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Structure of a lactic acid ether-containing and glycerol phosphate-containing O-polysaccharide from *Proteus mirabilis* O40

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Abstract—An O-polysaccharide was isolated by mild acid hydrolysis from the lipopolysaccharide of *Proteus mirabilis* O40 and studied by NMR spectroscopy, including 2D ^{1}H, ^{1}H COSY, TOCSY, ROESY, and ^{1}H, ^{13}C HMQC experiments, along with chemical methods. The polysaccharide was found to contain an ether of GlcNAc with lactic acid and glycerol phosphate in the main chain and to have the following structure:

→ 3)-β-D-Glc*p*NAc4(*R*-Lac)-(1 → 3)-α-D-Gal*p*-(1 → 3)-D-Gro-1-*P*-(O → 3)-β-D-Glc*p*NAc-(1 →

where D-Glc*p*NAc4(*R*-Lac) stands for 2-acetamido-4-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-D-glucose. This structure is unique among the known structures of the *Proteus* O-polysaccharides, which is in agreement with the classification of the strain studied into a separate O-serogroup. A serological relatedness of *P. mirabilis* O40 with some other *Proteus* strains was revealed and discussed in view of the O-polysaccharide structures.

Keywords: *Proteus mirabilis*; Lipopolysaccharide; O-polysaccharide structure; Glycerol phosphate; Lactic acid ether

1. Introduction

Bacteria of the genus *Proteus* from the family *Enterobacteriacae* are facultative human pathogens responsible for a number of infections in wounds, burns, skin, eyes, ears, nose, and throat, and are one of the most important urinary tract pathogens.[1,2] The role of *Proteus* surface antigens in the pathogenesis of rheumatoid arthritis has also been suggested.[3] The lipopolysaccharide (LPS) is considered as an important virulent factor of *Proteus*. The polysaccharide chain (O-polysaccharide or O-antigen) and sometimes the core region of the LPS define the serological specificity of these bacteria. Currently, *Proteus* strains are classified into about 80 O-serogroups. In most O-serogroups, the O-polysaccharide is acidic due to the presence of uronic acids, aldulosonic acids, amino acids, phosphate, and other acidic non-sugar components.[4] In this paper, we report a unique structure of the O-polysaccharide of *P. mirabilis* O40, which resembles glycerol teichoic acids and contains an ether of GlcNAc with lactic acid.

2. Results and discussion

The lipopolysaccharide was extracted from the bacterial cells of *P. mirabilis* O40 by the phenol/water procedure[5] and degraded with dilute acetic acid at 100 °C to give a

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Table 1. ^{1}H NMR data (δ, ppm)

Residue	H-1	H-2	H-3	H-4	H-5	H-6
→3)-β-Gal*p*-(1→	4.92	3.87	3.88	4.15	4.04	3.69
→3)-Gro-1-*P*-(O→	3.89	3.90	3.58, 3.73			
→3)-β-Glc*p*NAc^{I}-(1→	4.69	3.78	4.14	3.35	3.44	3.75, 3.95
→ 3)-β-Glc*p*NAc^{II}-(1→ 4 ↑	4.64	3.87	4.07	3.50	3.49	3.80, 3.93
Lac		4.70	1.47			

The chemical shifts for NAc are δ 2.03 and 2.07.

high-molecular mass O-polysaccharide isolated by GPC on Sephadex G-50.

The ^{1}H NMR spectrum of the polysaccharide (Table 1) contained, inter alia, signals for three anomeric protons at δ 4.64, 4.69 (both doublets, $J_{1,2}$ 8 Hz) and 4.92 (d, $J_{1,2}$ 3 Hz), a CH_3–CH group at δ 1.47 (d, J 7 Hz) and 4.70 (q) as well as two *N*-acetyl groups at δ 2.03 and 2.07. The ^{31}P NMR spectrum showed one signal for a phosphate group at δ −0.8. The spectrum of the polysaccharide (Fig. 1, Table 2) contained signals for three anomeric carbons at δ 99.7, 101.1, and 103.6, two nitrogen-bearing carbons at δ 56.4 and 56.5, five HO*C*H_2–C groups at δ 61.9–62.4, 67.6, and 69.4 (data of a DEPT experiment), a *C*H_3–C group at δ 19.2, 12 oxygen-bearing sugar-ring carbons at δ 68.5–80.5, and two *N*-acetyl groups at δ 23.5 and 23.6 (both CH_3) and δ 175.4 (2 CO). These data and, particularly, the total number of the ^{13}C NMR signals and the number of signals for the HO*C*H_2–C groups suggested that the repeating unit of the polysaccharide consists of three sugar residues and two non-sugar substituents, most likely, glycerol phosphate and an ether-linked lactic acid.

Monosaccharide analysis of the polysaccharide by GLC of the alditol acetates derived after full acidic hydrolyses revealed Gal and GlcN. GLC of the acetylated (*S*)-2-octyl glycoside[6] demonstrated that both monosaccharides have the D configuration. For determination of the absolute configuration of the glycerol residue (Gro), the polysaccharide was dephosphorylated, and Gro was oxidized to D-glyceric acid, which was identified by GLC of the (*S*)-2-octyl ester.[7] Analysis using an amino acid analyzer showed, in addition to

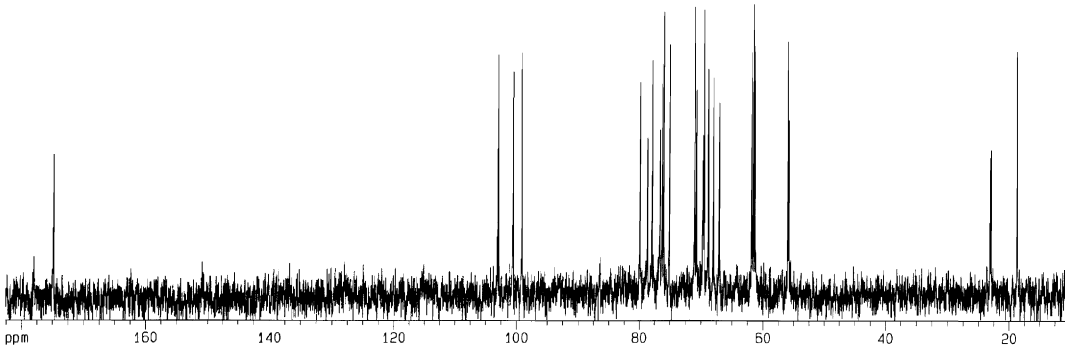


Figure 1. 125 MHz ^{13}C NMR spectrum of the O-polysaccharide of *P. mirabilis* O40.

Table 2. ^{13}C NMR data (δ, ppm)

Residue	C-1	C-2	C-3	C-4	C-5	C-6
→3)-β-Gal*p*-(1→	99.7	68.5	80.5	70.0	70.2	62.1
→3)-Gro-1-*P*-(O→	67.6	71.6	69.4			
→3)-β-Glc*p*NAc^{I}-(1→	101.1	56.4	79.2	71.3	76.6	62.4
→ 3)-β-Glc*p*NAc^{II}-(1→ 4 ↑	103.6	56.5	78.4	76.8	75.6	61.9
Lac	178.6	77.2	19.2			

The chemical shifts for NAc are δ 23.5 and 23.6 (Me), 175.4 (CO).

GlcN, the presence of another amino component, which had the same retention time as the authentic sample of 2-amino-4-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-D-glucose [D-GlcN4(*R*-Lac)] and different from that of D-GlcN4(*S*-Lac).

The ^{1}H and ^{13}C NMR spectra of the polysaccharide were assigned using 2D ^{1}H, ^{1}H COSY, TOCSY, and H-detected ^{1}H, ^{13}C HSQC experiments (Tables 1 and 2). Spin-systems for one Gal*p* and two Glc*p*NAc residues were identified based on the characteristic $J_{2,3}$, $J_{3,4}$ and $J_{4,5}$ coupling constant values estimated from the COSY and TOCSY spectra, and on the correlations of the protons at the nitrogen-bearing carbons to the corresponding carbons at δ 3.78/56.4 and δ 3.87/56.5 observed in the ^{1}H, ^{13}C HSQC spectrum. In accordance with their *gluco* configuration, both Glc*p*NAc residues showed correlations of H-1 with H-2,3,4,5,6 in the COSY and TOCSY spectra, whereas correlations with H-2,3,4 only was observed for Gal*p*. A $J_{1,2}$ coupling constant of 8 Hz showed that both Glc*p*NAc residues are β-linked, whereas a smaller $J_{1,2}$ value for Gal*p* (3 Hz) indicated its α configuration. Two more spin-systems were revealed for glycerol and lactic acid (Table 1).

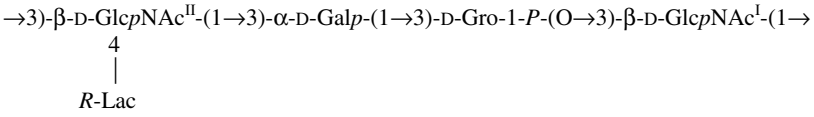
The ^{1}H, ^{31}P HMQC spectrum of the polysaccharide revealed correlations between the phosphate signal and the signals for H-1 of Gro at δ −0.8/3.89 and H-3 of one of the GlcNAc residues ($GlcNAc^{I}$) at δ −0.8/4.14. The corresponding ^{13}C NMR signals for Gro C-1 and $GlcNAc^{I}$ C-3 showed small but significant downfield displacements by 3–4 ppm due to phosphorylation as compared with their positions in the spectra of the corresponding non-substituted residues.[8] Therefore, Gro and $GlcNAc^{I}$ are interlinked via the phosphate group.

In the ROESY spectrum, the H-2 signal of the lactic acid residue showed a correlation to the H-4 signal of $GlcNAc^{II}$ at δ 4.70/3.50, and the $GlcNAc^{II}$ C-4 signal was shifted downfield by 5.5 ppm due to etherification. These data indicated that $GlcNAc^{II}$ bears the lactic acid residue at position 4 and thus confirmed the presence of GlcNAc4(*R*-Lac).

In addition, downfield displacements by 3–7 ppm were observed in the ^{13}C NMR spectrum for the signals of the linkage carbons, namely, for C-3 of Gal, C-3 of $GlcNAc^{II}$, and C-3 of Gro, which revealed the glycosylation pattern in the repeating unit. In the ROESY spectrum, the following intra-residue correlations were observed between anomeric protons and protons at the linkage carbons: $GlcNAc^{I}$ H-1, $GlcNAc^{II}$ H-3 at δ 4.69/4.07; $GlcNAc^{II}$ H-1, Gal H-3 at δ 4.64/3.88; and Gal H-1, Gro H-3a, 3b at δ 4.92/3.58, 3.73. These data are in agreement with the ^{13}C NMR chemical shift data and defined the monosaccharide sequence in the repeating unit.

The absolute configuration of $GlcNAc^{II}$, and thus the presence of D-GlcN4(*R*-Lac), was confirmed by the absence of any glycosylation effect on the C-4 signal of the 3-substituted Gal residue and a relatively large effect (7.5 ppm) on the C-1 signal of the glycosylating $GlcNAc^{II}$ residue, which are characteristic of the same configuration of these β-(1→3)-linked monosaccharides, that is, the D configuration of $GlcNAc^{II}$. Indeed, a relatively high by the absolute value (>2 ppm) negative effect on Gal C-4 and a much smaller (<4 ppm) effect on $GlcNAc^{II}$ C-1 would be observed in the case of different absolute configurations of the monosaccharides.[9] The same monosaccharide, D-GlcN4(*R*-Lac), has been earlier identified in the O-polysaccharide of *P. vulgaris* O15[10] and its isomer, D-GlcN4(*S*-Lac), in the O-polysaccharide of *P. penneri* 41 and 65 from serogroup O62,[11,12] whereas, to the best of our knowledge, neither of the derivatives have been reported as constituents of another bacterial polysaccharides.

Based on the data obtained, it was concluded that the O-polysaccharide of *P. mirabilis* O40 has the following structure:



A number of *Proteus* lipopolysaccharides with known O-polysaccharide structure were tested with rabbit polyclonal anti-*P. mirabilis* O40 serum in enzyme immunosorbent assay (EIA). As expected, the homologous LPS showed the strongest reaction (titer of antibodies 1:1,024,000), a weaker reaction was observed with the LPS of *P. vulgaris* O15 and *P. penneri* 19 (titers 1:256,000 and 1:64,000), and a very week cross-reactivity with *P. mirabilis* O57 and *P. vulgaris* O25 (titers 1:2000 and 1:1000, respectively). The LPS and isolated O-polysaccharide from *P. mirabilis* O40 inhibited the reaction in EIA in the homologous test-system *P. mirabilis* O40 LPS/anti-*P. mirabilis* O40 serum at minimal inhibiting doses of 2.2 and 78 ng, respectively. As expected, the heterologous antigens were significantly less active inhibitors (312 and 625 ng for *P. penneri* 19 and 625 and 1250 ng for *P. vulgaris* O15, respectively). In

Western immunoblotting, anti-*P. mirabilis* O40 serum reacted strongly with both slow- and fast-migrating bands of the homologous LPS, which correspond to high- and low-molecular-mass LPS species, respectively. The pattern of the former bands was characteristic for smooth-type LPS with a variable number of repeating units in the O-chain. A faint staining was observed also for high-molecular-mass LPS species of *P. vulgaris* O15 and *P. penneri* 19.

The serological relatedness of *P. mirabilis* O40 and *P. vulgaris* O15 could be accounted for by the occurrence in their O-polysaccharides of a common →3)-β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc4(*R*-Lac) disaccharide fragment (Fig. 2). The cross-reactivity between anti-O-*P. mirabilis* O40 serum and *P. penneri* 19 LPS (anti-Western blot, Fig. 3) could be due to similar trisaccharide fragments in the main chains of the O-polysaccharides, whereas structural similarities between the O-polysaccharides of *P. mirabilis* O40, *P. mirabilis* O57, and *P. vulgaris* O25 are more remote (Fig. 2). Therefore, the serological data combined with the uniqueness of the established O-polysaccharide structure show the expediency of classification of *P. mirabilis* O40 into a separate *Proteus* O-serogroup.

3. Experimental

3.1. Bacterial strain, growth, and isolation of the lipopolysaccharide

P. mirabilis O40 and O57, *P. vulgaris* O15 and O25 were from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague). *P. mirabilis* O40 (strain PrK 66/57) was cultivated under aerobic conditions in a fermenter (Chemap AG, Switzerland) in nutrient broth (BTL, Poland) under the controlled conditions (37 °C, pH 7.4–7.6, pO2 75–85%). Cells were harvested at the end of the logarithmic growth phase, centrifuged (5000*g*, 30 min), washed with distilled water and lyophilized. The LPS was isolated by the phenol–water procedure[5] and purified by treatment with DNase and RNase (Boehringer Mannheim, Germany) as described.[13] The LPS preparations obtained

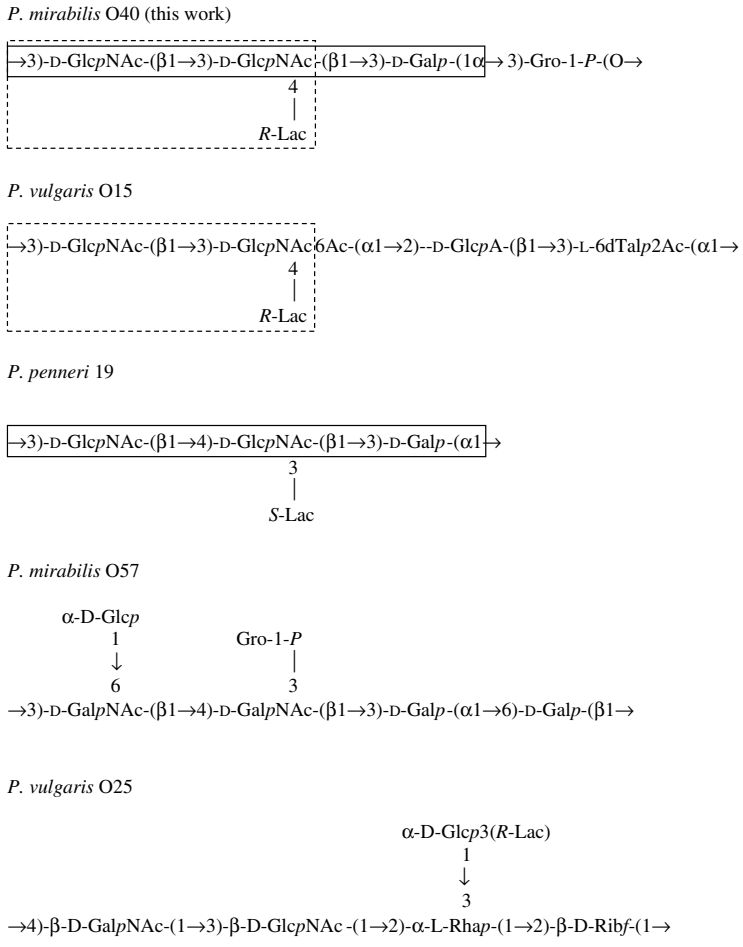


Figure 2. Structures of the *Proteus* O-polysaccharides. Putative common epitopes are shown in frames.

Figure 3. Anti-parallel Western blot of the lipopolysaccharides of *P. mirabilis* O40 (lane 1), *P. vulgaris* O15 (lane 2), and *P. penneri* 19 (lane 3) with anti-*P. mirabilis* O40 serum.

were essentially free of nucleic acid and contained <2.5% proteins.

3.2. Degradation of the lipopolysaccharide

The LPS (90 mg) was hydrolyzed with aq 2% HOAc at 100 °C for 1.5 h, and a lipid precipitate was removed by centrifugation at 13,000*g*. The carbohydrate portion was fractionated by GPC on Sephadex G-50 in 0.05 M pyridinium acetate buffer pH 4.5 with monitoring by a Knauer differential refractometer (Germany) to give a high-molecular mass polysaccharide (28.5 mg).

3.3. Sugar analysis

The polysaccharide was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h). Monosaccharides were identified by GLC of the alditol acetates[14] using a Hewlett–Packard 5989A instrument (USA) equipped with a HP-5 column and a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C min^{-1}. Amino sugars were identified using a Biotronic LC-2000 amino acid analyzer (Germany) equipped with a column (0.4 × 22 cm) of Ostion LG AN B cation exchange resin at 65 °C using 0.2 and 0.35 M sodium citrate buffers pH 3.25 and 5.25, respectively. Authentic samples of D-GlcNAc4(*R*-Lac) and D-GlcNAc4(*S*-Lac) were obtained from the O-polysaccharides of *P. vulgaris* O15[10] and *P. penneri* 41,[12] respectively.

The absolute configurations of the monosaccharides were determined by GLC of the acetylated (*S*)-2-octyl glycosides[6] using a Hewlett–Packard 5880 instrument (USA) with a DB-5 column and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C min^{-1}. The absolute configuration of glycerol was established by oxidation of the dephosphorylated (aq 48% HF, 4 °C, 16 h) polysaccharide in the presence of 2,2,6,6-tetramethyl-1-piperedinyloxy radical (TEMPO) as described[7] followed by acid hydrolysis and GLC of the acetylated (*S*)-2-octyl glycoside.

3.4. NMR spectroscopy

Samples were deuterium-exchanged by freeze-drying two times from D_2O and then examined as solutions in 99.96% D_2O at 50 °C. Spectra were recorded on a Bruker DRX-500 spectrometer (Germany) equipped with an SGI INDY computer workstation. 2D NMR spectra were obtained using standard Bruker software, and XWINNMR 2.6 program (Bruker, Germany) was used to acquire and process the NMR data. The parameters used in 2D experiments were essentially the same as described previously.[15] A mixing time of 200 and 100 ms was used in TOCSY and ROESY experiments, respectively. Chemical shifts are reported related to internal acetone (δ_H 2.225; δ_C 31.45).

3.5. Serological techniques

Rabbit polyclonal anti-*P. mirabilis* O40 serum was obtained, and EIA and inhibition tests were performed, as described.[16] LPS was used as antigen. Prior to use, an LPS stock solution (1 mg mL^{-1}) in phosphate-buffered saline was shaken thoroughly and sonicated. SDS-PAGE was performed according to the procedure of Laemmli[17] using 4% stacking gel and 12.5% resolving gel. The gels were stained with silver nitrate (Sigma, USA) according to Tsai and Frasch[18] or electroblotted onto nitrocellulose sheets (Schleicher & Schuell, Germany) by tank blotting as described[16] for immunoblotting with dilute (1:250) polyclonal anti-*P. mirabilis* O40 serum. The secondary antibodies used in immunoblotting were the same as in EIA, and the color development reagent was 4-chloro-1-naphtol (Sigma, USA) with H_2O_2 as substrate.

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