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Carbohydrate Research 312 (1998) 97–101

CARBOHYDRATE RESEARCH

Note

Structure of a new neutral O-specific polysaccharide of *Proteus penneri* 34

Filip V. Toukach [a], Nikolay P. Arbatsky [a], Alexander S. Shashkov [a], Yuriy A. Knirel [a]*, Krystyna Zych [b], Zygmunt Sidorczyk [b]

[a] *N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 117913 Moscow, Russian Federation*

[b] *Institute of Microbiology and Immunology, University of Lodz, 90-237 Lodz, Poland*

Received 4 June 1998; accepted 25 August 1998

Abstract

The O-specific polysaccharide of *Proteus penneri* strain 34 was studied using ^{1}H and ^{13}C NMR spectroscopy, including 2D COSY, TOCSY, NOESY, and H-detected $^{1}H,^{13}C$ HMQC experiments. The following structure was established, which is unique among the known structures of *Proteus* O-antigens: →4)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→4)-β-D-Gal*p*NAc-(1→4)-β-D-Gal*p*-(1→. Accordingly, no cross-reaction was observed between *P. penneri* 34 O-antiserum and O-antigens of other *Proteus* strains. Therefore, the strain studied should belong to a new *Proteus* serogroup O65.

Keywords: *Proteus penneri*; O-antigen; O-specific polysaccharide; Lipopolysaccharide; Structure

Bacteria of the genus *Proteus* are known to cause urinary tract infections which often result in severe complications, such as acute or chronic pyelonephritis and formation of bladder and kidney stones. *Proteus penneri* is a new bacterial species proposed for strains formerly described as *Proteus vulgaris* biogroup I [1,2]. Aiming at creation of a chemical basis for classification of this species, structures of the O-specific polysaccharide chains of lipopolysaccharide (LPS, O-antigens) have been elucidated for a number of *P. penneri* strains (refs. [3–12]. and refs. cited therein). With a few exceptions [11,12], the polysaccharides were found to be acidic due to the presence of various uronic acids and non-carbohydrate acidic groups. Based on the chemical and serological data, we proposed four new *Proteus* serogroups, O61–O64, which consist of *P. penneri* strains only [5,9,10,12]. Now we report immunochemical studies of *P. penneri* strain 34 LPS, which has a new neutral O-specific polysaccharide, and propose for this strain an additional *Proteus* serogroup O65.

LPS was isolated from dried bacterial cells of *P. penneri* 34 by the phenol–water extraction [13] and degraded with diluted acetic acid to give a high-molecular-mass O-specific polysaccharide. Sugar analysis of the polysaccharide revealed the presence of almost equal amounts of Glc and Gal as well as GalN, which were identified using sugar and amino acid analysers, respectively. Determination of the absolute configuration by GLC of

* Corresponding author. Fax: +7-095-135-5328; e-mail: knirel@ioc.ac.ru

0008-6215/98/$—see front matter
PII S0008-6215(98)00232-8

acetylated (*S*)-2-butyl glycosides showed that all monosaccharides have the D configuration.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) demonstrated a tetrasaccharide repeating unit. It contained signals for four anomeric carbons at δ 103.3–105.3, four unsubstituted CH_2OH groups at δ 61.6–62.4 (C-6 of hexoses and GalN, data of attached-proton test [14]), two carbons bearing nitrogen at δ 53.0 and 54.5 (C-2 of GalN), 14 sugar ring carbons bearing oxygen in the region δ 69.4–81.5, and two *N*-acetyl groups (CH_3 at δ 23.7 and 23.9, CO at δ 176.2 and 176.3). Accordingly, the ^1H NMR spectrum of the polysaccharide (Fig. 2) contained, inter alia, signals for four anomeric protons at δ 4.42–4.78 and two *N*-acetyl groups at δ 2.03 (6H). Therefore, the polysaccharide has a tetrasaccharide repeating unit containing two residues of D-GalNAc and one residue each of D-Glc and D-Gal; none of the monosaccharides is 6-substituted.

The ^1H NMR spectrum of the polysaccharide was assigned using 2D COSY and TOCSY experiments (Table 1). Despite a small $J_{4,5}$ coupling constants value, in the COSY spectrum a week H-4/H-5 cross-peak was observed for both GalNAc residues and for Gal. Based on the $^3J_{H,H}$ coupling constants values, the four sugar spin systems were assigned. The Glc*p* residue was identified by large $J_{3,4}$ and $J_{4,5}$ values of $\sim$10 Hz, as compared with values $\leq$3 Hz for the Gal*p* and GalNAc*p* residues. The GalNAc residues were distinguished from the Gal residue by correlation of the protons at carbons bearing nitrogen (H-2) to the corresponding carbons (C-2), which was revealed by a $^1H,^{13}C$ HMQC experiment.

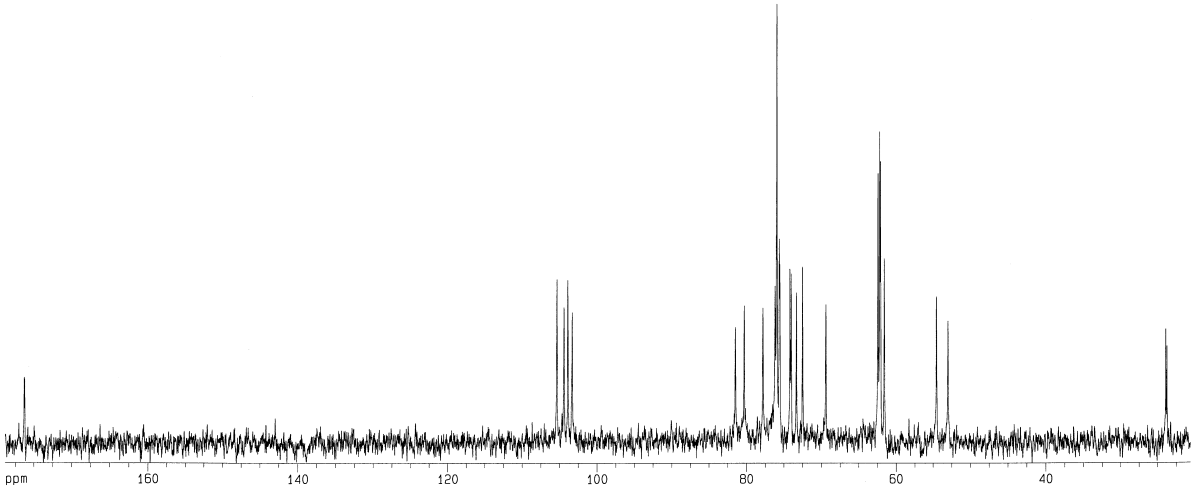

Fig. 1. ^{13}C NMR spectrum of the O-specific polysaccharide.

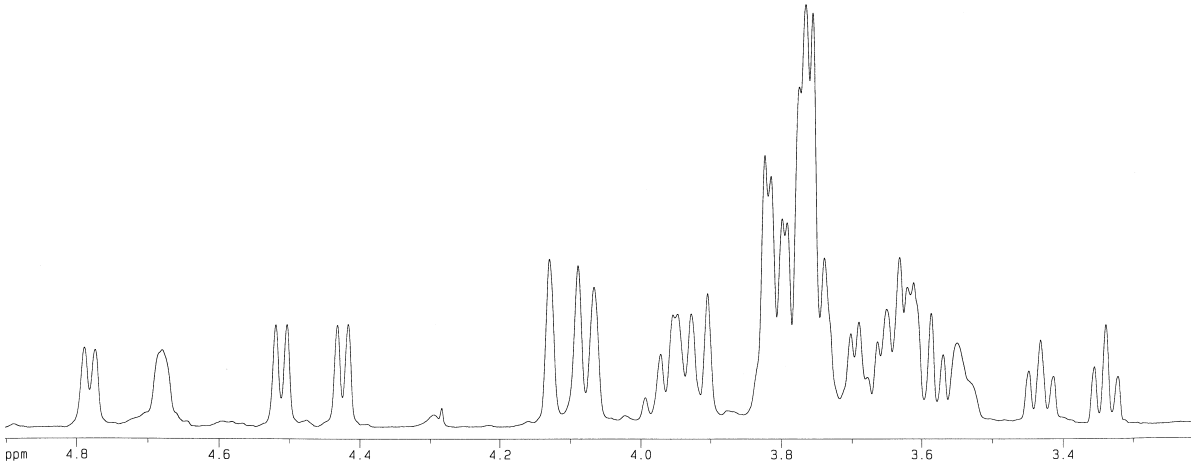

Fig. 2. ^1H NMR spectrum of the O-specific polysaccharide (the signal for the NAc groups at δ 2.03 is not shown).

Table 1
^{1}H NMR data (δ, ppm) for the O-specific polysaccharide. The chemical shift for NAc is δ 2.03

Sugar residue	Proton						
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
→4)-β-D-Glc*p*-(1→	4.51	3.34	3.63	3.59	3.54	3.91	3.78
→3)-β-D-Gal*p*NAc^{I}-(1→	4.78	3.97	3.94	4.13	3.66	3.79	
→4)-β-D-Gal*p*NAc^{II}-(1→	4.68	3.82	3.82	4.09	3.62	3.81	3.76
→4)-β-D-Gal*p*-(1→	4.42	3.43	3.76	4.07	3.70	3.76	

Table 2
^{13}C NMR data (δ, ppm) for the O-specific polysaccharide. Chemical shifts for NAc are δ 23.7, 23.9 (Me), 176.2, and 176.3 (CO)

Sugar residue	Carbon					
	C-1	C-2	C-3	C-4	C-5	C-6
→4)-β-D-Glc*p*-(1→	105.3	74.0	75.6[a]	80.3	76.0	61.6
→3)-β-D-Gal*p*NAc^{I}-(1→	103.3	53.0	81.5	69.4	76.0	62.1[b]
→4)-β-D-Gal*p*NAc^{II}-(1→	103.9	54.5	73.3	76.2	75.5[a]	62.4[b]
→4)-β-D-Gal*p*-(1→	104.4	72.5	74.2	77.8	76.0	62.2[b]

[a,b] Assignment could be interchanged.

As judged by relatively large $^3J_{1,2}$ coupling constants values of 7–8 Hz determined from the ^{1}H NMR spectrum for the anomeric protons at δ 4.42, 4.51, and 4.78, the residues of Glc, Gal, and one of the GalNAc residues (GalNAc^{I}) are β-linked. The H-1 signal for the second GalNAc residue (GalNAc^{II}) was not resolved owing to the coincidence of the signals for H-2 and H-3 at δ 3.82 (Fig. 2). Nevertheless, the position of this signal at δ 4.68 showed that it belongs to a β-linked residue as well. This conclusion as well as the assignment of the ^{1}H NMR spectrum in the whole were confirmed by a NOESY experiment, which revealed for all four monosaccharides a correlation between H-1 and H-3,5 of the same sugar residue that is typical of β-pyranosides.

In addition to the intraresidue NOE correlations, the NOESY experiment revealed the following interresidue correlations between the transglycosidic protons: Glc H-1,GalNAc^{I} H-3 at δ 4.51/3.94, GalNAc^{I} H-1,GalNAc^{II} H-4 at δ 4.78/4.09, and GalNAc^{II} H-1,Gal H-4 at δ 4.68/4.07. These data revealed the sequence and substitution pattern for three of the four monosaccharides. Two interresidue cross-peaks were observed for Gal H-1, one with Glc H-4 at δ 4.42/3.59 and the other with Glc H-6a at δ 4.42/3.91. Since 6-substitution was excluded by the ^{13}C NMR data (see above), the Glc residue is 4-substituted and, hence, the polysaccharide is linear. An H-1′,H-6 correlation is not uncommon for (1→4)-linked disaccharides (e.g., ref [15]).

The ^{13}C NMR spectrum was assigned using an H-detected ^{1}H,^{13}C HMQC experiment (Table 2). Low-field displacements of the signals for C-3 of GalNAc^{I} to δ 81.5 and C-4 of three other sugar residues to 76.2–80.3, as compared with their positions in the spectra of the corresponding unsubstituted monosaccharides at δ 72.0 and 68.8–70.7 [16], respectively, confirmed the mode of substitution and the linear character of the polysaccharide.

On the basis of these data, it was concluded that the O-specific polysaccharide of *P. penneri* 34 has the following structure, which is unique among the known structures of *Proteus* O-antigens: →4)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→4)-β-D-Gal*p*NAc-(1→4)-β-D-Gal*p*-(1→.

Rabbit polyclonal *P. penneri* 34 O-antiserum was tested with LPS from 68 strains of *P. penneri* as well from 37 strains of *P. mirabilis* and 28 strains of *P. vulgaris* which represent 49 *Proteus* O-serogroups. From them, only *P. penneri* 8 and 34 reacted in agglutination, passive hemolysis, and enzyme immunosorbent assay (EIA) (Table 3). However, the level of reaction in the heterologous system was much lower as compared with the homologous system.

Western blot after SDS/PAGE separation of the two serologically related LPSs is shown in Fig. 3. *P. penneri* 34 O-antiserum reacted with both slowly moving and fast moving *P. penneri* 34 LPS bands which corresponded to high-molecular-mass species (O-specific polysaccharide-core-lipid A) and low-molecular-mass species including unsubstituted core-lipid A, respectively. In contrast, only low-molecular-mass species of *P. penneri* 8 LPS

Table 3
Reactivity of *P. penneri* LPSs with rabbit polyclonal *P. penneri* 34 O-antiserum. Passive hemolysis and EIA were performed with IgM-rich or IgG-rich O-antiserum using alkali-treated LPS or LPS-BSA complex as antigen, respectively

Antigen *P. penneri* strain	Reciprocal titre in:		
	Agglutination	Passive hemolysis	EIA
8	80	6400	16 000
34	2560	51 200	256 000

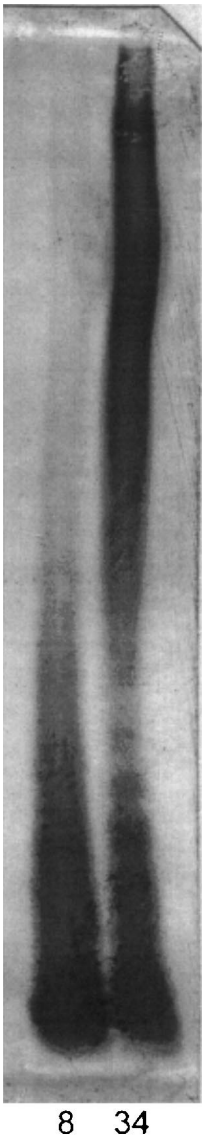

Fig. 3. Western blot of *P. penneri* 34 and *P. penneri* 8 LPSs with rabbit polyclonal *P. penneri* 34 O-antiserum.

were reactive. Such pattern indicated that the cross-reactive epitope is located in the LPS core region, whereas the polysaccharide chains of *P. penneri* 8 and 34 LPSs are serologically different. This conclusion is in agreement with the known structure of the O-specific polysaccharide of *P. penneri* 8, which is built up of branched acidic hexasaccharide repeating units [3] and has nothing in common with the polysaccharide of *P. penneri* 34.

Therefore, *P. penneri* strain 34 is separate with respect to the structure of the O-specific polysaccharide and the serological O-specificity and, thus, should belong to a new *Proteus* serogroup O65.

1. Experimental

Bacterial strains.—*P. penneri* strains were from the collection of the Institute of Microbiology and Immunology (Lodz, Poland). *P. penneri* strain 34 (TGH 1937) which was originally isolated in Toronto, was kindly provided by Professor D.J. Brenner (Centre for Diseases Control, Atlanta, GA). Strains of *P. mirabilis* and *P. vulgaris* were from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague).

Rabbit polyclonal O-antiserum and serological assays.—Rabbits were immunised intravenously with a suspension of 50, 100, 100, and 200 μL lyophilised bacterial cells in physiological saline at 1 mg/mL on days 0, 4, 7, and 11, respectively. Five days after the last injection, 20 mL of blood was obtained from ear vein (IgM-rich antiserum). Rabbits received a booster injection (500 mg) on day 51 and were exsanguinated on day 58 (IgG-rich antiserum).

Agglutination test [17], EIA using LPS–BSA complex as solid phase antigens [18], passive hemolysis, SDS/PAGE, and Western blot [4] were performed as described.

Isolation and degradation of lipopolysaccharide.—LPS was isolated from dried bacterial cells of *P. penneri* 34 grown as described [19], by extraction with hot aqueous phenol [13] and purified by treatment with cold aqueous 50% CCl_3CO_2H followed by dialysis of the supernatant.

Acid degradation of LPS was performed with 0.1 M sodium acetate buffer (pH 4.5) at 100 °C for 1.5 h. The O-specific polysaccharide was isolated by GPC on a column (3×65 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer (pH 4.5).

Alkali-treated LPS was prepared by saponification of LPS with 0.25 M sodium hydroxide (56 °C, 2 h) followed by precipitation with ethanol.

Sugar analysis.—The polysaccharide was hydrolysed with 3 M CF_3CO_2H (100 °C, 4 h), amino and neutral sugars were identified using a Biotronik LC-2000 amino acid and sugar analyser as described [10]. The absolute configuration of the monosaccharides was determined by GLC of acetylated (*S*)-2-butyl glycosides [20,21] using a Hewlett–Packard 5890 chromatograph equipped with an Ultra 2 capillary column.

NMR spectroscopy.—^{1}H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer in D_2O at 318 K using internal acetone (δ_H 2.225,

δ_c 31.45) as reference. 2D NMR experiments were performed using standard Bruker software. A mixing time of 120 and 300 ms was used in TOCSY and NOESY experiments, respectively.

Acknowledgements

This work was supported by grants no. 96-04-50460 and 96-15-97380 of the Russian Foundation for Basic Research.

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