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Carbohydrate Research 340 (2005) 2305–2310
Carbohydrate RESEARCH

Structure of the O-polysaccharide and serological studies of the lipopolysaccharide of *Proteus mirabilis* 2002

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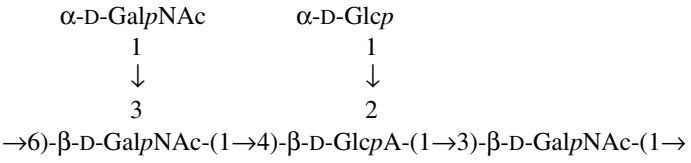
Received 31 May 2005; received in revised form 2 July 2005; accepted 2 July 2005
Available online 8 August 2005

Abstract—The structure of the O-polysaccharide of the lipopolysaccharide of *Proteus mirabilis* 2002 was elucidated by chemical methods and ^{1}H and ^{13}C NMR spectroscopy. It was found that the polysaccharide consists of branched pentasaccharide repeating units having the following structure:



The O-polysaccharide of *P. mirabilis* 2002 has a common tetrasaccharide fragment with that of *P. mirabilis* 52/57 from serogroup O29, and the lipopolysaccharides of the two strains are serologically related. Therefore, based on the structural and serological data, we propose to classify *P. mirabilis* 2002 into the *Proteus* O29 serogroup as a subgroup O29a,29b.

Keywords: Proteus mirabilis; Lipopolysaccharide; O-polysaccharide structure; O-serogroup; Epitope; Serological classification

1. Introduction

Proteus mirabilis is one of the species of the genus *Proteus*, Gram-negative bacilli from the family *Enterobacteriaceae*. These bacteria are facultative pathogens causing urinary tract infections, which can result in severe complications, such as catheter obstructions and the formation of bladder and kidney stones.[1,2] Currently, the genus *Proteus* is divided into five named species, including *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. myxofaciens* and *P. hauseri*, and three unnamed *P.* genomospecies 4, 5 and 6.[3,4]

The immunospecificity of these bacteria is defined by the O-polysaccharide chain (O-antigen) of the lipopolysaccharide (LPS), which is the major component of the outer membrane. Based on the O-antigens, two species, *P. mirabilis* and *P. vulgaris*, have been divided into 60 O-serogroups.[5–7] A number of further O-serogroups have been proposed for *P. penneri*[8–12] and *P. myxofaciens*.[13]

In this paper, we report the structure of an acidic O-polysaccharide from the nonclassified strain *P. mirabilis* 2002 and, based on structural and serological data, propose to classify it as a subgroup of an existing *Proteus* serogroup, O29.

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doi:10.1016/j.carres.2005.07.014

2. Results and discussion

2.1. Elucidation of the O-polysaccharide structure

Sugar analysis of the O-polysaccharide after full acid hydrolysis, including determination of the absolute configurations of monosaccharides, revealed D-glucose and 2-amino-2-deoxy-D-galactose (D-GalN) in a ratio 1:2.7 as well as D-glucuronic acid (D-GlcA).

The ^{13}C NMR spectrum of the O-polysaccharide (Fig. 1) contained signals for five anomeric carbons in the region δ 94.6–104.6, three carbons linked to nitrogen (C2 of GalN residues) in the region δ 50.8–52.2, 17 other sugar ring carbons in the region δ 69.4–81.6, four HO*C*H$_2$–C groups (C6 of Glc and GalN) at δ 61.9–62.4 and 67.9 (data of a DEPT experiment), one C–*C*OOH group (C6 of GlcA) at δ 174.3, and three *N*-acetyl groups at δ 23.5–23.9 (CH$_3$) and δ 176.0 (CO, 3C). Accordingly, the ^{1}H NMR spectrum of the O-polysaccharide contained, inter alia, signals for five anomeric protons in the region δ 4.56–5.28, three *N*-acetyl groups at δ 2.06 (3CH$_3$) and other sugar ring protons in the region δ 3.44–4.24. Therefore, the O-polysaccharide is composed of pentasaccharide repeating units containing one residue each of D-Glc and D-GlcA and three residues of D-GalNAc (designated as GalNAc^{I}–GalNAc^{III} according to their sequence in the repeating unit, see below).

The ^{1}H and ^{13}C NMR spectra of the O-polysaccharide were assigned using 2D homonuclear COSY, TOCSY, ROESY, and H-detected ^{1}H, ^{13}C HSQC and HMBC experiments (Table 1). The spin systems for Glc and GlcA were identified by correlations of H-1 with H-2 to H-6 and H-2 to H-5, respectively, in the TOCSY spectrum. For the GalNAc residues, the TOCSY experiment demonstrated correlations of H-1 with H-2 to H-4, the ROESY experiment of H-4 with H-3 and H-5. The HMBC experiment revealed the correlations of H-5 with C-6 for GalNAc^{II} and GalNAc^{III}. Therefore, the remaining signals were assigned to H-6 and C-6 of GalNAc^{I}.

The relatively small $J_{1,2}$ coupling constant values of ~3 Hz, determined from the ^{1}H NMR spectrum, showed that Gal and GalNAc^{III} are α-linked, whereas $J_{1,2}$ values of 7–8 Hz indicated that the other sugar residues are β-linked. The absence from the ^{13}C NMR spectrum of any signal for non-anomeric ring carbons at a lower field than δ 82 demonstrated the pyranose form of all monosaccharide residues.[14]

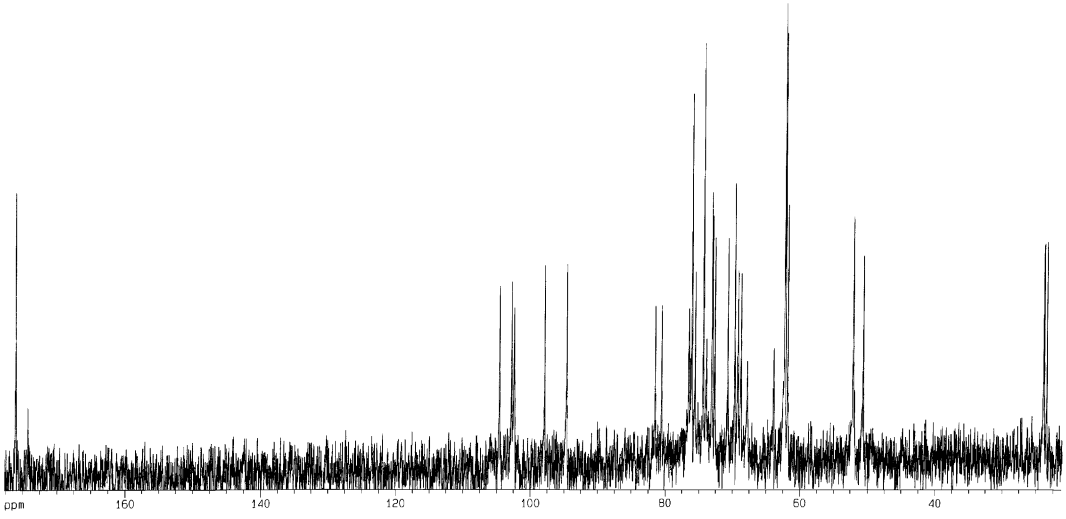


Figure 1. ^{13}C NMR spectrum of the O-polysaccharide of *P. mirabilis* 2002.

Table 1. ^{1}H and ^{13}C NMR data of the polysaccharide (δ, ppm)

Sugar residue	1	2	3	4	5	6
→3,6)-β-D-Gal*p*NAc^{I}-(1→	4.58	4.02	3.78	4.13	3.74	3.89, 3.97
	102.5	52.2	75.6	64.1	74.6	67.9
→2,4)-β-D-Glc*p*A-(1→	4.77	3.60	3.70	3.82	3.88	—
	104.6	76.8	74.2	81.6	76.1	174.3
→3)-β-D-Gal*p*NAc^{II}-(1→	4.56	4.04	3.95	4.15	3.69	3.72–3.79
	102.9	52.2	80.6	68.9	76.1	62.4
α-D-Gal*p*NAc^{III}-(1→	5.06	4.24	3.76	4.02	3.83	3.72–3.79
	94.6	50.8	69.4	69.9	72.7	62.3
α-D-Glc*p*-(1→	5.28	3.50	3.76	3.44	4.04	3.79, 3.84
	97.9	73.1	74.6	70.9	73.2	61.9

The chemical shifts for the *N*-acetyl groups are δ 2.06 (^{1}H$_{Me}$); δ 23.5, 23.8 and 23.9 (^{13}C$_{Me}$) and 176.0 (^{13}C$_{CO}$).

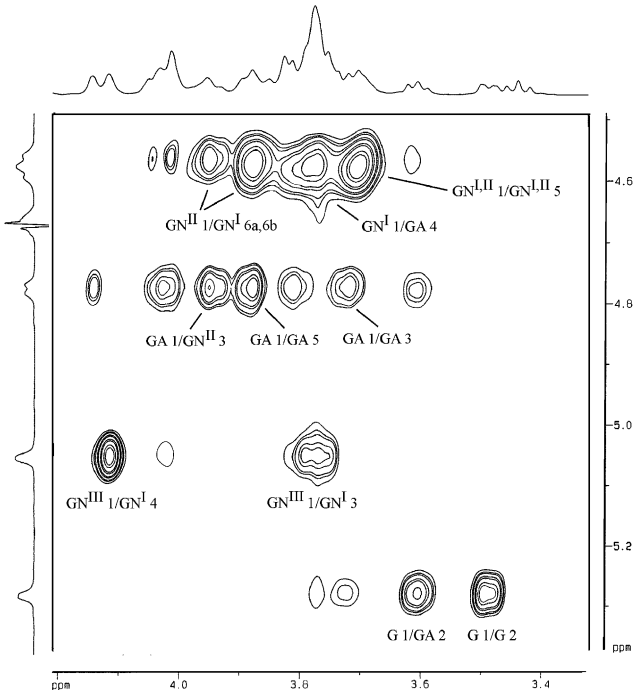


Figure 2. Partial 2D ROESY spectrum of the polysaccharide. The corresponding parts of the ^{1}H spectra are displayed along the axes. Arabic numerals refer to protons in the sugar residues denoted as follows: G, Glc; GA, GalA; GN^I–GN^{III}, GalNAc^I–GalNAc^{III}.

A ROESY experiment (Fig. 2) revealed inter-residue cross-peaks between the anomeric protons and protons at the linkage carbons at δ 4.58/3.82, 4.77/3.95, 4.56/3.89, 3.97, 5.06/3.78 and 5.28/3.60, which were assigned to GalNAc^I H-1, GlcA H-4; GlcA H-1, GalNAc^{II} H-3; GalNAcII H-1, GalNAcI H-6a, 6b; GalNAcIII H-1, GalNAcI H-3 and Glc H-1, GlcA H-2, correlations. Moreover, the HMBC experiment showed the following inter-residue cross-peaks between the atoms separated by three bonds: GalNAc^{II} H-1, GalNAc^I C-6; GalNAc^I H-1, GlcA C-4; GlcA H-1, GalNAc^{II} C-3; Glc C-1, GlcA H-2 and GalNAc^I C-1, GlcA H-4.

A relatively low-field position of the signals for C-3 and C-6 of β-GalNAc^I, C-2 and C-4 of β-GlcA and C-3 of GalNAc^{II} at δ 75.6, 67.9, 76.8, 81.6 and 80.6, respectively, as compared with their position in the corresponding non-substituted monosaccharides,[15] were due to glycosylation effects and confirmed the modes of substitution of the constituent sugar residues. Therefore, it was concluded that the O-polysaccharide of *P. mirabilis* 2002 has the structure:

Most likely, this structure represents the so-called biological repeating unit of the O-polysaccharide, which in the majority of the enterobacterial O-polysaccharides has either 3-substituted β-D-Glc*p*NAc or 3-substituted β-D-Gal*p*NAc at the reducing end.

2.2. Serological studies

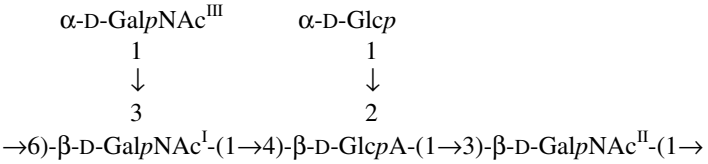
LPS from 94 *Proteus* strains with known O-polysaccharide structures representing all *Proteus* serogroups were tested with polyclonal rabbit O-antiserum against *P. mirabilis* 2002 in passive immunohemolysis (PIH) and enzyme immunosorbent assay (EIA). Only three of them were reactive, including the homologous LPS and those from *P. mirabilis* 52/57 (O29) and *P. mirabilis* S1959 (O3a,b) (Table 2). In both PIH and EIA, *P. mirabilis* 2002 O-antiserum cross-reacted strongly with the *P. mirabilis* O29 LPS, whereas the reaction with the *P. mirabilis* O3 LPS was significantly weaker.

In Western blots with *P. mirabilis* 2002 O-antiserum, the homologous LPS and the LPS of *P. mirabilis* O29 showed a similar pattern in respect to slow-migrating bands, which belong to the LPS molecules with a long-chain polysaccharide (Fig. 3). A faint staining was observed also for the slow-migrating bands of the *P. mirabilis* O3 LPS. The O-antiserum recognized also fast-migrating bands of the O-polysaccharide-lacking LPS species from *P. mirabilis* 2002 and *P. mirabilis* O29, the binding pattern being slightly different.

The reactivity of *P. mirabilis* 2002 O-antiserum in PIH was completely abolished when it was absorbed with the homologous LPS (Table 3). The absorption with *P. mirabilis* O29 LPS removed homologous antibodies, significantly decreased the reactivity with *P. mirabilis* 2002 LPS and did not influence the reaction with the *P. mirabilis* O3 LPS. Absorption with the *P. mirabilis* O3 LPS slightly decreased the reactivity with *P. mirabilis* 2002 LPS and had no influence on the reaction with the *P. mirabilis* O29 LPS.

Table 2. Reactivity of O-antiserum against *P. mirabilis* 2002 with the *Proteus* LPS

LPS from *Proteus* strain	Reciprocal titre for LPS in	
	PIH	EIA
P. mirabilis 2002	12,800	1,024,000
P. mirabilis O29	3200	128,000
P. mirabilis O3	800	4000



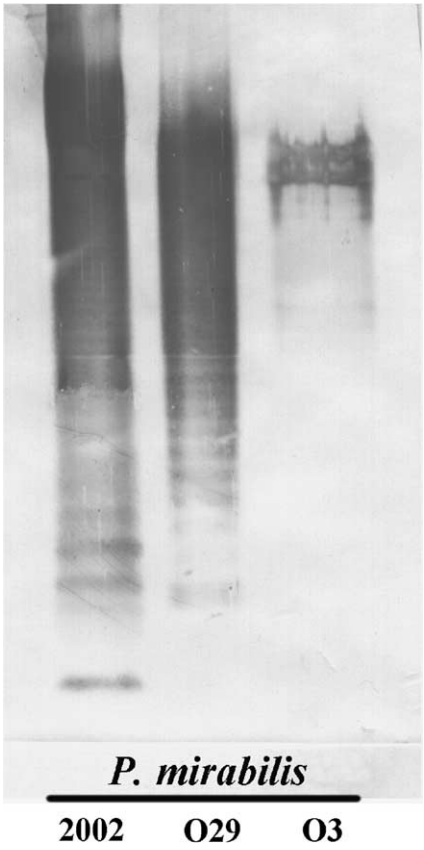


Figure 3. Western blot of *Proteus* lipopolysaccharides with *P. mirabilis* 2002 O-antiserum.

Table 3. Reactivity of absorbed O-antisera against *P. mirabilis* 2002 with the *Proteus* LPS in PIH

Alkali-treated LPS of strain	Reciprocal titre for alkali-treated LPS from *Proteus mirabilis* strains		
	2002	O29	O3
Control	12,800	3200	800
P. mirabilis 2002	<100	<100	<100
P. mirabilis O29	3200	<100	800
P. mirabilis O3	6400	3200	<100

Sheep red blood cells were used as control.

These data suggest the existence of two types of antibodies in *P. mirabilis* 2002 O-antiserum. One antibody recognizes an epitope shared by the LPS of *P. mirabilis* 2002 and *P. mirabilis* O29, while the other binds to another epitope shared by the LPS of *P. mirabilis* 2002 and *P. mirabilis* O3.

Comparison of the *P. mirabilis* 2002 and *P. mirabilis* O29 O-polysaccharide structures shown in Figure 4 suggested that they share an α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc disaccharide fragment, which, most likely, occupies the non-reducing end of the polysaccharide chain (see above). The terminal fragments are known

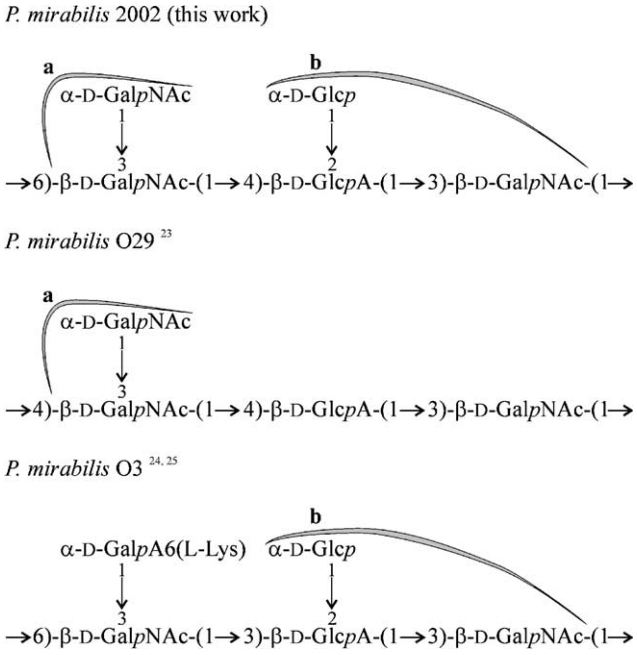


Figure 4. Structures of *P. mirabilis* 2002, *P. mirabilis* O29 (Ref. 23) and *P. mirabilis* O3 (Refs. 24 and 25) O-polysaccharide repeating units.

to contribute largely to the serospecificity of polysaccharide antigens, and the major epitope, **a**, seems to be associated with this disaccharide. The O-polysaccharides of *P. mirabilis* 2002 and *P. mirabilis* O3 possess another fragment in common, namely, an α-D-Glc*p*-(1→2)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc trisaccharides, which seems to be linked to epitope **b** (Fig. 4).

Therefore, on the basis of the similarity of the chemical structures of the O-polysaccharides (Fig. 4) and a close serological relatedness of the LPS, we propose to classify *P. mirabilis* 2002 into the same *Proteus* serogroup O29 as *P. mirabilis* 52/57 and to divide this serogroup into two subgroups, O29a for strain *P. mirabilis* 52/57 and O29a, 29b for *P. mirabilis* 2002.

3. Experimental

3.1. Bacterial strains and growth

P. mirabilis strain 2002 was isolated from an ear swab and 24 strains of *P. penneri* as well as *P. hauseri* strain were kindly provided by C. M. O'Hara and D. J. Brenner (Centres for Diseases Control and Prevention, Atlanta, Georgia, USA). The strains of *P. mirabilis* (39) and *P. vulgaris* (27) were from the Czech National Collection of Type Cultures (CNCTC, National Institute of Public Health, Prague, Czech Republic). A *P. myxofaciens* strain (CCUG 18769) was kindly provided by E. Falsen [Cultures Collection, University of Goeteborg (CCUG), Goeteborg, Sweden].

P. mirabilis strain 2002 was grown under aerobic conditions on nutrient broth (BTL, Łódź, Poland). Dry bacterial mass was obtained from an aerated, liquid culture as described previously.[16]

3.2. Isolation and degradation of the lipopolysaccharides

LPS was obtained by extraction of bacterial mass with phenol–water mixture[17] and purified by treatment with cold aqueous 50% CCl_3CO_2H followed by dialysis of the supernatant.[18]

Alkali-treated LPS were prepared by saponification of LPS with 0.2 M NaOH (56 °C, 2 h) followed by precipitation with ethanol. Acid degradation of *Proteus mirabilis* 2002 LPS was performed with aqueous 2% HOAc at 100 °C until lipid A precipitation. The precipitate was removed by centrifugation (13.000 × g, 20 min), and the supernatant was fractionated on a column (56 × 2.6 cm) of Sephadex G-50 (Pharmacia) in 0.05 M pyridinum acetate buffer pH 4.5 monitored using a Knauer differential refractometer (Germany). A high-molecular-mass polysaccharide was obtained in a yield of 16% of the lipopolysaccharide weight.

3.3. Rabbit antiserum and serological assays

Rabbit polyclonal O-antiserum against *P. mirabilis* 2002 was obtained by immunization of rabbits with bacterial suspension according to the published procedure.[19] Passive immunohemolysis (PIH), enzyme immunosorbent assay (EIA), absorption experiments, SDS-PAGE (using 12% acrylamide) as well as electrotransfer of LPS from gel to nitrocellulose sheets and immunostaining were performed as described previously.[20]

3.4. NMR spectroscopy

NMR spectra were recorded with a Bruker DRX-500 spectrometer for a solution in D_2O at 30 °C using internal acetone (δ_H 2.225, δ_C 31.45) as the reference. Standard Bruker software (XWINNMR 2.6) was used to acquire and process the NMR data. A mixing time of 200 and 300 ms was used in two-dimensional TOCSY and ROESY experiments.

3.5. Sugar analysis

The O-polysaccharide (1 mg) was hydrolyzed with 2 M CF_3COOH (120 °C, 2 h) or methanolyzed with 1 M HCl/MeOH (85 °C, 16 h), and the derived monosaccharides were analyzed by GLC of the acetylated alditols (for Glc and GlcNAc) or the acetylated methyl glycosides (for GlcA). The absolute configurations of the monosaccharides were determined by GLC of the acetylated (*S*)-2-butyl glycosides according to the published method.[21,22] GLC was performed on a DB-5 column (25 m × 0.25 mm) using a Hewlett-Packard 5880 instrument (USA) and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C min^{-1}.

Acknowledgements

This work was supported by RF grant NSh.1557.2003.3, University of Łódź (grant 801) and 02-04-48767 of the Russian Foundation for Basic Research.

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