core oligosaccharides derived from R-type LPS of *Providencia*.

Bacterium (fraction)	Highest molecular mass compound			Other compound(s)
	M_{exp} (Da)	M_{calc} (Da)	Fragment beyond the common fragment (M_{calc}, Da)	
P. alacalifaciens O8 (B)	1591.38	1591.37	**No**	M-*P*EtN
P. stuartii O18 (AB)	1915.48	1915.47	**Hex_2** (324.11)	**M-*P*EtN**, M-*P*EtN-HexA
	2401.64	2401.63	**Hex_5** (810.26)	**M-*P*EtN**, M-*P*-*P*EtN, **M-Hex**, **M-*P*EtN-Hex**, M-*P*-*P*EtN-Hex
P. alacalifaciens O16 (AB)	2038.49	2038.48	**Hex_2*P*EtN$_1$** (447.11)	**M-*P*EtN**, M-*P*EtN_2
P. alacalifaciens O35 (B)	2038.49	2038.48	**Hex_2*P*EtN$_1$** (447.11)	**M-*P*EtN**, M-*P*EtN_2
P. alacalifaciens O3 (AB)	2077.54	2077.53	**Hex_3** (486.16)	**M-*P*EtN**
P. alacalifaciens O24 (B)	2077.54	2077.53	Hex_3 (486.16)	**M-*P*EtN**, M-*P*-*P*EtN, M-*P*EtN-Hex, M-*P*-*P*EtN-Hex
	2118.57	2118.55	$Hex_2HexNAc_1$ (527.19)	**M-*P*EtN**, M-*P*-*P*EtN
P. alcalifaciens O23 (B)	2079.53	2079.51	$Hex_1HexNAc_1$*P*EtN_1 (488.14)	**M-*P*EtN**, M-*P*EtN_2, M-*P*-*P*EtN_2
P. alacalifaciens O42 (B)	2118.55	2118.55	**$Hex_2HexNAc_1$** (527.19)	M-*P*EtN, M-Hex, M-HexNAc
P. stuartii O61 (B)	2118.57	2118.55	**$Hex_2HexNAc_1$** (527.19)	**M-*P*EtN**
P. alacalifaciens O37 (B)	2118.57	2118.55	**$Hex_2HexNAc_1$** (527.19)	M-*P*EtN, M-HexNAc
P. alcalifaciens O19 (B)	2200.55	2200.53	Hex_3*P*EtN_1 (609.16)	**M-*P*EtN**, **M-*P*EtN_2**, M-*P*EtN_3, M-*P*EtN_2-Hex
P. alcalifaciens O21 (AB)	2200.55	2200.53	Hex_3*P*EtN_1 (609.16)	**M-*P*EtN**, M-*P*-*P*EtN, M-*P*EtN_2, M-*P*-*P*EtN_2
P. alcalifaciens O30 (AB)	2280.62	2280.61	**$Hex_3HexNAc_1$** (689.24)	M-*P*, M-*P*EtN, M-Hex, M-HexNAc, M-*P*EtN-HexNAc
P. alcalifaciens O5 (AB)	2280.63	2280.61	**$Hex_3HexNAc_1$** (689.24)	M-*P*, M-*P*EtN

P. alacalifaciens O32 (B)	2323.57	2323.54	Hex_3PEtN_2 (732.17)	M-*P*EtN, **M-*P*EtN$_2$,** M-*P*EtN$_2$-Hex, M-*P*EtN$_2$-HexA, M-*P*EtN$_2$-HexNAc, M-*P*EtN$_3$-Hex
P. rustigianii O14 (B)	2403.66	2403.62	$Hex_3HexNAc_1PEtN_1$ (812.24)	**M-*P*EtN**, **M-*P*EtN-Hex**, **M-*P*EtN$_2$-Hex**, **M-*P*EtN-HexNAc**, **M-*P*EtN$_2$-HexNAc**, **M-*P*EtN-Hex-HexNAc**, **M-*P*EtN$_2$-Hex-HexNAc**, M-*P*-*P*EtN$_2$-Hex-HexNAc
P. alcalifaciens O38 (B)	2442.66	2442.66	**$Hex_4HexNAc_1$** (851.29)	M-*P*EtN
P. rustigianii O34 (B)	2483.68	2483.68	$Hex_3HexNAc_2$ (892.32)	**M-HexNAc,** M-*P*EtN-HexNAc, M-*P*EtN-HexNAc$_2$, M-Hex-HexNAc$_2$, M-*P*EtN-Hex-HexNAc$_2$
P. stuartii O43 (B)	2526.63	2526.62	$Hex_3HexNAc_1PEtN_2$ (935.25)	M-*P*EtN
P. stuartii O47 (AB)	2526.64	2526.62	$Hex_3HexNAc_1PEtN_2$ (935.25)	**M-*P*EtN**, M-*P*EtN$_2$
P. stuartii O49 (B)	2526.63	2526.62	$Hex_3HexNAc_1PEtN_2$ (935.25)	**M-*P*EtN**, M-*P*EtN$_2$
P. stuartii O55 (B)	2526.63	2526.62	$Hex_3HexNAc_1PEtN_2$ (935.25)	M-*P*EtN, **M-*P*EtN$_2$**, **M-*P*EtN$_3$**, M-*P*EtN-HexNAc, M-*P*EtN$_2$-HexNAc
P. stuartii O57 (B)	2526.64	2526.62	$Hex_3HexNAc_1PEtN_2$ (935.25)	M-*P*EtN, M-*P*EtN$_2$, **M-*P*EtN-HexNAc**, M-*P*EtN$_2$-HexNAc
P. alcalifaciens O4	2606.72	2606.69	**$Hex_3HexNAc_2PEtN_1$** (1015.32)	**M-*P*EtN**
P. stuartii O33 (B)	2606.72	2606.71	$Hex_3HexNAc_2PEtN_1$ (1015.32)	**M-*P*EtN**, M-*P*EtN$_2$
P. stuartii O44 (B)	2606.72	2606.71	$Hex_3HexNAc_2PEtN_1$ (1015.32)	**M-*P*EtN**, M-*P*EtN$_2$, M-*P*EtN-HexNAc, M-*P*EtN$_2$-HexNAc
P. stuartii O56 (B)	2606.72	2606.71	**$Hex_3HexNAc_2PEtN_1$** (1015.32)	**M-*P*EtN**, M-*P*EtN$_2$

All oligosaccharides, except for those from *P. stuartii* O20 and O26, contain a common $Hex_1HexA_1Hep_3Kdo_1PenN_1P_1PEtN_2$ fragment with Kdo predominantly in an anhydro form (*m*/*z* 1591.37). M_{exp} and M_{calc} stand for experimental and calculated monoisotopic molecular mass. 'B' refers to fractions that contain only oligosaccharides from R-type LPS (unsubstituted core) and 'AB' to fractions containing oligosaccharides from both SR-type and R-type LPS. Na- and K-adducts are not indicated. Abbreviations: Hex, hexose; HexA, hexuronic acid; HexN, hexosamine. Predominant structural variants are indicated in bold type.

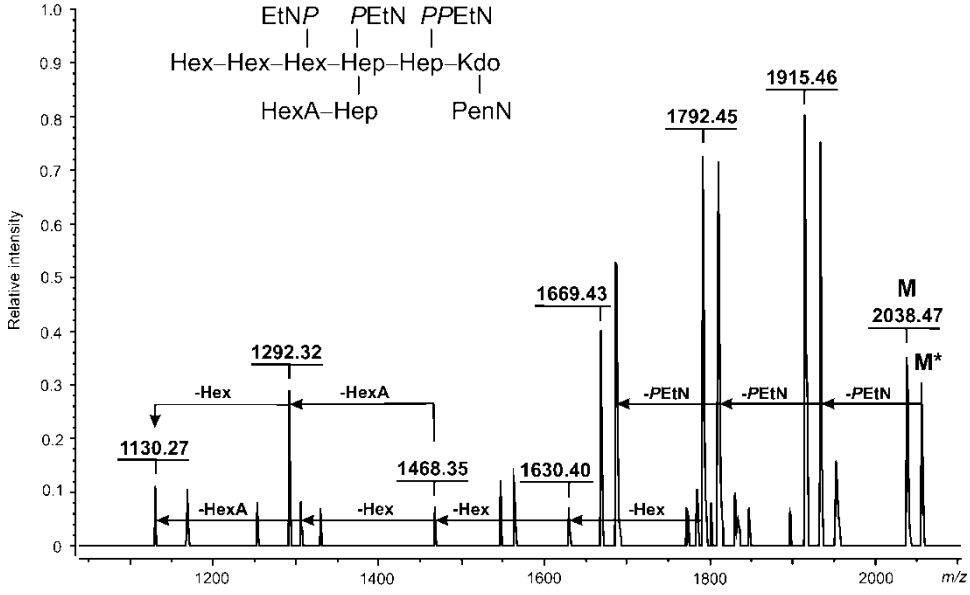

Figure 2: Charge deconvoluted positive ion CSD ESI FT-ICR mass spectrum of fraction B oligosaccharide from *P. alcalifaciens* O35. M and M* indicate peaks for oligosaccharides with anhydro-Kdo and Kdo at the reducing end, respectively.

data and was in agreement with the negative ion ESI FTICR MS data (Table 1). Most outer core sugars are Hex residues, which made impossible the CSD MS determination of the topology and monosaccharide sequence. However, when HexNAc is present, the CSD data enables discrimination between linear and branched structures. For instance, in the CSD mass spectrum of fraction B from LPS of *Providencia* O14, there was a pair of fragment ions at m/z 2077.57 and 2118.55 Da. These fragments were evidently derived from the parent ion at m/z 2403.66 by the cleavage of *P*EtN and either HexNAc or Hex, thus indicating a branching in the outer core.

Core oligosaccharides from the two LPSs of *Providencia* O20 and O26 were found to have an exceptional composition. Their negative ion ESI FTICR mass spectra showed the highest mass peak at m/z 2422.63, which could be assigned to a $Hex_4HexNAc_1Hep_3Kdo_2PenN_1P_2PEtN_2$ oligosaccharide (calculated molecular mass 2422.61 Da). If the assignment is correct, this core variant is distinguished from the typical *Providencia* core by the lack of HexA and the presence of the second Kdo residue.

Further information was obtained from GLC analysis of the alditol acetates derived from the fraction B oligosaccharides after full acid hydrolysis without and with dephosphorylation (Table 2). Particularly, the GLC data showed the presence or absence of galactose and in most cases enabled distinguishing between 2-amino-2-deoxyglucose and 2-amino-2-deoxygalactose. An increase

Table 2: Data of GLC of the alditol acetates from selected fraction B core oligosaccharides (content of monosaccharides related to Glc).

	Intact oligosaccharide					Dephosphorylated oligosaccharide				
Bacterium	**Glc**	**Gal**	**GlcN**	**GalN**	**Hep**	**Glc**	**Gal**	**GlcN**	**GalN**	**Hep**
P. alcalifaciens O23	1	2.4	0.5		0.6	1	1.9	0.6		1.4
P. stuartii O61	1	0.4	0.1		0.5	1	0.4	0.3		1.3
P. alcalifaciens O37	1		0.2		0.4	1		0.4		0.7
P. stuartii O43	1		0.2	0.2	0.5	1	0.3	0.2	0.2	0.5
P. stuartii O47 (AB)	1			0.2	0.5	1	0.3		0.2	0.7
P. stuartii O55	1			0.1	0.2	1			0.1	0.6
P. alcalifaciens O4	1	0.7	0.4		0.2	1	0.8	0.4		1
P. stuartii O33	1	0.5	0.3		0.4	1	1.0	0.4		0.7
P. stuartii O20	1				0.4	1				0.7

in the Hep content after dephosphorylation suggested that one or more Hep residues are phosphorylated. However, the exact composition of the core oligosaccharide was difficult to determine based on the MS and GLC data, and NMR spectroscopy studies were necessary to establish both composition and full structure of the core, including configurations of the monosaccharides and gycosidic linkages.

Full Structure Elucidation of the Core Oligosaccharides

Based on the MS studies, the fraction B core oligosaccharides from three strains, *Providencia* O8, O35, and O49, each of which was homogeneous in respect to monosaccharide composition, were selected for full structure elucidation. They were distinguished by different outer regions composed of one to five monosaccharides. The oligosaccharides were additionally purified by HiTrap Q anion-exchange chromatography and studied by sugar analysis and NMR spectroscopy.

***Providencia alacalifaciens* O8.** According to the MS data (Table 1), the major *P. alacalifaciens* O8 core oligosaccharide consists of the conserved Hex_1-$HexA_1Hep_3Kdo_1PenN_1P_1PEtN_2$ region only, a minority of the molecules (~20%) lacking one of the *P*EtN residues. GLC of the alditol acetates derived from the purified oligosaccharide showed a Glc:Hep ratio of 1:0.13 for the initial and 1:0.57 for the dephosphorylated oligosaccharide; a trace amount of Gal was also present.

Two-dimensional ^1H ^{13}C, and ^{31}P NMR spectroscopy, including $^1H^1H$ DQF COSY, TOCSY, NOESY, $^1H^{13}C$ HSQC, HSQC-TOCSY, gradient-enhanced HMBC, $^1H^{31}P$ HMQC, and HMQC-TOCSY experiments were employed for structure elucidation of the oligosaccharide. Assignment of the spectra was

performed essentially as described[17] using the computer program PRONTO,[12] and chemical shifts are tabulated in Table 3. The Kdo residue at the reducing end was found to occur in multiple forms, from which assignment is given for α-pyranose (**C**) and 4,7-anhydro-Kdo (**C**′). The identity of the other monosaccharides, including glucose (**H**), galacturonic acid (GalA, **K**), three heptose residues (Hep, **E**–**G**), and 4-amino-4-deoxyarabinose (Ara4 N, **Z**), was determined based on the vicinal coupling constants, which were in agreement with expected values for the sugar pyranosides[18] (the constituent monosaccharides in the *Providencia* LPS core were named as the corresponding monosaccharides in the structurally related *Proteus* LPS core[19]). The L-*glycero*-D-*manno*-configuration of the Hep residues was confirmed by the chemical shifts for C6 (δ 67.4–71.1 compared to typical δ_{C6} values of 72–73 in D-*glycero*-D-*manno*-heptose[20]).

Based on characteristic $J_{1,2}$ coupling constants values (<4 Hz for α-anomers and 7–8 Hz for ß-anomers) estimated from the two-dimensional NMR spectra, Glc **H** is α-linked, whereas GalA **K** and Ara4 N **Z** are β-linked. The α-configuration of Hep **E**–**G** was inferred based on intraresidue H1/H2 correlations in the two-dimensional NOESY spectrum, which are typical of α-linked pyranosides. The configurations of the glycosidic linkages were confirmed by the ^{13}C NMR chemical shift data, which enabled also determination of the glycosylation pattern in the core. The latter was based on downfield displacements of the linkage carbon signals, including those of Kdo **C** C8, Hep **E** C3, Hep **F** C3 and C7, and Hep **G** C7 (Table 3), which were essentially the same as in core oligosaccharides obtained by mild acid degradation of *Proteus* LPS.[20]

The following correlations between the neighboring monosaccharides were observed in the two-dimensional NOESY spectrum: Glc **H** H1/Hep **F** H3 (strong) and H-2 (weak), GalA **K** H1/Hep **G** H7 (weak), Hep **G** H1/Hep **F** H6 and H7 (both medium), Hep **F** H1/Hep **E** H3 (strong), Hep **E** H1/Kdo **C** H5 (strong) or **C**′ H5 (medium), and Hep **E** H1/Kdo **C** H7 or **C**′ H6 (both strong). Combined with the ^{13}C NMR chemical shift data, the NOESY data established the inner core carbohydrate backbone structure that is typical of many other enterobacterial LPS.[21]

The ^{1}H^{31}P HMQC spectrum of the major oligosaccharide showed correlation of a diphosphate group with H4 of Hep **E** and the CH_2O-group of one of the EtN residues at δ_P/δ_H −10.0/4.66 and −9.4/4.24, respectively. A phosphate group correlated with H6 of Hep **F** and the other EtN residue at δ_P/δ_H 1.13/4.76 and 1.13/4.19, respectively. Phosphorylation at position 6 accounts for a lower-field resonance of Hep **F** C6 at δ 71.1 compared to δ 68.4 and 67.4 for C6 of Hep **E** and **G**.

The data obtained suggested that the major core oligosaccharide obtained by mild acid degradation of the LPS of *P. alacalifaciens* O8 has the structure **1** shown in Figure 3.

Table 3: NMR chemical shifts of the major core oligosaccharides from R-type LPS.

Residue	Nucleus	1	2 (3ax)	3 (3eq)	4	5 (5a)	6 (6a, 5b)	7 (7a, 6b)	8a (7b)	8b
Oligosaccharide **1** (*P. alcalifaciens* O8)										
α-Kdo **C**	^{1}H	1.89		2.30	4.14	4.20		3.91	3.73	3.93
	^{13}C	35.2			66.9	74.2		71.7	67.3	
anhKdo **C′**	^{1}H	4.61				4.18	4.14	4.24	3.69	3.86
	^{13}C	79.7				87.4	78.1	83.7	68.8	
α-Hep4*PP* **E**	^{1}H	5.19	4.11	4.16	4.66	4.23	4.14	3.71	3.76	
	^{13}C	101.0	71.8	79.0	72.8	72.5	69.6	63.7		
α-Hep4*PP* **E′**	^{1}H	5.14	4.02	3.99	4.66	3.77	4.14	3.71	3.76	
	^{13}C	100.9	71.8	78.0	72.8	72.8	69.6	63.7		
α-Hep6*P* **F**	^{1}H	5.16	4.42	4.07	4.06	3.82	4.76	3.72	3.84	
	^{13}C	104.0	70.6	79.0	66.5	72.8	72.3	66.5		
α-Hep **G**	^{1}H	4.94	4.00	3.83	3.90	3.65	4.29	3.90	4.11	
	^{13}C	100.8	71.2	71.9	67.2	73.2	68.6	73.0		
α-Glc **H**	^{1}H	5.30	3.57	3.80	3.43	3.87	3.76	3.98		
	^{13}C	101.5	73.1	74.2	70.9	73.5	61.7			
β-GalA **K**	^{1}H	4.50	3.59	3.75	4.27	4.30				
	^{13}C	103.6	71.6	73.7	71.2	75.9	175.3			
β-Ara4N **Z**	^{1}H	5.02	3.76	4.19	3.73	3.86	4.13			
	^{13}C	99.5	69.2	66.8	53.2	59.3				
EtN on **E**	^{1}H	4.24	3.31							
	^{13}C	63.6	41.3							
EtN on **F**	^{1}H	4.19	3.31							
	^{13}C	63.4	41.3							
Oligosaccharide **2** (*P. alcalifaciens* O35)										
α-Kdo **C**	^{1}H	1.92		2.30	4.17	4.23				
	^{13}C	35.3			66.9	74.7				
anhKdo **C′**	^{1}H	4.64				4.20	4.16	4.27	3.72	3.87
	^{13}C	79.6				87.6	78.3	83.7	68.9	
α-Hep4*PP* **E**	^{1}H	5.20	4.11	4.18	4.67		4.17	3.73	3.78	
	^{13}C	101.2	71.9	79.1	72.6		69.6	63.7		

(*continued*)

Table 3: Continued.

Residue	Nucleus	1	2 (3ax)	3 (3eq)	4	5 (5a)	6 (6a, 5b)	7 (7a, 6b)	8a (7b)	8b
α-Hep4*PP* **E′**	^{1}H	5.16	4.03	4.01	4.67	3.78	4.17	3.73	3.78	
	^{13}C	100.9	71.9	77.9	72.6		69.6	63.7		
α-Hep6*P* **F**	^{1}H	5.20	4.42	4.11	4.09	3.84	4.79	3.76	3.85	
	^{13}C	103.9	70.5	78.9	66.7	72.8	72.6	66.7		
α-Hep **G**	^{1}H	4.96	4.03	3.85	3.92	3.67	4.30	3.92	4.11	
	^{13}C	100.7	71.1	72.0	67.4	73.1	68.7	72.9		
α-Glc4*P* **H**	^{1}H	5.52	3.81	4.07	4.06	3.99	3.85	4.02		
	^{13}C	98.4	76.6	71.9	75.4	72.4	61.6			
α-Glc **H′**	^{1}H	5.52	3.75	3.93	3.51					
	^{13}C	98.4	76.8	71.8	70.9					
β-GalA **K**	^{1}H	4.51	3.62	3.78	4.30	4.33				
	^{13}C	103.8	71.6	73.7	71.0	75.8	176.7			
α-Glc **I**	^{1}H	5.21	3.66	3.82	3.60	4.14	3.93	4.21		
	^{13}C	97.2	72.4	74.0	70.5	72.3	69.4			
β-Gal **J**	^{1}H	4.48	3.60	3.69	3.97	3.73	3.82	3.82		
	^{13}C	104.4	73.1	73.9	69.9	76.3	62.2			
β-Ara4 N **Z**	^{1}H	5.05	3.79	4.22	3.77	3.86	4.16			
	^{13}C	99.4	69.2	66.8	53.1	59.3				
EtN on **E** and **H**	^{1}H	4.22	3.36							
	^{13}C	63.2	41.3							
EtN on **F**	^{1}H	4.19	3.31							
	^{13}C	63.4	41.3							
Oligosaccharide **3** (*P. stuartii* O49)[a]										
anhKdo **C′**	^{1}H	4.59				4.16	4.13	4.21	3.70	3.84
	^{13}C	79.4				87.3	78.1	83.6	69.0	
α-Hep4*PP* **E**	^{1}H	5.12	4.01	3.97	4.66	3.75	4.13	3.70	3.75	
	^{13}C	100.7	71.7	77.9	72.6	72.8	69.5	63.6		

α-Hep4*P* **E′**	^{1}H			3.97	4.62	3.65			
	^{13}C			77.9	71.2	72.5			
α-Hep6*P* **F**	^{1}H	5.14	4.41	4.06	4.08	3.84	4.74	3.73	3.84
	^{13}C	103.8	70.6	78.6	66.6	73.2	72.3	66.5	
α-Hep **G**	^{1}H	4.93	3.99	3.82	3.90	3.64	4.28	3.90	4.08
	^{13}C	100.6	71.0	71.9	67.3	73.2	68.7	73.0	
α-Glc **H**	^{1}H	5.33	3.71	4.03	3.81	3.82	3.75	3.93	
	^{13}C	101.3	71.5	75.4	71.8	72.8	61.4		
β-GalA **K**	^{1}H	4.49	3.59	3.74	4.27	4.30			
	^{13}C	103.6	71.6	73.6	71.0	75.8	175.5		
α-Gal6*P* **I**	^{1}H	5.85	4.21	4.29	4.33	4.40	4.06	4.06	
	^{13}C	95.5	69.3	71.2	65.0	69.8	65.3		
α-Gal **I′**	^{1}H	5.89	4.21	4.27	4.27				
	^{13}C	95.1	69.3	72.5	65.6				
α-Glc4*P* **J**/**J′**	^{1}H	5.53	3.81	3.91	4.04	3.95	3.82	3.96	
	^{13}C	92.5	76.1/76.4	72.4	75.5	72.2	61.9		
α-Glc **L**	^{1}H	5.22	3.57	3.77	3.44	3.96	3.76	3.86	
	^{13}C	97.2	72.6	74.2	70.7	73.3	61.9		
α-GalNAc **M**	^{1}H	5.19	4.27	4.01	4.07	4.25	3.83	3.83	
	^{13}C	93.6	50.7	690	69.8	72.3	62.2		
β-Ara4N **Z**	^{1}H	5.01	3.76	4.18	3.74	3.84	4.11		
	^{13}C	99.5	69.1	66.8	53.1	59.3			
EtN on **E**, **I** and **J**	^{1}H	4.17	3.36						
	^{13}C	63.2	41.3						
EtN on **F**	^{1}H	4.23	3.31						
	^{13}C	63.5	41.3						

[a]No variant with α-Kdo was detected.
^{31}P NMR chemical shifts are: **F** P6 and EtN *P* δ 1.13 (in **1** and **2**) or δ 1.23 (in **3**), **E** *PP*4 δ −10.0 (in **1** and **2**) or δ −9.9 (in **3**), EtN *PP* −9.4 (in **1–3**), **H** *P*3 0.9 (in **2**), **I** *P*6 1.9, and **J** *P*4 1.0 (both in **3**). Chemical shifts for NAc in **3** are δ_H 2.06 and δ_C 23.3. Signals for H3 and C3 are seen for Kdo **C** but not seen for anhKdo **C′** (4,7-anhydro-Kdo) owing to exchange of protons with deuterium. All monosaccharides are in the pyranose form.

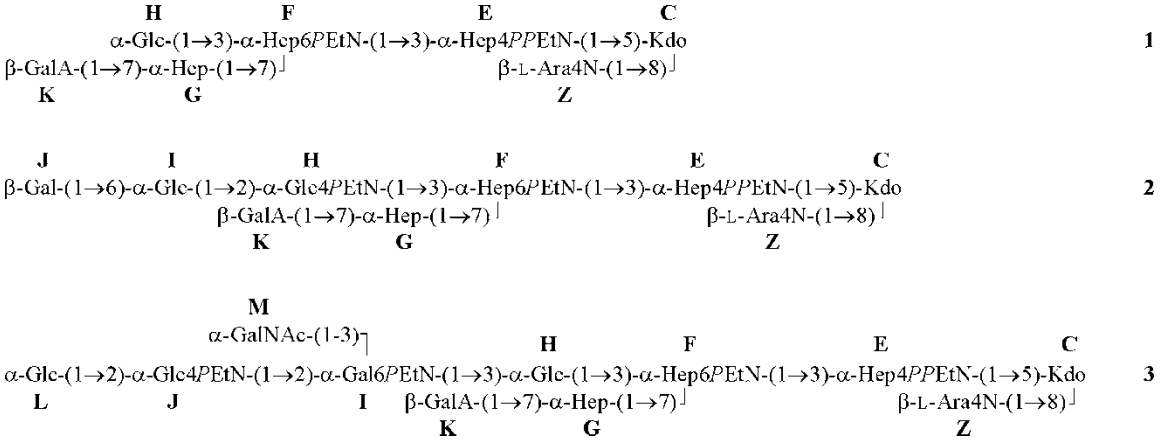

Figure 3: Structures of fraction B core oligosaccharides from R-type LPS of *P. alcalifaciens* O8 (**1**), *P. alcalifaciens* O35 (**2**), and *P. stuartii* O49 (**3**).

To ensure that no core component was lost during mild acid degradation of the LPS (except for the second Kdo residue, linked as a lateral monosaccharide to Kdo **C** in all enterobacterial LPS[21]), the LPS was degraded with 4 M KOH and the resultant core-lipid A carbohydrate backbone oligosaccharides were studied by ESI MS. The spectrum showed an abundant peak at m/z 2108.6, which corresponded to a $Glc_1Hep_3Ara4NGlcN_2GalA_1Kdo_2P_2PEtN_1$ oligosaccharide (calculated molecular mass 2108.54 Da). Taking into account the expected cleavage of the *PP*EtN group to afford a monophosphate group on Hep **E** and survival of the *P*EtN group on Hep **F** during alkaline degradation, this finding confirmed the established core structure. The presence of this oligosaccharide also demonstrated that the core is attached to a monophosphorylated lipid A backbone. In addition, a less intense peak at m/z 2239.8 indicated a compound having an additional PenN residue, whose position was not determined.

***Providenica alacalifaciens* O35.** ESI MS analysis (Table 1) showed the major core oligosaccharide having a $Hex_3HexA_1Hep_3Kdo_1PenN_1P_1PEtN_3$ composition and two minor oligosaccharides having one or two *P*EtN residues less. GLC analysis of the purified oligosaccharides showed Glc:Gal:Hep ratios of 1:0.7:0.1 for the initial and 1:0.5:0.4 for the dephosphorylated compound.

The structure of the *P. alcalifaciens* O35 core oligosaccharide was elucidated by two-dimensional $^1H^{13}C$, and ^{31}P NMR spectroscopy essentially as described above for the oligosaccharide from *P. alcalifaciens* O8 (see Table 3 for the assignment of chemical shifts). As a result, it was found that the former includes, as a part, the latter oligosaccharide, and differs in the presence of an additional terminal disaccharide fragment and an additional *P*EtN group. The disaccharide was found to consist of $1 \rightarrow 6$-interlinked β-Gal **J** and α-Glc **I** residues, from which Glc **I** is $1 \rightarrow 2$-linked to Glc **H**. This conclusion followed from downfield displacements of the signals for the Glc **I** C6 and Glc **H** C2 linkage carbons (Table 3) and the NOESY spectrum, which showed Gal **J** H1/Glc **I** H6 (medium) and Glc **I** H1/Glc **H** H2 (strong) correlations.

The $^1H^{31}P$ HMQC, and HMQC-TOSCY spectra (Fig. 4) showed a correlation of a phosphate group with either H3 or H4 of Glc **H** and CH_2O-group of EtN at δ 0.9/4.06–4.07 and 0.9/4.22, respectively, thus indicating the presence of an additional *P*EtN group in the outer core. A characteristic downfield displacement to δ 75.4 of the C4 signal of Glc **H** in the ^{13}C NMR spectrum, as compared to its position at δ 70.9 in the minor series corresponding to the nonphoshorylated Glc **H′**, allowed unambiguous determination of the additional phosphorylation site. Therefore, the major oligosaccharide from *P. alcalifaciens* O35 has the structure **2** shown in Fig. 3. About ~50% fraction B product was devoid of *P*EtN from Glc **H** (Table 3), and a minor portion lacks *P*EtN from the phosphate group of Hep **E**. The latter was confirmed by the occurrence of the major part of the Hep **E** H4/P4 cross-peak at

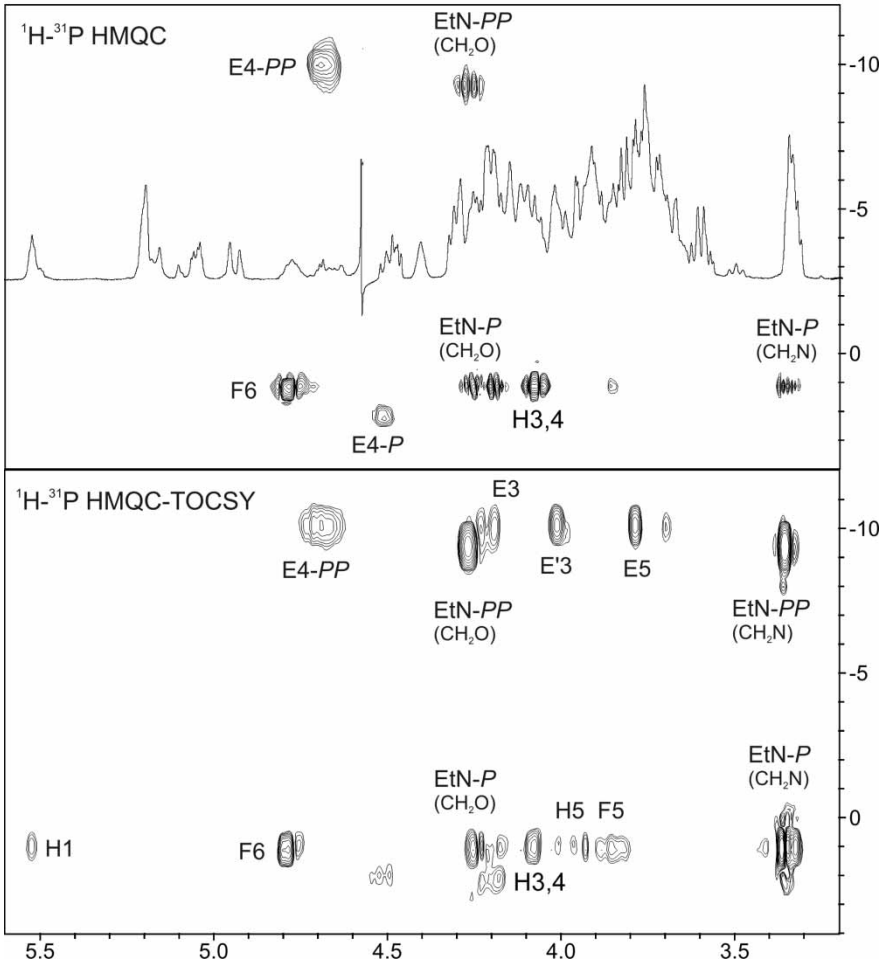

Figure 4: Parts of ^{1}H, ^{31}P HMQC, and ^{1}H, ^{31}P HMQC-TOSCY spectra of fraction B oligosaccharide **2** from *P. alcalifaciens* O35. For monosaccharide abbreviations see Table 3.

δ −10.0/4.67 and a minor part at δ 2.0/4.21, which corresponds to the *PP*EtN and *P* group at position 4, respectively.

The core structure **1** established in *P. alacalifaciens* O8 is a part of the structure **2** of the *P. alcalifaciens* O35 core.

The ESI MS of the alkaline degradation product derived form the LPS suggested an $Glc_2Gal_1Hep_3Ara4N_1GlcN_2GalA_1Kdo_2P_3PEtN_1$ oligosaccharide (mass peak at m/z 2512.8, the calculated molecular mass 2512.61 Da). Taking into account the cleavage of the *PP*EtN group on Hep **E** and *P*EtN group on Glc **H** (via formation of 3,4-cyclophosphate[22]), this finding confirmed the core structure. There was also a peak at m/z 2643.8 corresponding to a compound with an additional PenN residue at undetermined position as well

as those at m/z 2432.7 and 2563.8 due to a loss of a phosphate group from the above oligosaccharides.

***Providenica stuartii* O49.** The major *P. stuartii* O49 fraction B core oligosaccharide has a composition of $Hex_4HexNAc_1HexA_1Hep_3Kdo_1PenN_1P_1PEtN_4$. Two minor oligosaccharides were also present, one of which differs due to the presence of an additional *P*EtN group and the other one which lacks a *P*EtN group. GLC of the alditol acetates derived from the purified fraction B product showed Glc:Gal:GalN:Hep ratios of 1:0.5:0.4:0.1 for the initial and 1:0.2:0.2:0.2 for the dephosphorylated core.

Assignment of the NMR spectra of the purified product was performed as described above and the chemical shifts are summarized in Table 3. The 2D NMR correlation patterns, including the NOESY pattern, of the inner core region were essentially the same as those of *P. alcalifaciens* O8 and O35. The outer core region begins with α-Glc **H** and contains four more monosaccharides, which were identified based on coupling constants and $^1H^{13}C$ chemical shift correlation data as two more α-Glc residues (**J** and **L**), α-Gal (**I**) and α-GalN (**M**). The presence in the ^1H and ^{13}C NMR spectra of signals for an N-acetyl group at δ_H 2.06 and δ_C 23.3 indicated that GalN is N-acetylated.

The following interresidue cross-peaks were observed in the NOESY spectrum of the major oligosaccharide: Glc **L** H1/Glc **J** H1 and H2 (both medium), Glc **J** H1/Gal **I** H1 (strong) and H2 (weak), GalN **M** H1/Gal **I** H3 (overlapped with GalN **M** H2) and H4 (medium), and Gal **I** H1/Glc **H** H3 (medium). These data, which are in agreement with the ^{13}C NMR chemical shift data (Table 3), showed a branching structure with a GalNAc side chain and defined the monosaccharide sequence in the outer core region.

Two additional *P*EtN groups were found to be located at position 6 of Gal **I** and position 4 of Glc **J** as followed from correlations of the EtN-linked phosphorus to Gal **I** H6 and Glc **J** H4 at δ 1.9/4.06 and 1.0/4.04, respectively, in the $^1H^{31}P$ HMQC spectrum of the oligosaccharide. Therefore, the major core oligosaccharide from *P. alcalifaciens* O49 has the structure **3** shown in Figure 3. Again, essential part of *P*EtN was absent from position 4 of Hep **E** and position 6 of Gal **I**, the finding that accounts for the heterogeneity of the product revealed by ESI MS.

The core structure **3** includes the same inner core partial structure **1** as *P. alacalifaciens* O8 and O35, but the outer core region in the *P. alcalifaciens* O49 core (**3**) is different from that in the *P. alcalifaciens* O35 core (**2**).

The ESI mass spectrum of the alkaline treatment product from the LPS showed a mass peak at m/z 2959.2 corresponding to a $Glc_3Gal_1Hep_3Ara4N_1$-$GalN_1GlcN_2GalA_1Kdo_2P_3PEtN_2$ oligosaccharide (calculated molecular mass 2958.73 Da) with two unaffected *P*EtN groups on Hep **F** and Gal **I**. There were also three more mass peaks for oligosaccharides that lacked *P*EtN and/or contained an additional PenN residue at undetermined position.

DISCUSSION

All reference strains of *Providencia* O-serogroups studied contain R- and SR-type LPS molecules without O-antigen and with an O-antigen represented by a single O-unit, respectively. Phosphorylated oligosaccharides released from the R-type LPS by mild acid hydrolysis are heterogeneous owing to the occurrence of incomplete core variants that are devoid of some phosphate substituents or, less commonly, some monosaccharide constituents. The largest core oligosaccharide is a dodecasaccharide and the highest number of phosphate substituents is one *PP*EtN and three *P*EtN groups. The oligosaccharides share an inner core fragment, whereas the outer core region is represented by a number of structural variants. Full structures were established for the core oligosaccharides of various sizes from three selected strains.

The structural elucidation of the LPS core allowed some suggestions on the evolution of the genus *Providencia*. The data obtained showed intermediate position of the genus *Providencia* in respect to the core oligosaccharide structures. It shares some features with the so-called *Salmonella* core-type group, which is distinguished by phosphorylation of Hep **E** at position 4 with *PP*EtN (in the non-*Salmonella* group, this position is occupied by β-Glc) and attachment of α-Glc **H** at position 3 of Hep **F** (**H** is a sugar different from Glc in the non-*Salmonella* group[21]). However, the *Providencia* core resembles also that of the genus *Proteus* belonging to the non-*Salmonella* group, particularly in the glycosylation of Kdo **C** at position 8 with β-Ara4 N **Z**, phosphorylation of Hep **F** at position 6 with *P*EtN, and substitution of Hep **G** at position 7 with β-GalA **K**. This finding suggests a convergent evolution of the taxonomically related genera *Providencia* and *Proteus*, which both belong to the tribe *Proteae*.

The occurrence of a limited number of core variants within the genera *Providencia* and *Proteus* allows using the LPS core as a target for immunotherapeutic antibodies, and genes and enzymes responsible for synthesis of the conserved LPS structures as potential targets for therapeutics of new generation against a wide range of pathogens.

ABBREVIATIONS

Ara4 N	4-amino-4-deoxyarabinose
CSD	capillary skimmer dissociation
ESI ICR FT MS	electrospray ionisation ion cyclotrone resonance Fourier transform mass spectrometry
Hep	L-*glycero*-D-*manno*-heptose
Hex	hexose
HexA	hexuronic acid
HexN	hexosamine

Kdo	3-deoxy-D-*manno*-oct-2-ulosonic acid
LPS	lipopolysaccharide
PenN	pentosamine
*P*EtN	2-aminoethyl phosphate

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