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Carbohydrate Research 318 (1999) 146–153

CARBOHYDRATE RESEARCH

Structure of a new acidic O-antigen of *Proteus vulgaris* O22 containing O-acetylated 3-acetamido-3,6-dideoxy-D-glucose

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Received 29 January 1999; revised 12 April 1999; accepted 12 April 1999

Abstract

The acidic O-specific polysaccharide of *Proteus vulgaris* O22 was studied using ^{1}H and ^{13}C NMR spectroscopy, including 2D COSY, TOCSY, NOESY, and H-detected $^{1}H,^{13}C$ heteronuclear multiple-quantum coherence (HMQC) experiments, and the following structure for the branched pentasaccharide repeating unit was established:

α-D-Qui*p*3NAc2,4Ac$_2$
1
↓
2
→3)-β-D-Glc*p*NAc-(1→3)-β-L-Rha*p*-(1→4)-α-L-Rha*p*-(1→4)-β-D-Glc*p*A-(1→

where Qui3NAc is 3-acetamido-3,6-dideoxyglucose, O-acetylation of QuiNAc at position 4 is stoichiometric and at position 2 nonstoichiometric. Serological relationships of *P. vulgaris* O22 with some other *Proteus* strains were substantiated on the level of the O-antigen structures. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Proteus vulgaris*; O-Antigen; O-Specific polysaccharide; Lipopolysaccharide; O-Acetylation; 3-Acetamido-3,6-dideoxy-D-glucose

1. Introduction

Bacteria of the genus *Proteus* mainly cause wound and urinary tract infections, the latter sometimes leading to acute or chronic pyelonephritis and formation of bladder and kidney stones. Outer-membrane lipopolysaccharide (LPS, endotoxin) is considered as a virulence factor of *Proteus* [1]. The O-specific polysaccharide chain of LPS (O-antigen) defines the immunospecificity of strains. Based on the O-antigens, two species, *Proteus mirabilis* and *P. vulgaris*, were classified into 60 O-serogroups [2,3]. Recently, some more O-serogroups have been proposed for strains of a third species, *P. penneri* ([4–6] and references cited therein). Chemical and immunochemical studies of *Proteus* O-antigens are

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PII: S0008-6215(99)00088-9

important for understanding the molecular basis of the immunospecificity and classification of *Proteus* strains.

A peculiar feature of *Proteus* is the acidic character of the O-antigen in most strains studied. Among the acidic constituents found so far are phosphate groups, hexuronic acids, their amides with amino acids, sugar acetals with pyruvic acid, and ethers with lactic acid ([6,7] and references cited therein). The role of acidic polysaccharides in the pathogenicity of *Proteus* and, particularly, in urinary tract infections was discussed previously [1]. The acidic character of *Proteus* polysaccharides enables bacteria to bind metal cations, Mg^{2+} and Ca^{2+}, via electrostatic interaction. This may enhance the formation of struvite and carbonate apatite stones in the urinary tract under alkaline conditions in the presence of ammonia, one of the products of urea decomposition caused by *Proteus* [8]. Recently, the importance of an acidic polysaccharide rich in galacturonic acid and galactosamine was demonstrated for migration of *P. mirabilis* swarm cells by reduction of surface friction [9].

Of the *P. vulgaris* O-specific polysaccharides studied, some are neutral [6,10,11] and others acidic [6,10,12–15]. Now, we report the structure of a new acidic O-antigen of *P. vulgaris* O22 and its serological cross-reactivity with some other *Proteus* O-antigens with known structures.

2. Results and discussion

Chemical studies.—LPS was isolated from dried bacterial cells of *P. vulgaris* O22 by phenol–water extraction [16] and degraded with dilute acetic acid to give a high-molecular mass O-specific polysaccharide. Sugar analysis of the polysaccharide, using ion-exchange chromatography on sugar and amino acid analysers, revealed the presence of Rha, GlcA, GlcN and Qui3N. Determination of the absolute configurations by GLC of acetylated glycosides with chiral alcohols showed that GlcA, GlcN and Qui3N have D configuration, whereas Rha has L configuration.

In the ^{13}C NMR spectrum of the O-specific polysaccharide, there were signals of different integral intensities. They could originate from nonstoichiometric O-acetylation since the spectrum contained signals for *O*-acetyl groups at δ 21–22, which disappeared upon O-deacetylation of the polysaccharide with aqueous ammonia.

The ^{13}C NMR spectrum of the O-deacetylated polysaccharide (Fig. 1) contained signals for five anomeric carbons at δ 96.5–104.2, one nonsubstituted CH_2OH group (C-6 of GlcNAc) at δ 62.0, three methyl groups (C-6 of 6-deoxy sugars) at δ 17.7–18.1, two carbons bearing nitrogen (C-3 of Qui3N and C-2 of GlcN) at δ 55.3 and 55.6, 18 other sugar ring carbons at δ 68.6–84.8, and two *N*-acetyl groups at δ 23.7 and 23.9 (Me). In the region

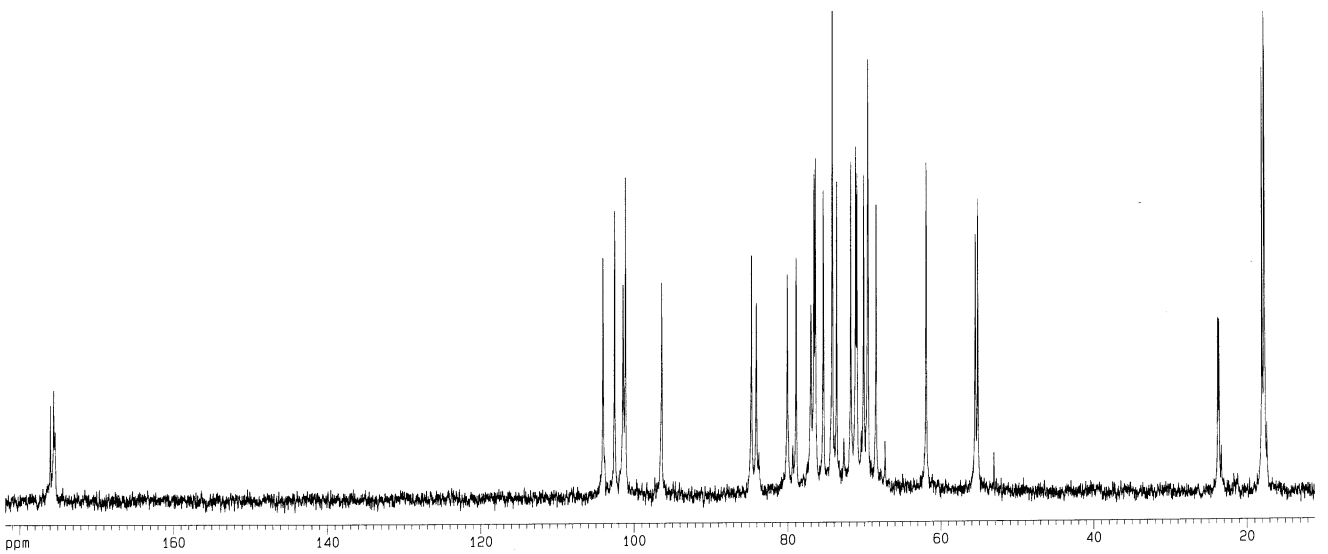

Fig. 1. ^{13}C NMR spectrum of the O-deacetylated polysaccharide.

Table 1
^{1}H NMR data (δ, ppm) for the O-deacetylated polysaccharide of *P. vulgaris* O22 [a]

Sugar residue	Proton					
	H-1	H-2	H-3	H-4	H-5	H-6a, H-6b
→4)-β-D-Glc*p*A-(1→	4.51	3.34	3.60	3.63	3.75	
→4)-α-L-Rha*p*^{I}-(1→	4.78	3.97	3.87	3.53	4.11	1.28
→3)-β-L-Rha*p*^{II}-(1→	4.68	4.38	3.71	3.31	3.45	1.32
→3)-β-D-Glc*p*NAc-(1→	5.01	3.91	3.51	3.55	3.32	3.83, 3.77
α-D-Qui*p*3NAc-(1→	5.06	3.72	4.21	3.21	4.18	1.22

[a] The chemical shifts for NAc are δ 2.10 and 2.16.

of δ 175.5–176.1 there were three signals for one carboxyl group (C-6 of GlcA) and two *N*-acetyl groups (CO). Accordingly, the ^{1}H NMR spectrum contained signals for five anomeric protons at δ 4.68–5.06, three methyl groups of 6-deoxy sugars at δ 1.22–1.32, and two *N*-acetyl groups at δ 2.10 and δ 2.16. Therefore, the polysaccharide has a pentasaccharide repeating unit containing one residue each of D-Qui3NAc, D-GlcNAc, D-GlcA, and two residues of L-Rha.

Methylation analysis of the polysaccharide using GLC-MS of partially methylated alditol acetates revealed 4-substituted Rha, 2,3-disubstituted Rha, terminal Qui3NAc, and 3-substituted GlcNAc. When the methylated polysaccharide was reduced with $LiBH_4$ prior to hydrolysis, 4,6-di-*O*-methylglucose was identified, which was evidently derived from 4-substituted GlcA. The content of the glucose derivative was low compared with other partially methylated monosaccharides, most likely due to degradation of 4-substituted GlcA by β-elimination under alkaline conditions of methylation. Therefore, the polysaccharide is branched, one of the Rha residues is at the branching point, the terminal position in the side chain is occupied by a Qui3NAc residue, and the remaining monosaccharides are monosubstituted at position 3 (GlcNAc) or 4 (Rha and GlcA).

The ^{1}H NMR spectrum of the O-deacetylated polysaccharide was assigned using 2D COSY and TOCSY experiments (Table 1). The TOCSY spectrum showed correlations between H-1 and H-2,3,4,5,6 of GlcNAc, Qui3NAc, and one of the Rha residues (Rha^{I}), and between H-1 and H-2,3,4,5 of GlcA. The only correlation exhibited by H-1 of Rha^{II} was that with H-2, but there were cross-peaks between H-2 and H-3,4,5,6 that allowed the full assignment to be performed. The assignment of the 6-deoxy sugars was confirmed by correlations between H-6 and H-1,2,3,4,5 of Rha^{I} and Qui3NAc and between H-6 and H-2,3,4,5 of Rha^{II}. The two Rha residues were distinguished by relatively small $J_{2,3}$ coupling constant values of 3–4 Hz, compared with those of $\sim$10 Hz for the three other sugars with the *gluco* configuration. The *N*-acetamido sugars were identified by correlation of protons at carbons bearing nitrogen to the corresponding carbons (C-2 of GlcNAc and C-3 of Qui3NAc) at δ 55.3 and δ 55.0, respectively, as revealed by a ^{1}H,^{13}C HMQC experiment.

The coupling constant values between the vicinal protons of the sugar rings were typical of the pyranose form in all five constituent monosaccharides [17]. A relatively small $J_{1,2}$ coupling constant value of 3.5 Hz demonstrated that Qui3NAc is α-linked, whereas larger values of 7.5–8.5 Hz showed that GlcNAc and GlcA are β-linked. The positions of the signals for H-5 (δ 4.11 and 3.45) and C-5 (δ 68.6 and 73.7) of Rha^{I} and Rha^{II}, respectively, indicated that the former is α-linked, and the latter β-linked (compare the H-5 and C-5 chemical shifts for α-Rha*p* (δ_H 3.86, δ_C 70.0) and β-Rha*p* (δ_H 3.39, δ_C 73.2) [18,19]). An upfield displacement of the signal for C-5 and a downfield displacement of the signal for H-5 of Rha^{I}, as compared with their positions in the spectra of α-Rha*p*, were due to the substitution of Rha^{I} at position 4 (see below). The linkage configurations were confirmed by

Table 2
^{13}C NMR data (δ, ppm) for the O-deacetylated polysaccharide of *P. vulgaris* O22 [a]

Sugar residue	Carbon					
	C-1	C-2	C-3	C-4	C-5	C-6
→4)-β-D-Glc*p*A-(1→	104.2	74.3	75.5	80.2	77.1	175.5
→4)-α-L-Rha*p*^{I}-(1→	101.5	71.1	70.2	84.8	68.6	17.8
→3)-β-L-Rha*p*^{II}-(1→	101.2	76.7	79.0	71.9	73.7	18.1
→3)-β-D-Glc*p*NAc-(1→	102.6	55.6	84.2	69.7	76.5	62.0
α-D-Qui*p*3NAc-(1→	96.5	71.3	55.3	74.3	69.7	17.7

[a] The chemical shifts for NAc are δ 23.7, 23.9 (Me), 175.6 and 176.1 (CO).

intraresidue H-1/H-2 correlation for the α-linked sugars and H-1/H-3,5 correlations for the β-linked sugars, which were revealed by a NOESY experiment.

The ^{13}C NMR spectrum of the O-deacetylated polysaccharide was assigned using a ^{1}H,^{13}C HMQC experiment (Table 2). The signals for C-4 of α-Rha^{I} and β-GlcA, C-3 of β-GlcNAc, C-2 and C-3 of β-Rha^{II} were shifted downfield to δ 84.8, 80.2, 84.2, 76.7 and 79.0, respectively, as compared with published data for the corresponding nonsubstituted monosaccharides [18,19]. These displacements (by 4–12 ppm) were due to the α effects of glycosylation and confirmed the substitution pattern of the polysaccharide. Close positions of the signals for α-Qui3NAc in the ^{13}C NMR spectra of the O-deacetylated polysaccharide and the nonsubstituted monosaccharide [20] were in agreement with the terminal position of this residue in the side chain.

A NOESY experiment revealed the following interresidue correlations between the transglycosidic protons: Rha^{II} H-1/Rha^{I} H-4 at δ 4.68/3.53, Rha^{I} H-1/GlcA H-4 at δ 4.78/3.63, and GlcA H-1/GlcNAc H-3 at δ 4.51/3.51. These data were consistent with the substitution pattern revealed by the ^{1}H,^{13}C HMQC experiment and demonstrated the sequence of monosaccharides in the oligosaccharide part structure Rha^{II}-(1→4)-Rha^{I}-(1→4)-Glc*p*A-(1→3)-Glc*p*NAc. H-1 of GlcNAc gave strong cross-peaks with both H-2 and H-3 of Rha^{II} at δ 5.01/4.38 and δ 5.01/3.71, respectively. H-1 of Qui3NAc gave a likewise strong cross-peak with H-2 of Rha^{II} at δ 5.06/4.38, but an additional correlation with H-3 of Rha^{II} at δ 5.06/3.71 could not be excluded (if present, the Qui3NAc H-1/Rha^{II} H-3 cross-peak would overlap with the intraresidue Qui3NAc H-1/H-2 cross-peak at δ 5.06/3.72). However, the attachment of Qui3NAc at position 2 of Rha^{II} could be demonstrated by the presence of the Qui3NAc H-1/Rha^{II} H-1 at δ 5.06/4.68. Hence, GlcNAc, which gave no H-1/H-1 cross-peak, is attached at position 3 of Rha^{II}. The unusual NOE pattern in Rha^{II} may be accounted for by a deviation from the regular conformation around the glycosidic linkages caused by the attachment of two *N*-acetamido sugars at the vicinal positions.

The data obtained allowed the structure for the pentasaccharide repeating unit of the O-deacetylated polysaccharide (Scheme 1) to be determined.

The ^{1}H and ^{13}C NMR spectra of the initial polysaccharide were assigned as described above for the O-deacetylated polysaccharide. Analysis of the ^{1}H,^{13}C HMQC spectrum (Fig. 2) showed that O-acetylation caused most significant changes in Qui3NAc. In particular, part of the H-2/C-2 cross-peak shifted downfield to δ 4.97/72.1, as compared with its position at δ 3.72/71.3 in the ^{1}H,^{13}C HMQC spectrum of the O-deacetylated polysaccha-

α-D-Qui*p*3NAc
1
↓
2
→3)-β-D-Glc*p*NAc-(1→3)-β-L-Rha*p*^{II}-(1→4)-α-L-Rha*p*^{I}-(1→4)-β-D-Glc*p*A-(1→

Scheme 1. Structure of the branched pentasaccharide repeating unit of the acidic O-deacetylated polysaccharide of *P. vulgaris* O22.

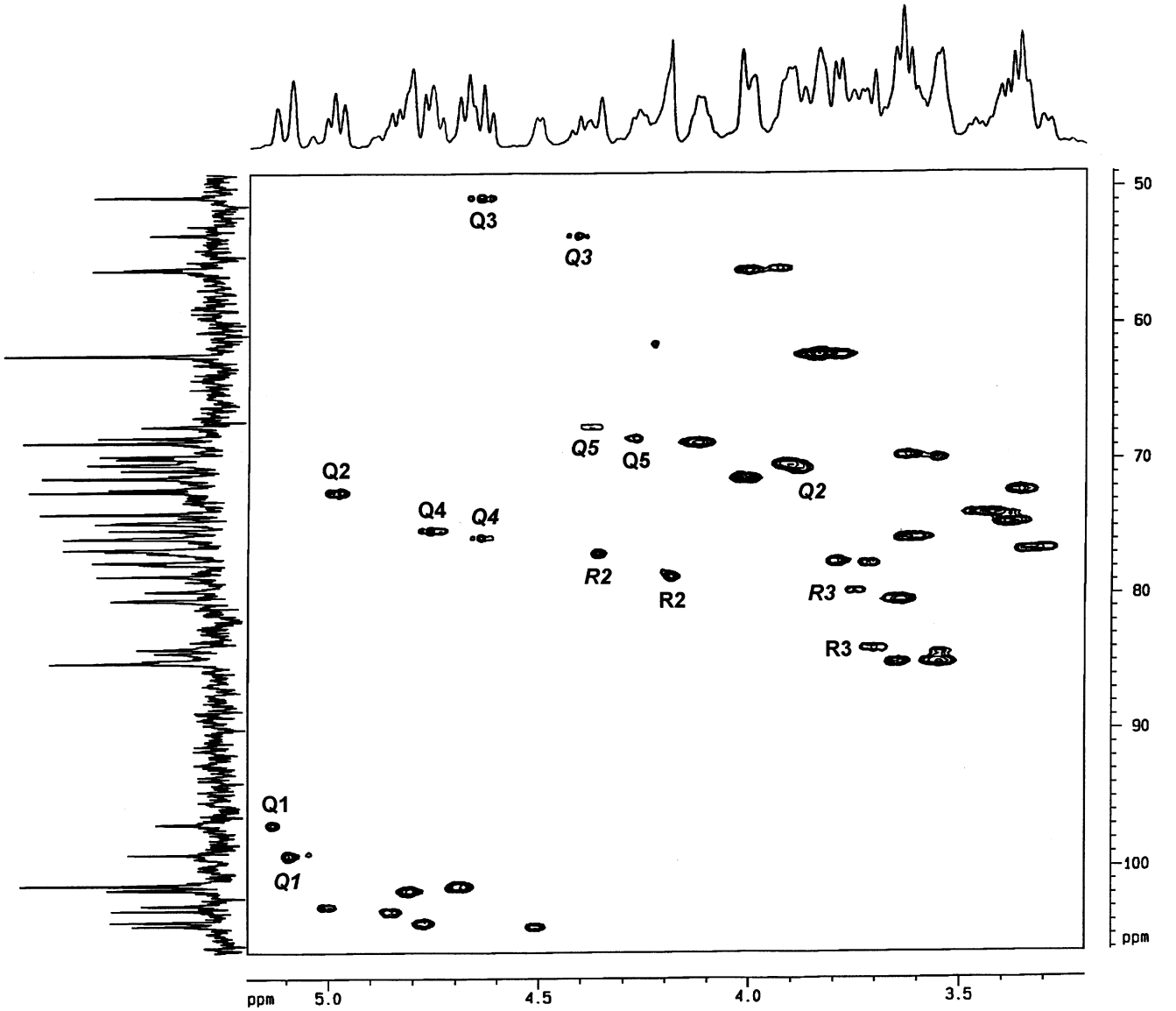

Fig. 2. Part of a $^{1}H,^{13}C$ HMQC spectrum of the O-specific polysaccharide. The corresponding parts of the ^{1}H and ^{13}C NMR spectra are shown along the horizontal and vertical axes, respectively. Arabic numerals refer to the atoms in Qui3NAc2,4Ac$_2$ (Q) or Qui3NAc4Ac (*Q*) and Rha^{II} (R or *R*, respectively).

ride. This displacement was due to a strong deshielding effect of the *O*-acetyl group at position 2 and, therefore, one of the *O*-acetyl groups is attached at O-2 of a part of the Qui3NAc residues. The Qui3NAc H-4/C-4 cross-peak shifted from δ 3.21/74.3 to δ 4.75/75.0 and δ 4.63/75.6 in the 2-O-acetylated and non-2-O-acetylated Qui3NAc residue, respectively. This displacement demonstrated full O-acetylation of Qui3NAc at O-4. The O-acetylation pattern was confirmed by an upfield shift of the signal for C-3 from δ 55.3 in Qui3NAc to δ 53.2 and 50.5 in Qui3NAc4Ac and Qui3NAc2,4Ac$_2$, respectively, which was caused by β effects of O-acetylation at O-2 and O-4 [21]. Unexpectedly, the C-1 signal shifted downfield from δ 96.5 in Qui3NAc to δ 98.9 in Qui3NAc2,4Ac$_2$, which could be accounted for by a strong influence of O-acetylation on the conformation around the glycosidic linkage in the Qui3NAc-(1→2)-Rha^{II} disaccharide fragment. O-Acetylation of Qui3NAc also affected, most likely for the same reason, some signals of the neighbouring sugars. In particular, the H-2/C-2 and H-3/C-3 cross-peaks of Rha^{II} shifted from δ 4.38/76.7 and δ 3.71/79.0 in the repeating unit with Qui3NAc to δ 4.19/78.4 and δ 3.70/83.8 in the

Table 3
Serological reactivity of *Proteus* LPS with anti-*P. vulgaris* O22 serum (reciprocal titres) [a]

Antigen of	Passive hemolysis	EIA
P. vulgaris O22	12,800	512,000
P. vulgaris O32	3200	250
P. penneri 14	3200	256,000
P. penneri 52	<100	8000

[a] Passive haemolysis was performed with early (16 days) serum using alkali-treated LPS as antigen. EIA was performed with late (56 days) serum using LPS as antigen; the end titre was taken as the highest dilution of antiserum yielding $A_{405} > 0.2$.

repeating unit with Qui3NAc2,4Ac$_2$, respectively. None of the signals for the other monosaccharides shifted significantly in the carbon dimension, thus indicating that Qui3NAc is the only O-acetylated sugar. The ratio of the integral intensities of the signals for Qui3NAc2,4Ac$_2$ and Qui3NAc4Ac allowed estimation of the degree of O-acetylation at position 2 as $\sim 65\%$.

The data obtained showed that the majority of the pentasaccharide repeating units of the O-specific polysaccharide of *P. vulgaris* O22 has the structure shown below, and the minority lacks the *O*-acetyl group at position 2 of Qui3NAc.

α-D-Qui*p*3NAc2,4Ac$_2$
1
↓
2
→3)-β-D-Glc*p*NAc-(1→3)-β-L-Rha*p*^{II}-(1→4)-α-L-Rha*p*^{I}-(1→4)-β-D-Glc*p*A-(1→

P. vulgaris O22

Serological studies.—Anti-*P. vulgaris* O22 serum was tested with *Proteus* LPS with known O-antigen structures and found to cross-react with LPS of three strains, namely *P. vulgaris* O32 and *P. penneri* strains 14 and 52 [14,22–24] (Table 3, Fig. 3). Anti-*P. vulgaris* O22 serum cross-reacted with LPS of *P. vulgaris* O32 in passive hemolysis, and earlier it had been shown that anti-*P. vulgaris* O32 serum cross-reacted with *P. vulgaris* O22 LPS [14]. The two-way cross-reactivity suggested that *P. vulgaris* O22 and O32 share an epitope on the LPS. This may be associated with structurally similar trisaccharide fragments of the O-antigens, namely α-L-Rha*p*^{I}-(1→4)-β-D-Glc*p*A-(1→3)-β-D-GlcpNAc in *P. vulgaris* O22 and α-L-Rha*p*^{I}-(1→4)-β-D-Gal*p*A-(1→3)-β-D-Glc*p*NAc in *P. vulgaris* O32 (see the full structure of the latter O-antigen below). The only difference between the putative cross-reactive epitopes is the configuration at C-4 of β-hexuronic acids, but β-D-GalpA was found to be of no importance for manifesting

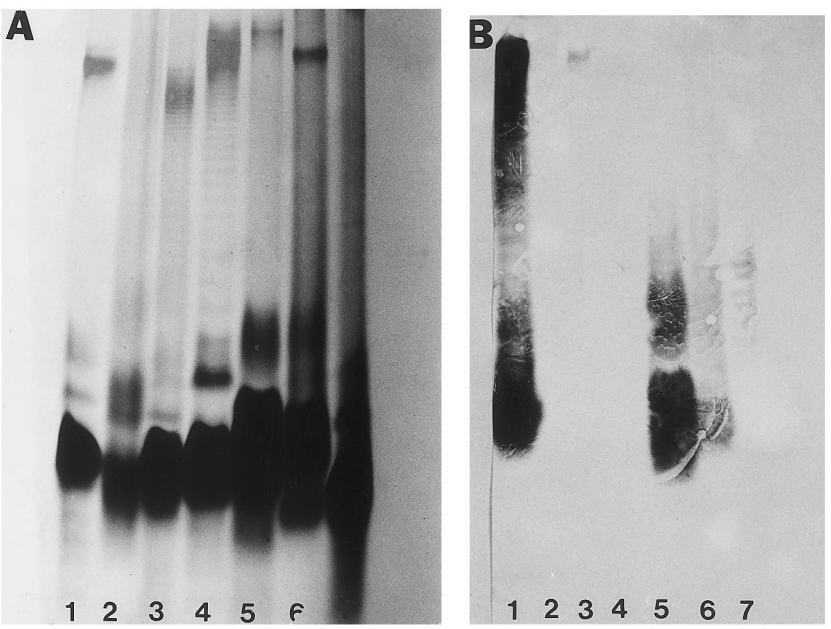

Fig. 3. Silver-stained sodium deoxycholate-PAGE (A) and parallel Western blot with anti-*P. vulgaris* O22 serum (B) of *Proteus* LPS. Lane 1, *P. vulgaris* O22; lane 2, *P. mirabilis* O28; lane 3, *P. vulgaris* O32; lane 4, *P. vulgaris* O45; lane 5, *P. penneri* 14; lane 6, *P. penneri* 52; lane 7, *P. penneri* 26.

the immunospecificity of the *P. vulgaris* O32 O-antigen [14]. That no significant cross-reactivity was observed in enzyme immunosorbent assay (EIA) and Western blot could be accounted for by a different exposure of LPS (on the polystyrol surface and nitrocellulose membrane, respectively, rather than on the erythrocyte membrane).

P. vulgaris O32

The O-antigens of *P. vulgaris* O22 and cross-reactive *P. penneri* 52 (in EIA and Western blot) and *P. penneri* 14 (in all assays used, Table 3, Fig. 3) share only one sugar residue, →3)-β-D-Glc*p*NAc. The importance of the single →3)-β-D-Glc*p*NAc unit in manifesting the immunospecificity of two *P. mirabilis* O-antigens has been reported [25].

3. Experimental

Bacterial strain, isolation and degradation of lipopolysaccharide.—*P. vulgaris* O22, strain PrK 40/57, was from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague). The bacterium was grown as described in Ref. [26].

LPS was isolated from dried bacterial cells of *P. vulgaris* by extraction with hot aq phenol [16] and purified by treatment with cold aq 50% CCl_3CO_2H followed by dialysis of the supernatant.

Acid degradation of LPS was performed with aq 1% AcOH 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). O-Deacetylation of the polysaccharide was performed with aq 12% ammonia for 37 °C overnight. Alkali-treated LPS was prepared by saponification of LPS with 0.25 M NaOH (37 °C, 15 h); the solution was evaporated in vacuo, the residue washed with methanol, dissolved in water, and lyophilised.

Sugar analysis.—The polysaccharide was hydrolysed with 2 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 previously [14]. The absolute configurations of the monosaccharides were determined by GLC of acetylated (*R*)-2-octyl glycosides (for Rha and Qui3NAc) and (*S*)-2-butyl glycosides (for GlcA and GlcNAc) [27–29] using a Hewlett–Packard 5890 chromatograph equipped with a DB-5 fused-silica capillary column. D-Qui3NAc from the O-specific polysaccharide of *Providencia alcalifaciens* O5 [30] served as the reference sugar.

Methylation analysis.—The polysaccharide was methylated according to the Hakomori procedure [31]. A part of the methylated polysaccharide was hydrolysed as in sugar analysis, and partially methylated alditol acetates derived were identified by GLC-MS on a NERMAG R10-10L mass spectrometer (France) equipped with a DB-5 fused-silica capillary column using published data [32,33]. Another part was reduced with $LiBH_4$ in aq 70% 2-propanol (20 °C, 16 h), then hydrolysed and analysed as above.

NMR spectroscopy.—^1H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer in D_2O at 60 °C using internal acetone (δ_H 2.225, δ_c 31.45) as reference. Standard Bruker software (XWINNMR 1.2) was used to acquire and process the NMR data. Mixing times of 120 and 300 ms were used in TOCSY and NOESY experiments, respectively.

Serological techniques.—Preparation of O-antisera, EIA using LPS as coating antigen, SDS-PAGE, and Western blot were carried out as described earlier [14]. Passive haemolysis and passive haemagglutination were performed as described [14], using sheep red blood cells sensitised with the optimal amount of alkali-treated LPS. In the case of *P. vulgaris* O22, 64 μg alkali-treated LPS per 0.2 ml cells was used.

Acknowledgements

This work was supported by grants 99-04-48279 of the Russian Foundation for Basic

Research and grant 4 PO5A 140 14 of the Sciences Research Committee (KBN, Poland).

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