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Structure of the O-polysaccharide of a serologically separate strain of *Proteus mirabilis,* TG 332, from a new proposed *Proteus* serogroup O50

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Abstract

The O-polysaccharide was obtained by mild acid degradation of the lipopolysaccharide of *Proteus mirabilis* TG 332 strain. The following structure of the O-polysaccharide was determined by chemical methods along with NMR spectroscopy, including 2D COSY, TOCSY, ROESY and ^{1}H, ^{13}C HMQC experiments:

β-D-Glc*p*
1
↓
4
→3)-β-D-Glc*p*NAc-(1→3)-β-D-Glc*p*A-(1→4)-β-D-Gal*p*NAc-(1→4)-β-D-Gal*p*-(1→

The O-polysaccharide studied has a unique structure among *Proteus* O-antigens. Accordingly, *P. mirabilis* TG 332 is serologically separate, and we propose to classify this strain into a new *Proteus* serogroup, O50. The nature of minor epitopes that provide a cross-reactivity of *P. mirabilis* TG 332 O-antiserum with the LPS of *P. mirabilis* O30 and *Proteus penneri* 34 (O60) is discussed.

Keywords: Lipopolysaccharide; O-Antigen; Bacterial polysaccharide; Structures; Serological classification; *Proteus mirabilis*

1. Introduction

Bacteria of the genus *Proteus* are known to cause nosocomial and urinary tract infections, which often result in severe complications, such as acute or chronic pyelonephritis and formation of bladder and kidney stones.[1,2] Potential virulence factors of *Proteus* that mediate the infection processes are swarming growth, fimbriae, flagella, enzymes (urease, IgA proteases), siderophores, hemolysins, capsular polysaccharides and lipopolysaccharide (endotoxin, LPS).[3,4] The polysaccharide chain (O-antigen) of the LPS defines the serological O-specificity of the bacteria. Based on the immunospecificity of the O-antigens, two medically important Gram-negative bacteria, *Proteus mirabilis* and *Proteus vulgaris*, are divided in the classification scheme of Kauffmann and Perch into 49 O-serogroups.[5] Further O-serogroups for some other strains of these *Proteus* species have been proposed more recently.[6] Recently, based on serological and structural studies of the O-antigens, the classification of *Proteus* strains has been improved, and new serogroups have been created, including those for strains of *Proteus penneri*.[7–9]

Now we report on the structure of the O-polysaccharide and serological properties of the LPS of *P. mirabilis*

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TG 332, which is one of the strains that were considered earlier as candidates for new *Proteus* serogroups.[6] Based on the data obtained, we propose classification of this strain into a new *Proteus* serogroup, O50.

2. Results and discussion

The LPS was isolated from dried bacterial cells of *P. mirabilis* TG 332 by the phenol–water procedure. Mild acid degradation of the LPS with dilute acetic acid, followed by GPC on Sephadex G-50; resulted in a high-molecular-mass O-polysaccharide.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1, Table 1) contained signals for five anomeric carbons at δ 100.7–104.0, two nitrogen-bearing carbons (C-2 of amino sugars) at δ 53.7 and 55.3, 18 oxygen-bearing carbons in the region δ 69.3–82.9, four unsubstituted HO*C*H$_2$–C groups (C-6 of hexoses and hexosamines) at δ 61.4, one COOH group (C-6 of a hexuronic acid) at δ 174.6, and two *N*-acetyl groups [CH$_3$ at δ 23.6 (2C) and CO at δ 175.8 and 175.9].

The ^{1}H NMR spectrum of the polysaccharide (Table 1) contained, inter alia, signals for five anomeric protons in the region δ 4.42–5.09 and two *N*-acetyl groups at δ 2.05 (2CH$_3$). These data suggest that the polysaccharide has a pentasaccharide repeating unit containing two residues each of hexoses and *N*-acetylhexosamines and one residue of hexuronic acid.

Monosaccharide analysis by GLC of the alditol acetates showed that the polysaccharide contains glucose, galactose, GlcN, and GalN. GLC of the acetylated (*S*)-2-octyl glycosides confirmed the D configuration of the monosaccharides. Methylation analysis of the polysaccharide revealed terminal Glc, 4-substituted Gal, 3-substituted GlcN, and 4-substituted GalN. When the methylated polysaccharide was reduced with LiBH$_4$ prior to hydrolysis, in addition to the monosaccharides listed above, 3,4,6-trisubstituted Glc was demonstrated, which was evidently derived from 3,4-disubstituted GlcA (see below).

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 HMQC experiments (Table 1), and five sugar spin systems were identified. The Glc*p*, Glc*p*NAc and Glc*p*A spin systems were distinguished from those of Gal*p* and Gal*p*NAc on the basis of the relatively large $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ coupling constant values. Gal*p*NAc and Glc*p*NAc were demonstrated by correlations of the protons at the nitrogen-bearing carbons at δ 3.98 and 4.07 to the corresponding carbons at δ 53.7 and 55.3, respectively. $J_{1,2}$ coupling constant values of 7–8 Hz showed that all sugar residues are β-linked.

The following signals were shifted downfield as compared with their positions in the spectra of the corresponding non-substituted monosaccharides:10 C-4 of GalNAc to δ 76.1 (+7.0 ppm), C-4 of Gal to δ 76.5 (+6.5 ppm), C-3 of GlcNAc to δ 82.9 (+7.8 ppm), C-3 and C-4 of GlcA to δ 79.6 and 74.9 (+3.1 and +2.2 ppm, respectively). The ^{13}C NMR chemical shifts for the glucose residue were close to those for the unsubstituted β-Glc*p*,[10] thus indicating the terminal position of this sugar. These data revealed in the repeating unit the glycosylation pattern, which is in agreement with the methylation analysis data (see above).

The ROESY spectrum showed the following correlations between the anomeric protons and protons at the linkage carbons: Gal H-1,GlcNAc H-3 at δ 4.42/3.82; GlcNAc H-1,GlcA H-3 at δ 5.09/3.95; Glc H-1,GlcA H-4 at δ 4.60/3.96; GlcA H-1,GalNAc H-4 at δ 4.65/4.12; and GalNAc H-1,Gal H-4 at δ 4.72/4.10. Based on these data, it was concluded that the pentasaccharide repeating unit of the O-polysaccharide of *P. mirabilis* TG 332 has the structure shown in Fig. 2. As most other *Proteus*

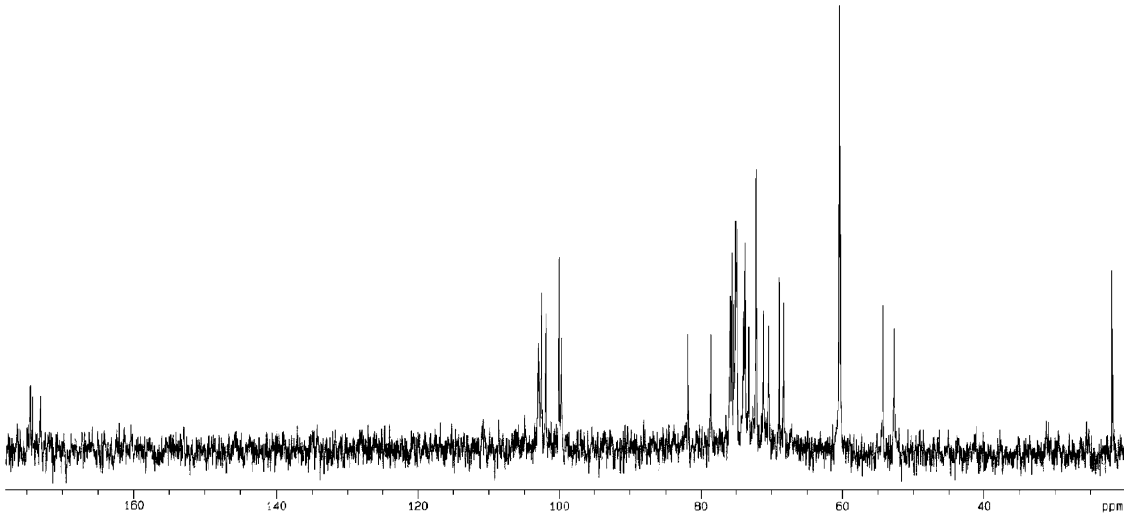

Fig. 1. ^{13}C NMR (125 Mhz) spectrum of the O-polysaccharide of *P. mirabilis* TG 332.

Table 1
^{13}C and ^{1}H NMR data (δ, ppm) [a]

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
→4)-β-D-Gal*p*NAc-(1→	102.9	53.7	72.1	76.1	74.2	61.4
→4)-β-D-Gal*p*-(1→	104.0	71.4	73.1	76.5	74.9	61.4
→3)-β-D-Glc*p*NAc-(1→	100.7	55.3	82.9	69.3	75.9	61.4
→3,4)-β-D-Glc*p*A-(1→	103.6	74.8	79.6	74.9	76.9	174.6
β-D-Glc*p*-(1→	101.1	74.2	75.9	69.9	76.0	61.4
	H-1	H-2	H-3	H-4	H-5	H-6a,6b
→4)-β-D-Gal*p*NAc-(1→	4.72	3.98	3.85	4.12	3.65	3.78
→4)-β-D-Gal*p*-(1→	4.42	3.43	3.78	4.10	3.71	3.77
→3)-β-D-Glc*p*NAc-(1→	5.09	3.89	3.82	3.55	3.51	3.75, 3.92
→3,4)-β-D-Glc*p*A-(1→	4.65	3.62	3.95	3.96	3.82	
β-D-Glc*p*-(1→	4.60	3.36	3.49	3.43	3.44	3.74, 3.87

[a] The chemical shifts for NAc are δ_C 23.6 (CH_3, 2C), 175.8 and 175.9 (both CO); δ_H 2.05.

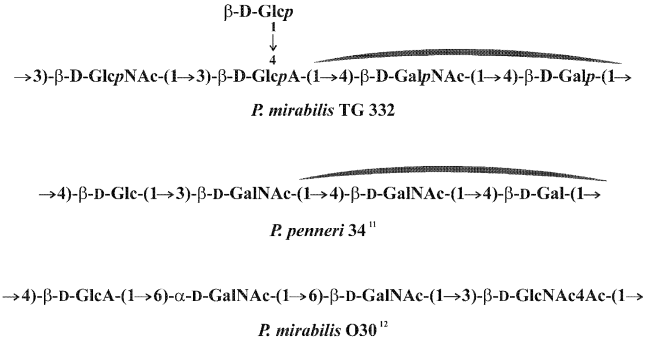

Fig. 2. Structures of the O-polysaccharides of the cross-reactive *Proteus* strains.

O-antigens,[9] the polysaccharide studied is acidic and has a unique structure.

The LPS of 130 *Proteus* strains, including 28 strains of *P. vulgaris*, 37 strains of *P. mirabilis* and 65 strains of *P. penneri*, were tested with *P. mirabilis* TG 332 O-antiserum. Only two LPS, those of *P. mirabilis* O30 and *P. penneri* 34 (O65), cross-reacted but their reactivity was weak (reciprocal titers 4000 and 8000 in enzyme immunosorbent assay; 3200 and 6400 in passive immunohemolysis, respectively), as compared with the reactivity of the homologous LPS (reciprocal titers 512,000 and 51,200, respectively). The inhibitory activity of the LPS of *P. mirabilis* O30 and *P. penneri* 34 (O65) in passive immunohemolysis was negligible.

The reactivity of *P. mirabilis* TG 332 O-antiserum with all tested antigens in passive immunohemolysis was completely abolished when it was absorbed with the homologous LPS (Table 2). Absorption of the O-antiserum with the LPS from either *P. mirabilis* O30 or *P. penneri* 34 removed only those antibodies that react with the LPS used for absorption (Table 2).

In Western blot (Fig. 3), *P. mirabilis* TG 332 O-antiserum strongly reacted with both slow- and fast-migrating bands of the homologous LPS, which correspond to the high- and low-molecular-mass LPS species with and without O-polysaccharide chain, respectively. It recognized also slow-migrating bands of the *P. penneri* 34 LPS and fast-migrating bands of the *P. mirabilis* O30 LPS (Fig. 3). These data suggest the presence of at least three fractions of *P. mirabilis* TG 332 O-antibodies. The major fraction is specific to a unique epitope on the homologous O-polysaccharide, whereas minor fractions recognize common epitopes on the O-polysaccharides of *P. mirabilis* TG 332 and *P. penneri* 34 and on the LPS core of *P. mirabilis* TG 332 and O30.

The *P. mirabilis* TG 332 and *P. penneri* 34 O-polysaccharides have a common β-D-Gal*p*NAc-(1→4)-β-D-Gal*p* disaccharide fragment (Fig. 2), which could be a minor epitope that is responsible for the cross-reactivity of these strains. The O-polysaccharides of *P. mirabilis* TG 332 and O30 have no structural similarity (Fig. 2), which is in agreement with the Western blot data. The cross-reactive epitope of these strains cannot be assigned to any particular LPS fragment since the core structure of *P. mirabilis* TG 332 and O30 remain unknown.

Based on the finding that the O-polysaccharide of *P. mirabilis* TG 332 has a unique structure, and that the LPS of this strain shows no or a weak cross-reactivity with other *Proteus* strains, we suggest classifying *P. mirabilis* TG 332 into a new, separate *Proteus* serogroup, O50, in which at present this strain is the single representative.

3. Experimental

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

P. mirabilis TG 332 and 11 more *P. mirabilis* and *P. vulgaris* strains[6] were kindly provided by Professor J.L.

Table 2
Passive immunohemolysis data with *P. mirabilis* TG 332 O-antiserum absorbed with *Proteus* LPS [a]

Alkali-treated LPS used for absorption	Reactivity (reciprocal titer) with alkali-treated LPS from		
	P. mirabilis TG 332	*P. penneri* 34	*P. mirabilis* O30
Control	51,200	1600	6400
P. mirabilis TG 332	<100	<100	<100
P. penneri 34	51,200	<100	6400
P. mirabilis O30	25,600	1600	<100

[a] Sheep red blood cells were used as control.

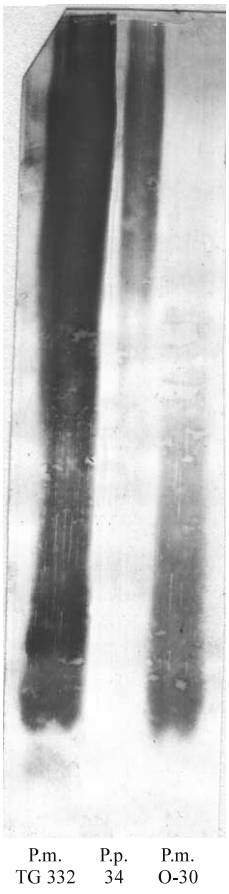

Fig. 3. Western blot of the *Proteus* LPS with *P. mirabilis* TG 332 O-antiserum. P.m., *P. mirabilis*; P.p., *P. penneri*.

Penner (Department of Medical Genetics, University of Toronto, Canada). Sixty-five *P. penneri* strains were from the Collection of the Department of General Microbiology (University of Łódz, Poland), and 37 *P. mirabilis* and 28 *P. vulgaris* strains were from the Czech National Collection of Type Cultures (Prague, The Czech Republic). Dry bacterial cells were obtained from aerated liquid cultures as described.[13] The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with water, and lyophilised. The LPS was isolated from dried cells by the phenol–water method[14] and purified using the CCl_3CO_2H precipitation procedure as described.[15]

3.2. Degradation of the lipopolysaccharide

The LPS (103 mg) was hydrolysed with aq 2% HOAc at 100 °C for 2 h, and a lipid precipitate was removed by centrifugation at 13,000*g* for 20 min. The carbohydrate portion was fractionated by GPC on a column (56 × 2.6 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer pH 4.5 with monitoring by a Knauer differential refractometer to give a high-molecular-mass polysaccharide (28 mg).

3.3. Monosaccharide analysis

The polysaccharide was hydrolysed with 2 M CF_3COOH (120 °C, 2 h), the monosaccharides were identified as the alditol acetates by GLC using a Hewlett–Packard 5989A instrument equipped with an HP-5 column and a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C min^{-1}. The absolute configurations of the monosaccharides were determined by GLC of the acetylated (*S*)-2-octyl glycosides prepared as described[16,17] using a Hewlett–Packard 5880 instrument with a DB-5 column and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C min^{-1}.

3.4. Methylation analysis

Methylation of the polysaccharide was performed with CH_3I in dimethyl sulfoxide in the presence of sodium methylsulfinylmethanide.[18] The partially methylated monosaccharides were derivatized after hydrolysis under the same conditions as in monosaccharide analysis, reduced with $NaBH_4$, acetylated and analysed by GLC–MS on a Hewlett–Packard 5890 chromatograph equipped with a NERMAG R10-10L mass spectrometer (France), using a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C min^{-1}. A portion of the methylated polysaccharide was reduced with $LiBH_4$ in aq 70% 2-propanol for 16 h at 20 °C, the reduced polysaccharide

was recovered using a SepPack cartridge, hydrolysed and analysed as above.

3.5. 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 60 °C. Spectra were recorded on a Bruker DRX-500 MHz spectrometer (Germany) equipped with an SGI INDY computer workstation. Two-dimensional spectra were obtained using standard Bruker software, and XWINNMR 2.6 program (Bruker) was used to acquire and process the NMR data. The parameters used in 2-D experiments were essentially the same as described previously.[19] A mixing time of 200 ms was used in TOCSY and ROESY experiments.

3.6. Rabbit antiserum and serological assays

Polyclonal O-antiserum was obtained by immunization of rabbits with heat-inactivated bacteria of *P. mirabilis* TG 332 according to the published procedure.[20] SDS-PAGE, electrotransfer of LPS from gels to nitrocellulose sheets, immunostaining and absorption experiments were carried out as described earlier[21] LPS was used as the antigen in the enzyme immunosorbent assay. Passive immunohemolysis was performed with increasing amounts (from 2 to 200 μg) of alkali-treated LPS.[21]

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