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Structure of the Core Part of the Lipopolysaccharide from *Proteus mirabilis* Genomic Strain HI4320

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Abstract—The structure of the core part of the lipopolysaccharide from *Proteus mirabilis* genomic strain HI4320 was studied. Core oligosaccharide was isolated by mild acid hydrolysis of the lipopolysaccharide and analyzed by NMR spectroscopy and mass spectrometry as well as chemical methods. The structure of the oligosaccharide was established.

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Key words: lipopolysaccharide, *Proteus mirabilis*, core structure, NMR spectroscopy

Gram-negative bacteria of the genus *Proteus* belong to the Enterobacteriaceae family. The genus consists of five species, including *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. hauseri*, and *P. myxofaciens*, as well as three unnamed genomospecies 4, 5, and 6 [1, 2]. *Proteus* is widely distributed in nature and is an opportunistic pathogen causing mainly wound, urinary tract, and nosocomial infections. *Proteus* infections are often complicated by stone formation, which is enhanced by its surface polysaccharides [3].

Antigenic properties of *Proteus* strains are defined by the lipopolysaccharide (LPS) on the cell surface. The LPS contains a carbohydrate core region, which has a structurally conserved inner part substituted by various oligosaccharides. Most of the known *Proteus* core structures have been reviewed [4]. Recently, the full genome sequence has been reported for *P. mirabilis* strain HI4320 [5]. This opens new possibilities for studies of LPS biosynthesis. Here we present results of structural analysis of the LPS core of the genomic strain *P. mirabilis* HI4320, which will facilitate the identification of gene functions. The polysaccharide chain of the LPS of *P. mirabilis* HI4320 has the same structure as *P. mirabilis* O10 [6].

Abbreviations: anhMan, 2,5-anhydromannose; Ara4N, 4-amino-4-deoxy-L-arabinose; DDHep, D-*glycero*-D-*manno*-heptose; GalALys, amide of D-galacturonic acid with the 2-amino group of L-lysine; Hep, L-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LPS, lipopolysaccharide; NOE, nuclear Overhauser effect; *P*EtN, 2-aminoethyl phosphate.

MATERIALS AND METHODS

Bacterial strain and growth. *Proteus mirabilis* strain HI4320 was kindly provided by the University of Michigan Medical School, Department of Microbiology and Immunology, Mobley Lab (Ann Arbor, MI) and assigned NRCC number 6655. The culture (24 liters) was grown using Brain Heart Infusion Broth (Difco, USA) in a MBR 30-liter fermenter (Multiple Bioreactors, Switzerland) with dissolved oxygen control at 20% using variable agitation and air/oxygen blending. The 4-h culture yielded 496 g wet weight cells. Cells were inactivated by the addition of 4% phenol. Bacterial mass was harvested with a Z41 Cepa continuous centrifuge (New Brunswick, USA) to obtain a cell pellet.

Isolation and degradation of lipopolysaccharide. One hundred grams wet biomass was stirred in 400 ml water until homogenous mixture was obtained, 500 ml 95% aqueous phenol was added, and stirring was continued at 75°C for 20 min. The mixture was cooled, centrifuged at 5000 rpm, the water layer was dialyzed for one week then acidified by addition of AcOH (10% v/v), the precipitate was removed by centrifugation, and the solution was dialyzed and dried. The LPS was purified by ultracentrifugation at 120,000*g* for 4 h.

Acid degradation of the LPS was performed with 2% AcOH (100°C, 1.5 h). The polysaccharide and core fractions were separated by gel-permeation chromatography on a column (2.5 × 65 cm) of Biogel P10 (BioRad, USA) using 0.05 M pyridinium–acetate buffer, pH 4.5, as eluent; monitoring was performed using a differential refractometer. The core oligosaccharide was purified by anion-

exchange chromatography on a HiTrap Q column (5 ml; Amersham) eluted with a linear gradient of NaCl (0-1 M, 1 h). Desalting was performed on Sephadex G-15.

Deamination. LPS (60 mg) was dissolved in 4 ml water, and 0.4 ml AcOH and 100 mg $NaNO_2$ were added, then the mixture was stirred overnight and the remaining LPS was removed by ultracentrifugation. The supernatant was fractionated on a Biogel P10 column to give a polysaccharide and OS1.

NMR spectroscopy. NMR experiments were carried out on a Varian (USA) INOVA 600 MHz (^1H) spectrometer at 25°C with acetone as internal reference (2.225 ppm for ^1H and 31.45 ppm for ^{13}C) using standard pulse sequences DQ COSY, TOCSY (mixing time 120 msec), NOESY (mixing time 500 msec), and ^1H,^{13}C HSQC and HMBC (100 msec long range transfer delay). AQ time was kept at 0.8-1 sec for H–H correlations and 0.25 sec for HSQC, and 256 increments were acquired for t1.

Monosaccharide analysis. A sample (0.5 mg) was hydrolyzed with 0.5 ml 3 M CF_3CO_2H (120°C, 2 h), and the hydrolysate was evaporation to dryness under a stream of air. The residue was dissolved in water (0.5 ml), reduced with $NaBH_4$ (~5 mg, 1 h), neutralized with AcOH (0.3 ml), dried, and MeOH (1 ml) was added. The mixture was evaporated twice with the addition of MeOH, and the residue was acetylated with Ac_2O in pyridine (0.5 ml each, 100°C, 30 min), dried, and analyzed by GLC on a DB17 capillary column (30 m × 0.25 mm) using an Agilent 6850 chromatograph (Agilent Technologies, USA) equipped with a flame ionization detector using a temperature gradient from 170 to 260°C at 4°C/min and by GC-MS on a Varian Saturn 2000 ion-trap instrument (LabX, Canada) on the same column.

Methylation analysis. A core sample (0.5 mg) was dissolved in anhydrous DMSO (1 ml), dry powdered NaOH (~50 mg) was added, the mixture was stirred for 1 h, MeI (0.3 ml) was added, and after stirring for 30 min excess MeI was removed by an air stream, the remainder was diluted with water (5 ml), passed through a SepPak C18 cartridge, which was washed with water (10 ml), the methylated product was eluted with MeOH (5 ml), and converted conventionally into the alditol acetates using $NaBD_4$ and analyzed by GC-MS.

Determination of the absolute configuration. OS1 was treated with 0.2 ml of (*R*)-2-butanol and 0.02 ml of acetyl chloride (90°C, 3 h), dried under the stream of air, acetylated, and the products analyzed by GC-MS in a temperature gradient from 150 to 250°C at 2°C/min. Standards were prepared from L-lysine, D-GalA, and D-GlcNAc with (*R*)- and (*S*)-2-butanol in the same way.

RESULTS AND DISCUSSION

LPS was isolated from bacterial mass by phenol–water extraction and purified by ultracentrifugation. Mild acid hydrolysis of the LPS afforded a polysaccharide and a core oligosaccharide. The core was significantly contaminated by protein and nucleic acid fragments and was further purified by anion-exchange chromatography. Monosaccharide analysis of the core using GC of the alditol acetates revealed similar amounts of glucose, L-*glycero*-D-*manno*-heptose (Hep), D-*glycero*-D-*manno*-heptose (DDHep), and a ~5 times smaller amount of glucosamine (GlcN). Methylation analysis using GC of the partially methylated alditol acetates showed the presence of terminal glucose, terminal DDHep, 7-substituted Hep, 3,4-disubstituted Hep, and 3-substituted GlcN.

For structure elucidation, two-dimensional NMR spectra of the core (DQ COSY, TOCSY, NOESY, ^1H-^{13}C HSQC, ^1H-^{13}C HMBC) were recorded. Owing to the exact overlap of three H-1 signals (residues I, T, and Y), the core structure could not be determined straightforwardly from these spectra. A high-field position at 3.28 ppm of the signal for H-2 of GlcN (residue X) in the NMR spectra showed that its amino group is not acylated. This opened a possibility to cleave a part of the structure by deamination. LPS was treated with $NaNO_2$ in 10% AcOH, insoluble products containing a lipid part were removed by ultracentrifugation, and the soluble material was fractionated by gel chromatography on Biogel P10 to give a minor amount of the polysaccharide and an oligosaccharide (OS1), which was further purified by anion-exchange chromatography.

OS1 showed clear NMR spectra without any overlaps, and spin systems of three monosaccharides were recognized, including β-GalA (T), β-GlcNAc (Y), and 2,5-anhydromannose (X, a deamination product of GlcN). In addition, OS1 contained a derivative of lysine with the amino group at position 6 replaced by a hydroxyl group by deamination. The spectra were assigned (table) and the structure presented in Fig. 1 was inferred. Identities of GalA and GlcNAc were deduced from characteristic vicinal coupling constants and ^{13}C NMR signal positions. Lysine was found to be linked to the carboxyl group of β-GalA by its 2-amino group, which was confirmed by a correlation between H-2 of Lys and C-6 of GalA revealed by an HMBC experiment.

After the identification of the outer core structure, which included two sugars with overlapping H-1 signals, the spectra of the core were completely assigned (table), except for signals of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), which was present in multiple degraded forms (Fig. 2). The location of 4-amino-4-deoxy-L-arabinose (Ara4N) at position 8 of Kdo followed from the fact that there was no other attachment site in this molecule and was confirmed by a comparison with published data of other *Proteus* strains having a Ara4N-8-Kdo fragment in the core ([4] and references cited therein). Vicinal coupling constants, signal positions, and NOE patterns of all spin systems in the spectra were in agreement with the proposed structure. Phosphorylation of O-6 of Hep F was

NMR data (chemical shifts, ppm) for the core and, where indicated, OS1 (D_2O, 25°C, 600 MHz)

Unit	H1 C1	H2 C2	H3 C3	H4 C4	H5 C5	H6a, b C6	H7a, b C7
GalA T, OS1	4.52 104.5	3.57 69.8	3.72 73.3	4.22 70.1	4.23 75.5	 172.1	
GlcNAc Y, OS1	4.61 102.5	3.89 56.0	3.87 82.5	3.56 71.4	3.54 76.8	3.80, 3.96 61.9	
anhMan X, OS1	5.07 90.7	3.72 85.3	4.17 78.3	4.04 77.7	3.99 82.5	3.74, 4.07 70.1	
Lys*, OS1	 176.9	4.32 55.1	1.77, 1.87 32.0	1.40 22.5	1.57 32.0	3.59 62.6	
Hep E	5.13-5.21 99.6	3.97 71.1	4.01 74.8	4.29 74.7	4.42 75.3	4.14 69.2	3.75, 3.75 63.8
Hep F	5.31-5.35 102.4	4.19 70.5	4.07 78.0	4.06 67.1	3.74 72.3	4.69 73.4	3.72, 3.86 69.5
Hep G	4.92-4.96 101.1	3.99 71.1	3.84 71.9	3.90 67.1	3.66 73.1	4.31 68.4	3.87, 4.09 73.2
Glc I	4.52-4.56 103.7	3.29-3.32 74.9	3.52 76.6	3.37 70.9	3.41 77.5	3.73, 3.89 62.6	
GalA H	5.71 98.2	4.11 71.6	4.25 67.7	4.65 79.6	4.62 71.4	 176.0	
DDHep L	5.14 97.9	3.98 71.1	3.87 71.7	3.75 68.3	3.93 74.4	4.02 72.6	3.73, 3.83 62.8
GlcN X	5.17 97.2	3.28 55.3	3.89 70.4	3.49 70.4	4.46 72.2	3.83, 4.13 68.9	
GlcNAc Y	4.55 102.5	3.92 56.0	3.85 83.2	3.59 69.8	3.52 76.6	3.80, 3.96 62.0	
GalA T	4.55 104.8	3.56 71.4	3.74 73.4	4.22 70.2	4.28 75.6	 172.3	
GalA K	4.50 103.7	3.59 71.4	3.76 73.4	4.29 70.8	3.74 73.4	 171.8	
Ara4N Z	5.01-5.05 99.6	3.76 69.0	4.18 66.7	3.72 53.0	3.81, 4.12 59.3		
Lys	 176.7	4.51 53.4	1.82, 1.97 31.5	1.46 22.9	1.70 27.3	3.00 40.3	
EtN	4.20 63.4	3.32 41.3					

Note: The signals for NAc are at 2.07/23.5 ppm. Lys* stands for $HO(CH_2)_4CH(NH)CO_2H$.

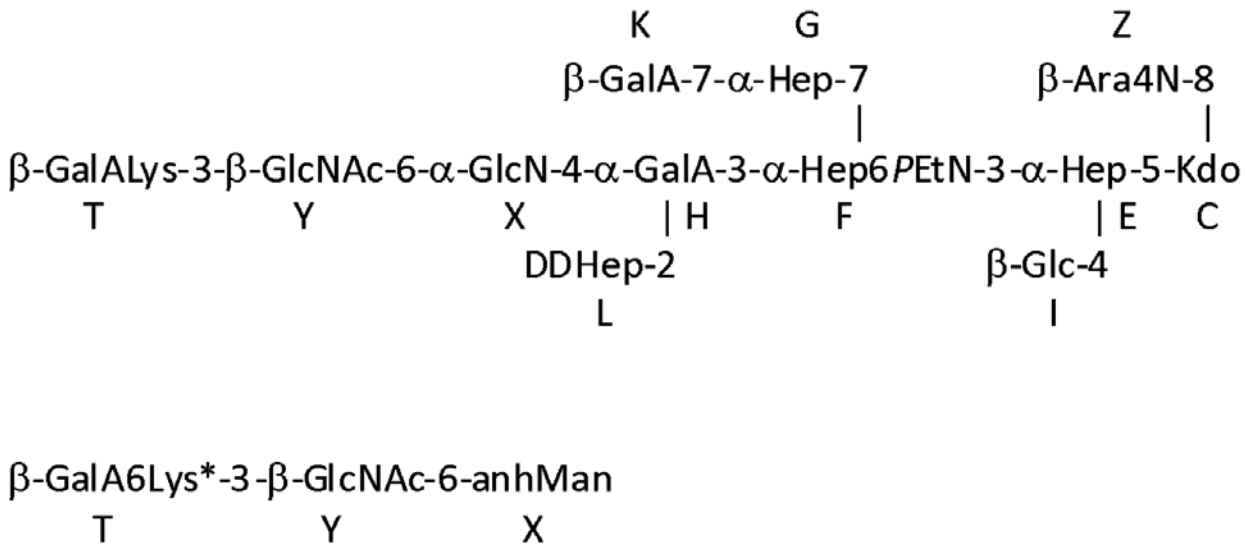

Fig. 1. Structure of the core oligosaccharide released by mild acid degradation (top) and OS1 obtained by deamination (bottom) of the LPS of *P. mirabilis* HI4320. All monosaccharides are in the pyranose form. Lys* stands for $HO(CH_2)_4CH(NH)CO_2H$.

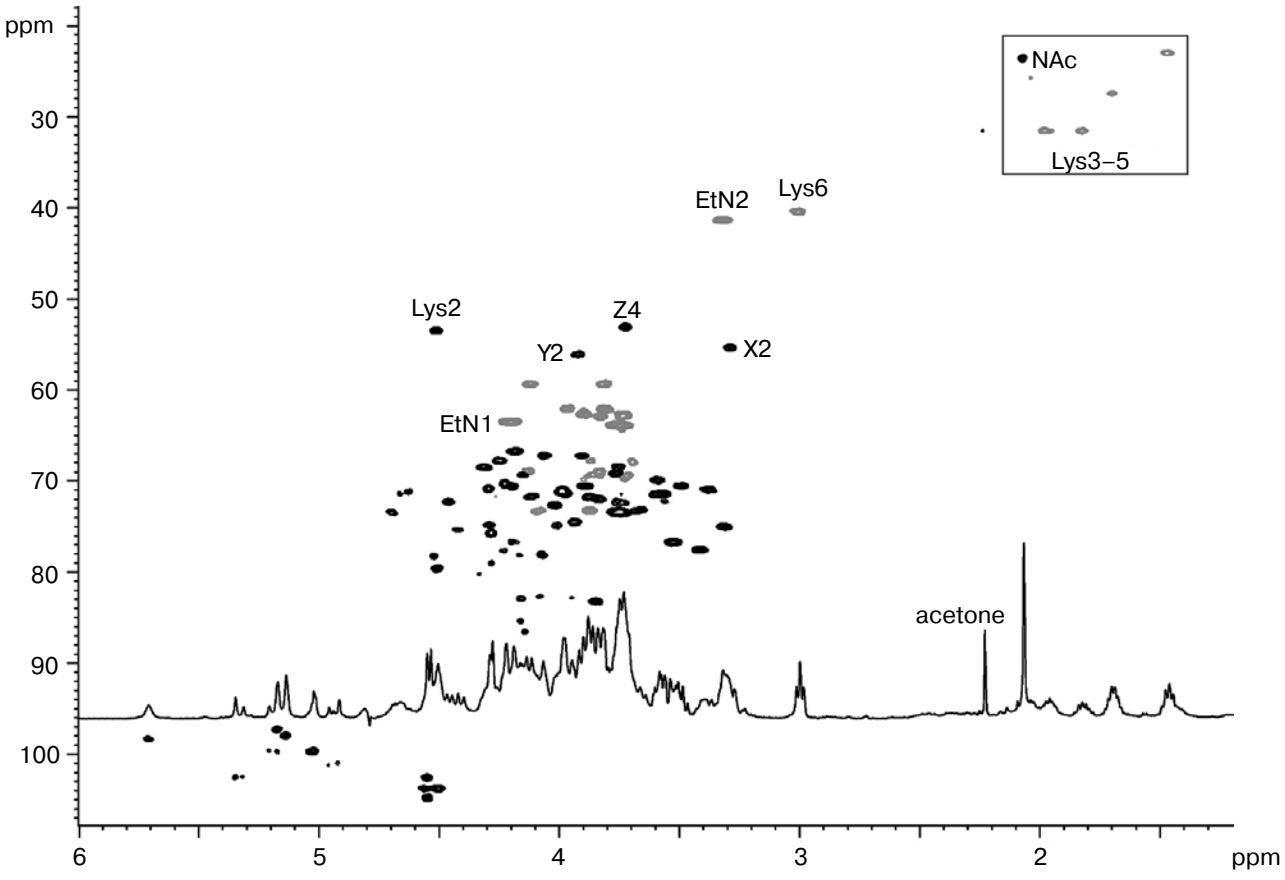

Fig. 2. ^1H NMR spectrum and two-dimensional $^1H,^{13}C$ HSQC spectrum of the core oligosaccharide obtained by mild acid hydrolysis of the LPS of *P. mirabilis* strain HI4320. Cross-peaks of CH and CH_3 groups are shown in black, CH_2 groups in gray.

confirmed by a ^{1}H-^{31}P correlation (a ^{31}P signal was at −1.6 ppm) and a low-field position of the H-6 signal of Hep F (table).

The core structure was confirmed by methylation data (see above). The absence of any derivatives of Hep F among methylation products is accounted for by phosphorylation; no Ara4N derivative was seen too, which is usual for this sugar; no attempt to identify derivatives of uronic acids was made. The structure was in agreement also with the negative mode ESI mass spectrum of the core, which showed one main component with the molecular mass of 2425.2 Da (the calculated molecular mass is 2424.77 Da).

Absolute configuration of D-GalA, D-GlcN, and L-Lys in the OS1 were determined using GC-MS of acetylated glycosides and/or esters with optically active 2-butanol. Absolute configurations of the monosaccharides from inner core region are presented as they were identified before [4].

The structure of the core part of *P. mirabilis* HI4320 contains a specific β-GalALys-3-β-GlcNAc (T-Y) disaccharide, which has not been found in other *Proteus* strains analyzed to date. The rest of the structure is typical of the LPS core of *Proteus* [4]. Deamination of the LPS gave a small amount of a free polysaccharide, which had the same ^{1}H NMR spectrum as the polysaccharide released in a much higher quantity by mild acid hydrolysis of the LPS [6]. Therefore, the polysaccharide chain of the LPS is not linked to the core part that was released by deamination.

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