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New Structures of the O-Specific Polysaccharides of *Proteus*. 3. Polysaccharides Containing Non-carbohydrate Organic Acids*

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Abstract—Four new *Proteus* O-specific polysaccharides were isolated by mild acid degradation from the lipopolysaccharides of *P. penneri* 28 (**1**), *P. vulgaris* O44 (**2**), *P. mirabilis* G1 (O3) (**3**), and *P. myxofaciens* (**4**), and their structures were elucidated using NMR spectroscopy and chemical methods. They were found to contain non-carbohydrate organic acids, including ether-linked lactic acid and amide-linked amino acids, and the following structures of the repeating units were established:

→3)-α-L-Qui*p*NAc-(1→3)-α-D-Glc*p*NAc-(1→6)-α-D-Glc*p*NAc-(1→ **(1)**
(S)-Lac-(2–3)┘

→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→ **(2)**
L-Ala-(2–6)┘

→3)-β-D-Gal*p*NAc-(1→6)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*A-(1→ **(3)**
L-Lys-(2–6)-α-D-Gal*p*A-(1→4)┘

→4)-β-D-Glc*p*A-(1→6)-α-D-Gal*p*NAc-(1→6)-β-D-Glc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→ **(4)**
(R)-aLys-(2–6)┘

where (S)-Lac and (R)-aLys stand for (S)-1-carboxyethyl (residue of lactic acid) and N^{ε}-[(R)-1-carboxyethyl]-L-lysine ("alaninolysine"), respectively. The data obtained in this work and earlier serve as the chemical basis for classification of the bacteria *Proteus*.

Key words: lipopolysaccharide, O-antigen, bacterial polysaccharide, structure, lactic acid, amino acid, *Proteus*

Three species of the genus *Proteus*, *P. vulgaris*, *P. mirabilis*, and *P. penneri*, are a common cause of urinary tract infections and often found in clinical material [2, 3]. Recently, it has been suggested that *P. mirabilis* plays an etiopathogenic role in rheumatoid arthritis [4]. Based on the immunospecificity of the cell-wall lipopolysaccharides, two species, *P. mirabilis* and *P. vulgaris*, have been classified into 60 O-serogroups [5, 6], and recently new O-serogroups have been proposed for *P. penneri* strains [7, 8]. A *Proteus* strain producing a copious amount of slime was isolated in 1996 from living and dead larvae of the gypsy moth (*Porthetria dispar*) and called *P. myxofaciens* [9]. The medical importance of this strain and its position in the serological classification of *Proteus* is yet to be determined.

With the goal of the creation of the molecular basis for classification of *Proteus* strains, we have elucidated

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structures of the O-specific polysaccharide chains of the lipopolysaccharides of a number of serologically distinguishable strains of *P. vulgaris*, *P. mirabilis*, and *P. penneri* [1, 7, 10]. Most *Proteus* polysaccharides studied contain non-sugar components, such as residues of lactic, pyruvic, and amino acids, various N-acyl groups of amino sugars [7], O-acetyl groups [10], phosphate groups, and phosphate-linked amino alcohols [1]. Here we report on the determination of structures of a new O-specific polysaccharide containing lactic acid, which was isolated from *P. penneri* 28, and two O-specific polysaccharide from *P. vulgaris* O44 and *P. mirabilis* G1, which contain amino acids. In addition, we studied, for the first time, the O-specific polysaccharide of the only representative of *P. myxofaciens* and found it to contain a 1-carboxyethyl derivative of lysine.

MATERIALS AND METHODS

Bacterial strains, growth, and isolation of lipopolysaccharides and O-specific polysaccharides. *P. penneri* 28 was supplied by Prof. D. J. Brenner (Center for Disease Control, Atlanta, USA). *P. myxofaciens* was from the Culture Collection of the University of Göteborg (CCUG, Sweden). *P. vulgaris* O44 was from the Czech National Collection of Type Cultures (CNCTC, Institute of Epidemiology and Microbiology, Prague). *P. mirabilis* G1 was kindly provided by Dr. J. Gmeiner (Institute for Microbiology and Genetics, Darmstadt, Germany). The bacteria were grown on nutrient broth (Warsaw Laboratory of Sera and Vaccines) supplemented with 1% glucose. Bacterial cells were separated by centrifugation, washed with distilled water, and lyophilized.

Lipopolysaccharides were isolated by extraction of dried cells with hot aqueous phenol [11] and purified by treatment with ribonuclease and deoxyribonuclease and by ultracentrifugation [12] as described. The lipopolysaccharides were degraded with 2% acetic acid at 100°C until the precipitation of lipid. High-molecular-mass O-specific polysaccharides were isolated from the water-soluble portion by gel-permeation chromatography on Sephadex G-50 as described [12] using a differential refractometer Knauer (Germany) for monitoring.

NMR spectroscopy. NMR spectra were recorded on Bruker WM-250 and Bruker DRX-500 spectrometers (Germany) in solutions in D_2O (internal standard acetone, δ_H 2.225 ppm, δ_C 31.45 ppm). Assignment of the NMR spectra was performed using two-dimensional (2D) experiments (COSY, TOCSY, H-detected $^1H,^{13}C$ HMQC) as described previously [1]. Linkage and sequence analyses were performed using 2D nuclear Overhauser effect (ROESY or NOESY) and $^1H,^{13}C$ heteronuclear multiple-bond correlation (HMBC) experiments.

Chemical methods. The polysaccharides were hydrolyzed with 2 M CF_3COOH (120°C, 3 h), and the monosaccharides were converted into the acetylated alditols [10] and analyzed by GLC using a Hewlett-Packard 5890 chromatograph equipped with an Ultra 2 capillary column and a temperature gradient of 160→290°C at 3°C/min. Amino and neutral sugars were identified using a Biotronik LC-2000 amino acid and sugar analyzer equipped with a column (0.4 × 22 cm) of Ostion LG AN B cation-exchange resin in 0.2 M sodium citrate buffer (pH 3.25) at 65°C. The absolute configurations of the monosaccharides and amino acids were determined by GLC of the acetylated (S)-2-octyl or (S)-2-butyl glycosides [13-15] and (S)-2-butyl esters, respectively, under the conditions described above. Synthetic N^{ε}-[(R)- and (S)-1-carboxyethyl]-L-lysine [16], muramic acid from purchased N-acetylmuramic acid and isomuramic acid from the O-polysaccharide of *P. penneri* 19 [17] were used as references.

RESULTS

***Proteus penneri* 28.** Analysis using an amino acid analyzer after hydrolysis of the polysaccharide revealed GlcN and 2-amino-2,6-dideoxyglucose (quinovosamine, QuiN) in the ratio ~1 : 1 as well as another amino component, which had the same retention time as isomuramic acid and different from that of muramic acid. The identification of the amino sugars was confirmed and the D configuration of GlcN and the L configuration of QuiN were determined by GLC of the acetylated (+)-2-octyl glycosides. The structure of isomuramic acid, including the presence and position of the lactic acid residue (Lac) and the absolute configuration of the sugar residue, were confirmed by NMR spectroscopic studies of the polysaccharide (see below).

The ^{13}C-NMR spectrum of the polysaccharide (Fig. 1) contained signals for three anomeric carbons at δ 98.0-98.8, one unsubstituted and one substituted O*C*H_2-C group at δ 61.9 and 66.2, respectively, one *C*H_3-C group of a 6-deoxy sugar at δ 17.8, three nitrogen-bearing carbons at δ 54.0-54.9, 11 oxygen-bearing carbons in the region δ 69.0-82.4, a lactic acid residue (CH_3 at δ 19.8 and COOH at δ 179.9), and three N-acetyl groups of amino sugars (CH_3 at δ 23.3, 23.4, and 23.6; CO at δ 174.5, 174.8, and 175.2).

Accordingly, the ^1H-NMR spectrum of the polysaccharide contained signals for three anomeric protons at δ 4.92-5.07, two CH_3-C groups at δ 1.34 and 1.43, and three N-acetyl groups at δ 2.03-2.16. Therefore, it was concluded that the polysaccharide has a trisaccharide repeating unit containing one residue each of D-GlcNAc, L-QuiNAc, and N-acetylisomuramic acid (Fig. 2, structure *1*).

The ^1H-NMR spectrum of the polysaccharide was assigned using a 2D COSY experiment, which showed correlations between all protons within each sugar spin

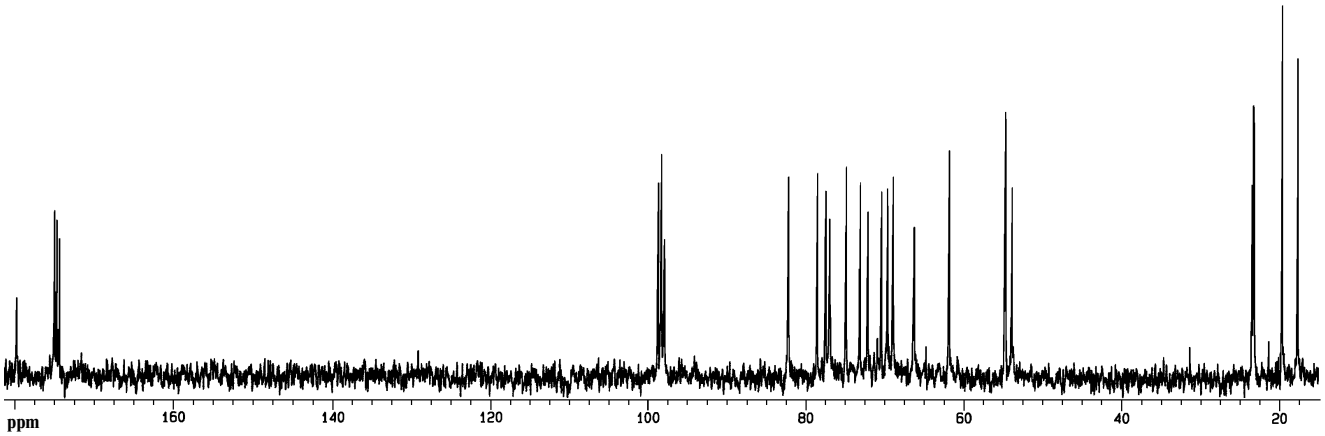

Fig. 1. ^{13}C-NMR spectrum of the O-specific polysaccharide of *P. penneri* 28.

system; this was confirmed by a 2D TOCSY experiment. The spin system of Qui*p*NAc was distinguished from those of Glc*p*NAc (Glc*p*NAc^{I} and Glc*p*NAc^{II}) by a H5/H6 correlation at δ 4.26/1.34 in the COSY spectrum. An H2/H3 cross-peak at δ 4.29/1.43 was observed for the lactic acid residue in both 2D NMR spectra. With the ^{1}H-NMR spectrum assigned (Table 1), the ^{13}C-NMR spectrum was assigned using a 2D ^{1}H,^{13}C HMQC experiment (Table 2).

The appearance of the signals for the anomeric protons as broadened singlets indicated small $J_{1,2}$ coupling constants and thus the α-configuration of all three sugar residues. Accordingly, a 2D ROESY experiment showed an H1/H2 correlation for each sugar residue typical of the 1,2-*cis* orientation of these protons but no correlation of H1 with H3 and H5, which would be observed for β-linked monosaccharides. The ^{1}H,^{13}C HMBC spectrum showed a correlation between Lac H2 and GlcNAc^{II} C3 at δ 4.29/82.4, thus demonstrating the location of the lactic acid residue at position 3 of GlcNAc^{II}.

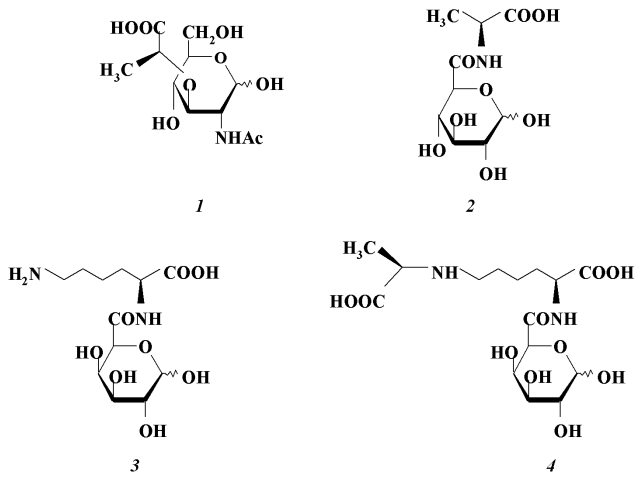

Fig. 2. Components of the O-specific polysaccharides of *Proteus* containing non-sugar organic acids. *1*) 2-Acetamido-3-O-[(S)-1-carboxyethyl]-2-deoxy-D-glucose (N-acetylisomuramic acid); *2*) N-(D-glucuronoyl)-L-alanine; *3*) N$^{\alpha}$-(D-galacturonoyl)-L-lysine; *4*) N$^{\varepsilon}$-[(R)-1-carboxyethyl]-N$^{\alpha}$-(D-galacturonoyl)-L-lysine.

Low-field displacements of the signals for C3 of Qui*p*NAc and Glc*p*NAc^{I} and C6 of Glc*p*NAc^{II} to δ 77.5, 76.9, and 66.2, respectively, i.e., by 4-6 ppm as compared with their positions in the spectra of the corresponding non-substituted monosaccharides [18, 19], revealed the substitution pattern of the monosaccharide residues and showed that the polysaccharide is linear.

The 2D ROESY experiment showed Glc*p*NAc^{II} H1/Qui*p*NAc H3 and Qui*p*NAc H1/Glc*p*NAc^{I} H3 cross-peaks at δ 5.07/3.81 and 5.06/3.93, respectively. Both Glc*p*NAc^{I} H1 at δ 4.92 and Lac H2 signals at δ 4.29 gave cross-peaks with a signal at δ 3.66, which were interpreted as Glc*p*NAc^{I} H1/Glc*p*NAc^{II} H6a and Lac H2/Glc*p*NAc^{II} H3 correlations, respectively. These data were in agreement with positions of substitution of the monosaccharides and indicated a →QuiNAc→ GlcNAc^{I}→GlcNAc^{II}→ sequence in the repeating unit.

A relatively large effect (−2.2 ppm) on C4 of L-QuiNAc caused by its glycosylation by α-GlcNAc^{II} indicated different absolute configurations of the linked sugar residues, i.e., the D configuration of GlcNAc^{II} (compare the corresponding glycosylation effects of −2 and +0.7 ppm in α1→3-linked disaccharides of 2-acetamido-2-deoxy-L- and -D-galacturonic acid with D-QuiNAc, respectively [19]). This finding confirmed finally the presence of 2-acetamido-3-O-[(S)-1-carboxyethyl]-2-deoxy-D-glucose (N-acetylisomuramic acid).

On the basis of these data, it was concluded that the O-specific polysaccharide of *P. penneri* 28 has the structure shown in Table 3.

***Proteus vulgaris* O44.** Sugar analysis of the polysaccharide, including determination of the absolute configu-

Table 1. ^{1}H-NMR data (δ, ppm)*

Strain	H1	H2	H3	H4	H5	H6a, H6b
Proteus penneri 28						
→3)-α-L-Qui*p*NAc-(1→	5.06	4.16	3.81	3.37	4.26	1.34
→3)-α-D-Glc*p*NAc^{I}-(1→	4.92	4.13	3.93	3.64	3.84	3.86, 3.93
→6)-α-D-Glc*p*NAc^{II}-(1→	5.07	4.11	3.66	3.82	4.28	3.66, 4.03
(S)-Lac		4.29	1.43			
Proteus vulgaris O44						
→4)-β-D-GlcpA-(1→	4.53	3.36	3.59	3.87	3.90	
→3)-β-D-Gal*p*NAc^{I}-(1→	4.56	3.98	3.85	4.07	3.69	3.70, 3.75
→4)-β-D-Glc*p*-(1→	4.67	3.37	3.65	3.56	3.52	3.59, 3.81
→3)-α-D-Gal*p*-(1→	4.96	3.98	4.07	4.23	4.29	3.65, 3.65
→4)-β-D-Gal*p*NAc^{II}-(1→	4.44	3.89	3.72	4.01	3.66	3.81, 3.90
L-Ala		4.33	1.43			
Proteus mirabilis G1						
→3)-β-D-Gal*p*NAc^{I}-(1→	4.55	4.02	3.85	4.12	3.70	3.78, 3.78
→6)-β-D-Gal*p*NAc^{II}-(1→	4.51	3.89	3.80	4.03	3.85	3.89, 4.16
4 ↑						
→4)-β-D-Glc*p*A-(1→	4.53	3.36	3.56	3.73	3.76	
α-D-Gal*p*A-(1→	5.20	3.86	4.05	4.30	4.96	
L-Lys		4.38	1.79, 1.91	1.43	1.68	3.00
Proteus myxofaciens						
→6)-α-D-Gal*p*NAc-(1→	4.92	4.20	3.97	4.01	4.13	3.87, 4.01
→6)-β-D-Glc*p*NAc^{I}-(1→	4.62	3.73	3.58	3.60	3.68	3.71, 3.98
→3)-β-D-Glc*p*NAc^{II}-(1→	4.40	3.77	3.65	3.51	3.41	3.73, 3.90
→4)-β-D-Glc*p*A-(1→	4.58	3.36	3.63	3.95	3.97	
(R)-aLys**		4.34	1.76, 1.93	1.48	1.72	3.06

Note: (S)-Lac is (S)-1-carboxyethyl (residue of lactic acid); (R)-aLys is N$^{\varepsilon}$-[(R)-1-carboxyethyl]-L-lysine ("alaninolysine").

* Signals for NAc-groups: δ 1.94-2.16.

** Signals for 1-carboxyethyl group: δ 1.47 (CH_3) and 3.72 (CHN).

ration of the monosaccharides, revealed D-Glc, D-Gal, and D-GalN in the ratios ~1 : 1 : 2, respectively. In addition, there were found L-Ala, GlcA, and another acidic component, which was either a GlcA-containing disaccharide or an amide of GlcA with Ala (see below). The D configuration of GlcA was determined by the glycosylation effects in the ^{13}C-NMR spectrum of the polysaccharide.

The ^{13}C-NMR spectrum of the polysaccharide (Fig. 3) contained signals for five anomeric carbons at δ 101.5-105.2, four unsubstituted O*C*H_2-C groups at δ 61.2, 61.6, 61.8, and 62.0 (data of a DEPT-135 experiment), one carboxyl group of GlcA (δ 170.1), three nitrogen-bearing carbons at δ 50.9, 52.5, and 53.8, 18 sugar-ring oxygen-bearing carbons in the region δ 68.7-81.3, an alanine residue (CH_3 at δ 17.9, CO at δ 178.0), and two N-acetyl groups (CH_3 at δ 23.4 and 23.5, CO at δ 175.8 and 175.9).

Accordingly, the ^{1}H-NMR spectrum of the polysaccharide contained signals for five anomeric protons at δ 4.53-4.96, one CH_3-C group of Ala at δ 1.43, and two N-acetyl groups at δ 1.99 and 2.00. Therefore, the polysaccharide has a pentasaccharide repeating unit consisting of

Table 2. ^{13}C-NMR data (δ, ppm)*

Strain	C1	C2	C3	C4	C5	C6
Proteus penneri 28						
→3)-α-L-Qui*p*NAc-(1→	98.0	54.8	77.5	74.9	69.0	17.8
→3)-α-D-Glc*p*NAc^{I}-(1→	98.4	54.9	76.9	69.6	73.2	61.9
→6)-α-D-Glc*p*NAc^{II}-(1→	98.8	54.0	82.4	70.4	72.2	66.2
(S)-Lac	179.9	78.7	19.8			
Proteus vulgaris O44						
→4)-β-D-GlcpA-(1→	105.2	73.4	74.8	78.9	75.3	170.1
→3)-β-D-Gal*p*NAc^{I}-(1→	102.5	52.5	81.3	68.9	76.0	62.0
→4)-β-D-Glc*p*-(1→	104.5	74.2	75.4	80.3	75.4	61.2
→3)-α-D-Gal*p*-(1→	101.5	68.7	80.3	69.9	71.7	61.8
→4)-β-D-Gal*p*NAc^{II}-(1→	101.6	53.8	71.8	78.3	76.5	61.6
L-Ala	178.0	50.9	17.9			
Proteus mirabilis G1						
→3)-β-D-Gal*p*NAc^{I}-(1→	102.4	52.3	81.3	69.0	76.1	62.4
→6)-β-D-Gal*p*NAc^{II}-(1→	102.8	53.2	71.1	75.9	73.4	66.7
4 ↑						
→4)-β-D-Glc*p*A-(1→	105.5	73.7	74.9	81.3	76.9	174.6
α-D-Gal*p*A-(1→	100.9	69.5	70.1	71.0	72.6	172.3
L-Lys	177.9	54.3	31.9	23.0	27.4	40.5
Proteus myxofaciens						
→6)-α-D-Gal*p*NAc-(1→	98.7	50.9	68.5	69.7	71.0	70.1
→6)-β-D-Glc*p*NAc^{I}-(1→	102.7	56.8	74.8	70.7	75.3	66.9
→3)-β-D-Glc*p*NAc^{II}-(1→	100.6	55.2	84.1	70.0	76.7	61.9
→4)-β-D-Glc*p*A-(1→	104.0	73.7	74.6	77.6	75.4	170.1
(R)-aLys**	177.9	55.7	31.9	23.4	26.5	47.0

Note: (S)-Lac is (S)-1-carboxyethyl (residue of lactic acid); (R)-aLys is N$^{\varepsilon}$-[(R)-1-carboxyethyl]-L-lysine ("alaninolysine").
* Signals for NAc-groups: δ 23.3-23.7 (CH_3) and 174.5-176.2 (CO).
** Signals for 1-carboxyethyl group: δ 16.2 (CH_3), 58.8 (CHN), and 175.8 (CO).

two residues of D-GalNAc and one residue each of D-Glc, D-Gal, D-GlcA, and L-Ala.

The ^{1}H-NMR spectrum of the polysaccharide was assigned using 2D COSY, TOCSY, and ^{1}H,^{13}C HMQC-TOCSY experiments (Table 1). Spin systems of Glc*p* and Glc*p*A were identified by a relatively large $J_{3,4}$ coupling constant of ~10 Hz typical of sugar pyranosides with the gluco configuration (compare with the values ≤3 Hz for Gal*p* and Gal*p*NAc having the galacto configuration). The signal for C6 of Glc*p*A was assigned by H4/C6 and H5/C6 correlations at δ 3.87/170.1 and 3.90/170.1, respectively, which were observed in the 2D HMBC spectrum. Two Gal*p*NAc residues (Gal*p*NAc^{I} and Gal*p*NAc^{II}) were distinguished by correlations of the protons at nitrogen-bearing carbons (H2) to the corresponding carbons (C2) at δ 3.98/52.5 and 3.89/53.8, which were revealed by a 2D ^{1}H,^{13}C HMQC experiment.

As judged by relatively large $^{3}J_{1,2}$ coupling constants values of 8-8.5 Hz determined from the ^{1}H-NMR spectrum for four anomeric protons at δ 4.44-4.67, all sugar residues but Gal*p* are β-linked. A smaller $^{3}J_{1,2}$ value of 3.5 Hz showed that Gal*p* is α-linked. This conclusion was

Table 3. New structures of the O-specific polysaccharides of *Proteus* containing non-carbohydrate organic acids and of structurally related polysaccharides

Strain	Structure of O-specific polysaccharide	Reference
Proteus penneri 28 (O31)	(S)-Lac | 3 →3)-α-L-Qui*p*NAc-(1→3)-α-D-Glc*p*NAc^{I}-(1→6)-α-D-Glc*p*NAc^{II}-(1→	this paper
Proteus penneri 26 (O31)	→3)-α-L-Qui*p*NAc-(1→3)-α-D-Glc*p*NAc-(1→6)-α-D-Glc*p*NAc-(1→	[20]
Proteus vulgaris O44	L-Ala | 6 →4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc^{I}-(1→4)-β-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→4)-β-D-Gal*p*NAc^{II}-(1→	this paper
Proteus mirabilis G1 (O3)	L-Lys-(2–6)-α-D-Gal*p*A 1 ↓ 4 →3)-β-D-Gal*p*NAc^{I}-(1→6)-β-D-Gal*p*NAc^{II}-(1→4)-β-D-Glc*p*A-(1→	[21], this paper
Proteus mirabilis S1959 and OXK (O3)	L-Lys-(2–6)-α-D-Gal*p*A α-D-Glc*p* 1 1 ↓ ↓ 4 2 →3)-β-D-Gal*p*NAc-(1→6)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*A-(1→	[22, 23]
Proteus myxofaciens	(R)-aLys 2 | 6 →4)-β-D-GlcA-(1→6)-α-D-Gal*p*NAc-(1→6)-β-D-Glc*p*NAc^{I}-(1→3)-β-D-Glc*p*NAc^{II}-(1→	this paper
Providencia alcalifaciens O23	(R)-aLys 2 | 6 →4)-β-D-Glc*p*A-(1→6)-β-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→	[16, 24]
Providencia alcafaciens O14	(S)-aLys 2 | 6 →4)-α-D-Gal*p*NAc-(1→3)-α-D-Glc*p*NAc-(1→3)-α-D-Gal*p*A-(1→	[25]
Proteus mirabilis O13	(R)-aLys-(2–6)-α-D-Gal*p*A 1 ↓ 4 →3)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*-(1→	[26, 27]

Note: (S)-Lac is (S)-1-carboxyethyl (residue of lactic acid); (R)-aLys is N^{ε}-[(R)-1-carboxyethyl]-L-lysine (“alaninolysine”).

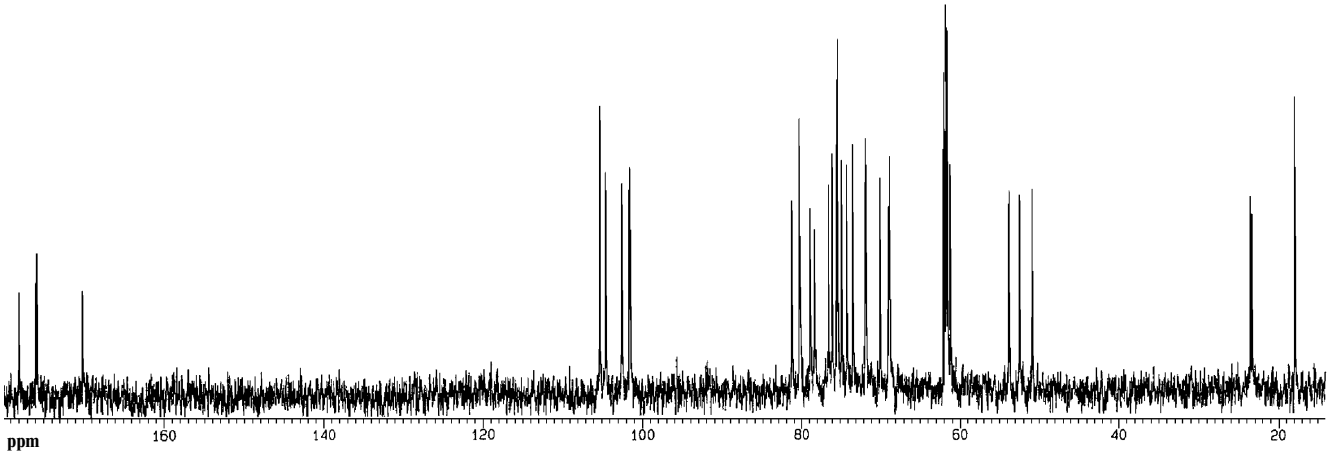

Fig. 3. ^{13}C-NMR spectrum of the O-specific polysaccharide of *P. vulgaris* O44.

confirmed by a 2D ROESY experiment, which revealed intraresidue H1/H3 and H1/H5 correlations for Glc*p*, Glc*p*A, and both Glc*p*NAc residues but no such correlation for Gal*p*.

The ^{13}C-NMR spectrum of the polysaccharide was assigned using 2D ^{1}H,^{13}C HMQC and HMQC-TOCSY experiments (Table 2). Significant downfield displacements of the signals for C4 of Glc, GlcA, and GalNAc^{II} and C3 of Gal and GalNAc^{I} to 78.3-81.3, as compared with their positions in the corresponding unsubstituted sugars at δ 69.1-72.4 [18, 28], enabled determination of the substitution pattern of the monosaccharides.

In addition to the intraresidue correlations, the 2D ROESY experiment showed the following interresidue correlations between the anomeric protons and protons at the linkage carbons: GlcA H1/GalNAc^{I} H3 at δ 4.53/3.85, GalNAc^{I} H1/Glc H4 at δ 4.56/3.56, Glc H1/Gal H3 at δ 4.67/4.07, Gal H1/GalNAc^{II} H4 at δ 4.96/4.01, and GalNAc^{II} H1/GlcA H4 at δ 4.44/3.87. These data defined the sugar sequence in the repeating unit.

Correlations between H2 of both GalNAc residues and CO of the N-acetyl groups at δ 3.98/175.8 and 3.89/175.9, which were observed in the 2D HMBC spectrum, showed that both amino sugars are N-acetylated and, hence, the alanine residue is attached to the carboxyl group of GlcA. An Ala H2/GlcA C6 cross-peak at δ 4.33/170.1 confirmed the presence of an amide of GlcA with Ala (Fig. 2, structure *2*).

Analysis of the glycosylation effects in the ^{13}C-NMR spectrum of the polysaccharide showed that all monosaccharides have the same absolute configuration, which is in agreement with the D configuration of Glc, Gal, and GalNAc determined by sugar analysis. The effect on C1 of the GlcA residue was relatively large (7.9 ppm), thus indicating the same absolute configuration of this sugar and the neighboring D-GalNAc residue (compare the effect of ~8 ppm for the same and ~3.5 ppm for β1→3-linked disaccharides with the galacto configuration of the glycosylated sugar and different absolute configurations of the constituent monosaccharides [29]).

These data showed that the O-specific polysaccharide of *Proteus vulgaris* O44 has the structure shown in Table 3.

***Proteus mirabilis* G1.** Sugar analysis of the polysaccharide after acid hydrolysis revealed GlcA and GalA in the ratio ~1 : 5. Analysis using an amino acid analyzer showed the presence of GalN and lysine. GalA and GalN have the D configuration and lysine has the L configuration. The D configuration of GlcA was established by analysis of ^{13}C-NMR chemical shift data of the polysaccharide (see below).

The ^{13}C-NMR spectrum of the polysaccharide (Fig. 4) contained signals for four anomeric carbons at δ 100.9-105.5, one unsubstituted (δ 62.4) and one substituted (δ 66.7) O*C*H$_2$-C group (C6 of GalN, data of attached-proton test), two carboxyl groups at δ 172.3 and 174.6 (C6 of GlcA and GalA), two nitrogen-bearing carbons at δ 52.3 and 53.2 (C2 of GalN), 14 oxygen-bearing sugar ring carbons in the region δ 69.0-81.3, two N-acetyl groups (CH$_3$ at δ 23.6, CO at δ 175.9 and 176.2), and six carbons of lysine at δ 23.0, 27.4, 31.9, 40.5, 54.3, and 177.9 (Table 2, compare published data [30]). Accordingly, the ^{1}H-NMR spectrum of the polysaccharide contained signals for four anomeric protons at δ 4.51-5.20, two N-acetyl groups at δ 2.02 and 2.05, and six signals for lysine at δ 1.43-1.91, 3.00, and 4.38 (Table 1).

Therefore, the polysaccharide has a tetrasaccharide repeating unit containing one residue each of D-GlcA, D-GalA, and L-Lys and two residues of D-GalNAc. A smaller than expected relative content of GlcA in the polysaccharide hydrolyzate could be accounted for by its retention in an oligosaccharide(s) with GalN (see the polysaccharide structure below).

The ^{1}H- and ^{13}C-NMR spectra of the polysaccharide were assigned using 2D COSY, TOCSY, ROESY, ^{1}H,^{13}C

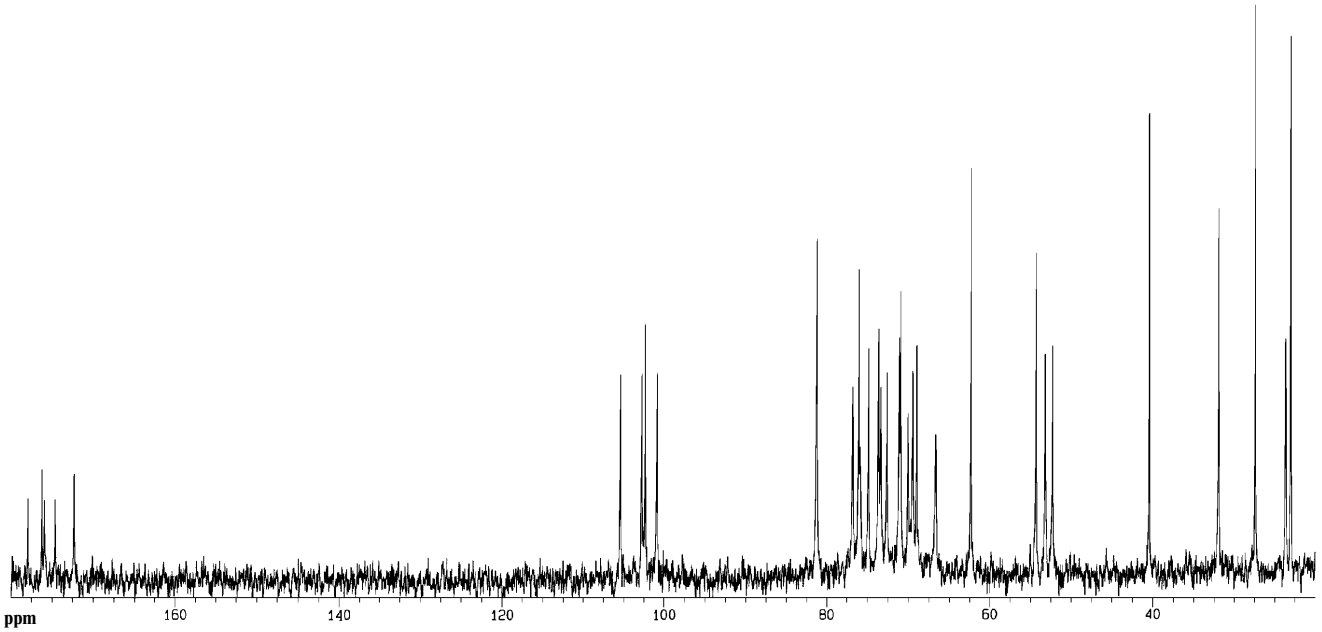

Fig. 4. ^{13}C-NMR spectrum of the O-specific polysaccharide of *P. mirabilis* G1.

HMQC, and HMQC-TOCSY experiments (Tables 1 and 2). The TOCSY spectrum showed correlations of H1 with H2-H5 for Glc*p*A and Gal*p*A and with H2-H4 for both Gal*p*NAc residues (Gal*p*NAc^{I} and GalNAc^{II}). The signals for H5 and H6 of Gal*p*NAc^{I} were assigned by H4/H5 correlation in the 2D ROESY spectrum and H5/H6 correlation in the COSY spectrum. The corresponding ^{13}C-NMR signals were found by correlations in the 2D ^{1}H,^{13}C HMQC spectrum, and three remaining cross-peaks in this spectrum were assigned to H5/C5, H6a/C6, and H6b/C6 correlations of Gal*p*NAc^{II}.

The Glc*p*A residue was identified by a large $J_{3,4}$ coupling constant value of ~10 Hz, as compared with a smaller $J_{3,4}$ values of ≤3 Hz for the other sugars that have the galacto configuration. The Gal*p*NAc residues were distinguished by correlation of protons at nitrogen-bearing carbons (H2) with the corresponding carbons (C2), which were revealed by the ^{1}H,^{13}C HMQC experiment. The signals for the carboxyl groups (C6 of Glc*p*A and Gal*p*A and C1 of Lys) were assigned by H5/C6 and H2/C1 correlations, respectively, that were observed in the HMBC spectrum. The spectrum showed also a correlation between H2 of Lys and C6 of Gal*p*A, thus demonstrating the presence of N$^{\alpha}$-galacturonoyllysine (Fig. 2, structure *3*). This conclusion was confirmed by typical ^{13}C-NMR chemical shifts for the free carboxyl group of lysine (δ 177.9) and the amidated carboxyl group of GalA (δ 172.3) (compare published data [30]).

Relatively large $J_{1,2}$ coupling constant values of 7-8 Hz determined from the ^{1}H-NMR spectrum for the anomeric protons at δ 4.51-4.55 showed that Glc*p*A and both Gal*p*NAc residues are β-linked. The α-linkage was suggested for a poorly resolved H1 signal of Gal*p*A, which appeared at low field at δ 5.20, and was confirmed by the ^{13}C-NMR chemical shift data (Table 2).

Significant downfield displacements of the signals for C3 of Gal*p*NAc^{I}, C4 of Glc*p*A, C4 and C6 of Gal*p*NAc^{II} to δ 81.3, 81.3, 75.9, and 66.7, respectively, as compared with their positions in the corresponding unsubstituted sugars [28], demonstrated the glycosylation pattern. The ^{13}C-NMR chemical shifts for the GalA residue were close to those for the unsubstituted monosaccharide [28] and, hence, this residue is terminal.

The 2D ROESY spectrum of the polysaccharide showed a GalA H1/GalNAc^{II} H4 correlation at δ 5.20/4.03, and, hence, GalA is attached to the disubstituted GalNAc^{II} residue as a monosaccharide side chain. Correlations of the β-linked sugars in the main chain were difficult to interpret because of close positions of the H1 resonances and multiple coincidences of intraresidue H1/H3,H5 and interresidue cross-peaks. The HMBC spectrum of the polysaccharide contained a GalNAc^{I} H1/GalNAc^{II} C6 cross-peak at δ 4.55/66.7 and two overlapping cross-peaks at 4.51-4.53/81.3, which could be assigned to GalNAc^{II} H1/GlcA C4 and GlcA H1/GalNAc^{I} C3 correlations. In addition, GalA C1/GalNAc^{II} H4 and GalNAc^{II} C1/GlcA H4 cross-peaks were present at δ 100.9/4.03 and 102.8/3.73, respectively, whereas other expected interresidue correlations were either not observed (for Glc*p*A C1) or difficult to interpret unambiguously (for Gal*p*A H1 and Glc*p*A C1). Nevertheless, the ROESY and HMBC data were in

accordance with the ^{13}C-NMR chemical shift data and were sufficient for determination of the full monosaccharide sequence in the repeating unit.

A relatively large effect (>8 ppm) on C1 of GlcA [29] indicated that GlcA and the neighboring GalNAc residue in the β1→3-linked disaccharide fragment have the same absolute configuration (in case of their different absolute configuration the effect on C1 would be <5 ppm [29]). Hence, GlcA has the D configuration.

On the basis of the data obtained, it was concluded that the O-specific polysaccharide of *P. mirabilis* G1 has the structure shown in Table 3.

***Proteus myxofaciens*.** Sugar analysis of the polysaccharide showed the presence of D-GlcN and D-GalN in the ratio ~2 : 1 as well as another amino component and GlcA. The D configuration of GlcA was established by analysis of ^{13}C-NMR chemical shift data of the polysaccharide (see below). The amino component was isolated from the polysaccharide hydrolyzate by gel chromatography on TSK HW-40 and, after conversion into the ammonium salt, identified as N^{ε}-[(R)-1-carboxyethyl]-L-lysine ("alaninolysine", aLys) (Fig. 2, structure *4*) by the specific optical rotation value and the ^{13}C-NMR spectrum compared with the spectra of synthetic stereoisomers as described [16].

The ^{13}C-NMR spectrum (Fig. 5) of the polysaccharide contained signals for four anomeric carbons at δ 98.7-104.0, five nitrogen-bearing carbons at δ 47.0-58.8, one unsubstituted (δ 61.9) and one substituted (δ 66.9) O*C*H$_2$-C group (data of attached-proton test), one *C*H$_3$-C group at δ 16.2, three C-*C*H$_2$-C groups at δ 31.9, 26.5, and 23.4, three N-acetyl groups (CH$_3$ at δ 23.5-23.8), and carbonyl groups at δ 170.1-177.9.

The ^{1}H-NMR spectrum of the polysaccharide contained signals for four anomeric protons at δ 4.40-4.92, three N-acetyl groups at δ 2.01-2.10, one *C*H$_3$-C group at δ 1.47, and three C-CH$_2$-C groups at δ 1.48-1.93. These data together showed that the polysaccharide is regular and has a tetrasaccharide repeating unit consisting of two D-GlcNAc residues (Glc*p*NAc^{I} and Glc*p*NAc^{II}) and one residue each of D-GalNAc, D-GlcA, and aLys.

The ^{1}H- and ^{13}C-NMR spectra of the polysaccharide were assigned using 2D NMR spectroscopy, including 2D ^{1}H,^{1}H COSY, TOCSY, ROESY, and ^{1}H,^{13}C HMQC experiments (Tables 1 and 2). As a result, the spin systems of β-Glc*p*NAc^{I}, β-Glc*p*NAc^{II}, α-Gal*p*NAc, and β-Glc*p* were identified based on the characteristic $J_{H,H}$ coupling constants values. Particularly, the configurations of the glycosidic linkages were determined by the $J_{1,2}$ coupling constant values of 7-8 and ~4 Hz for β- and α-linked monosaccharides, respectively. The remaining, non-sugar signals belonged to an aLys residue. The ^{13}C-NMR chemical shifts of aLys were close to those of alanine and lysine, but the signals for C2 of alanine and C6 of lysine were shifted to δ 58.8 and 47.0, as compared with their position at δ 51.6 and 40.6 in the free amino acids.

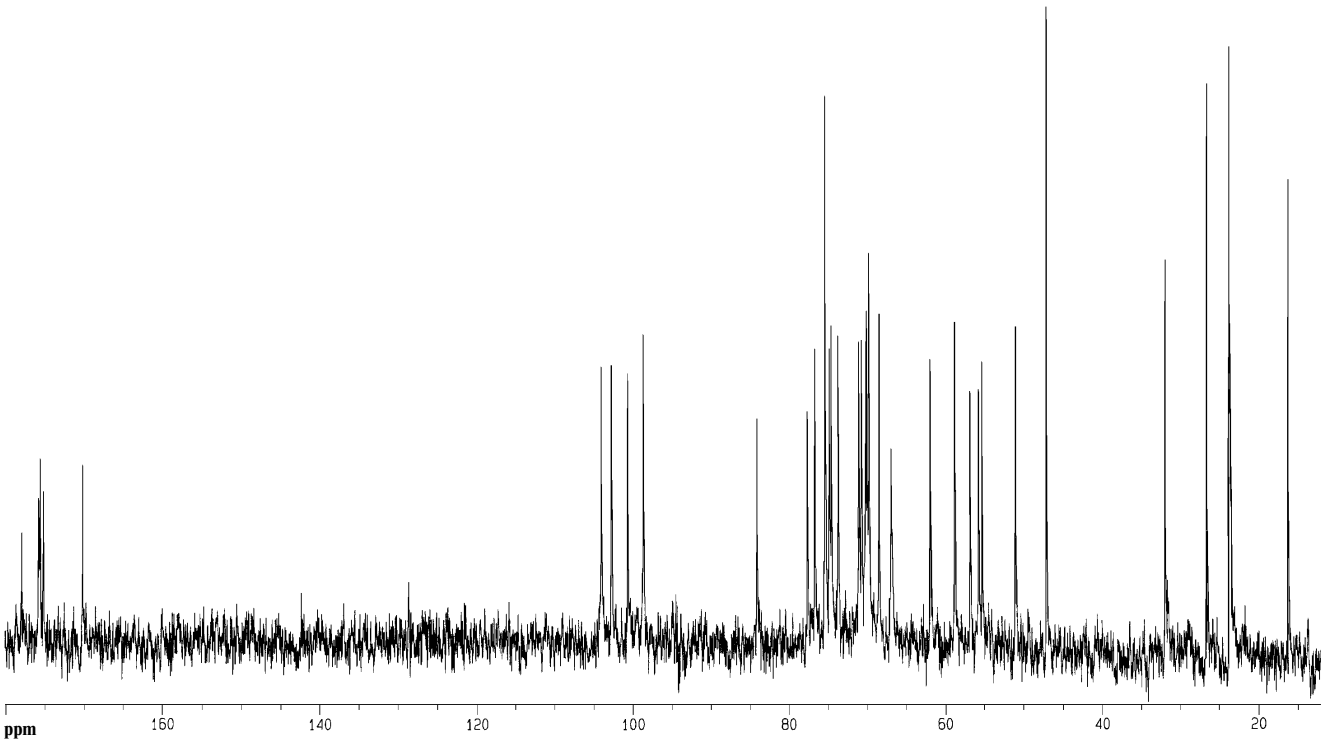

Fig. 5. ^{13}C-NMR spectrum of the O-specific polysaccharide of *P. myxofaciens*.

Low field displacements of the signals for C3 of Glc*p*NAc^{II}, C4 of GlcA, C6 of GalNAc and Glc*p*NAc^{I} to δ 77.6, 70.1, 66.9, and 84.1, respectively, i.e., by ~4.5-9.5 ppm, as compared with their position in the spectra of the corresponding unsubstituted monosaccharides [18, 28], demonstrated the modes of glycosylation in the repeating unit.

The glycosylation pattern was confirmed and the sequence of the monosaccharides was determined using a 2D ROESY experiment, which revealed the following correlations between transglycosidic protons: GalNAc H1/GlcNAc^{I} H6a,6b at δ 4.92/3.71 and 4.92/3.98, GlcNAc^{I} H1/GlcNAc^{II} H3 at δ 4.62/3.65, GlcNAc^{II} H1/GlcA H4 at δ 4.40/3.95, and GlcA H1/GalNAc H6a,6b at δ 4.58/3.87 and 4.58/4.01. Accordingly, the HMBC spectrum of the polysaccharide contained GalNAc H1/GlcNAc^{I} C6, GlcNAc H1/GlcNAc^{II} C3, GlcNAc H1/GlcA C4, and GlcA H1/GalNAc C6 cross-peaks at δ 4.92/66.9, 4.40/84.1, 4.40/77.6, and 4.58/70.1, respectively.

In the ^{13}C-NMR spectrum of the polysaccharide, a relatively large β-effect on the chemical shift of the C3 signal of GlcA (ca. −2 ppm) caused by its glycosylation at position 4 with β-D-GlcNAc showed that both monosaccharides have the same absolute configuration and, hence, GlcA has the D configuration (in case of the L configuration of GlcA the β-effect on C3 would be close to zero) [29].

These data established the structure of the repeating unit of the polysaccharide of *P. myxofaciens*, which is shown in Table 3.

DISCUSSION

Unlike O-specific polysaccharides of most other Gram-negative bacteria, *Proteus* O-antigens are often highly charged. Most of the *Proteus* polysaccharides studied contain either acidic or both basic and acidic groups with sometimes as many as three charged groups per repeating unit. This feature is assumed to play a role in adaptation of bacteria to growth at different pH of the environment. The most common negatively charged components are uronic acids, from which D-glucuronic and D-galacturonic acids are widespread in the *Proteus* O-antigens and L-alturonic acid has been found only in the polysaccharide of *P. mirabilis* O10 [31, 32]. Another common way of incorporating of both negatively and positively charged groups to the *Proteus* O-antigens residues is phosphorylation and attachment of amino alcohols, e.g., 2-aminoethanol and 2-(1-carboxyethylamino)-ethanol, to the phosphate groups [1]. A number of *Proteus* polysaccharides include residues of non-carbohydrate organic acids with the free carboxyl group, such as lactic acid, pyruvic acid, and various amino acids [7].

In the polysaccharide of *P. penneri* 28 studied in this work, (S)-lactic acid is ether-linked to N-acetylglucosamine to form 2-acetamido-3-O-[(S)-1-carboxyethyl]-2-deoxy-D-glucose (N-acetylisomuramic acid) (*1*). An isomeric ether with (R)-lactic acid (N-acetylmuramic acid) is a common component of bacterial cell-wall peptidoglycan and has been found in a few bacterial polysaccharides, including the capsular polysaccharide of *Vibrio vulnifias* ATCC 27562 [33] and the O-antigens of *Yersinia ruckerii* [34] and *Providencia alcalifaciens* O16 [25]. N-Acetylisomuramic acid has been first discovered in the O-specific polysaccharide of *P. penneri* 62 [35] and then in the structurally and serologically related polysaccharide of *P. penneri* 19 [36], in which it forms one of the cross-reactive epitopes recognized by polyclonal O-antisera. The O-antigens of *P. penneri* O62 [37] and *P. vulgaris* O15 (A. V. Perepelov, Y. A. Knirel, and A. Rozalski, unpublished data) contain positional isomers of N-acetylisomuramic acid and N-acetylmuramic acid, respectively, bearing a lactic acid residue at position 4 of the monosaccharide. In addition, ethers of (R)- and (S)-lactic acid with other amino sugars, neutral sugars, and hexuronic acids have been found in a number of bacterial polysaccharides [38], including 3-O-[(S)-1-carboxyethyl]-D-glucose in the O-antigen of *P. vulgaris* O25 [39].

The ^{13}C-NMR spectra showed that the O-specific polysaccharide of *P. penneri* 28 has the same structure as that of *P. vulgaris* O31, and, based on these data and the serological identity of the O-antigens, *P. penneri* 28 was classified into the same *Proteus* serogroup O31. The polysaccharide of *P. penneri* 28 resembles also that of *P. penneri* 26 studied by us earlier [20], which has the same carbohydrate backbone and differs only in the absence of lactic acid residue from the residue of N-acetylglucosamine. Serological studies, which will be reported elsewhere, suggested that it is reasonable to classify *P. penneri* 26 into *Proteus* serogroup O31 as the second subgroup.

Although amino acids are often found in lipopolysaccharide preparations, in most cases they either come from protein contaminations or are components of the lipopolysaccharide core, including core of some *Proteus* strains [40]. The first amino acid that was proved to be a component of an O-antigen was lysine in *P. mirabilis* S1959 [41]. Since that time lysine, as well as alanine, serine, and threonine have been found in a number of *Proteus* strains [7]. Amides of hexuronic acids with L-lysine play an important role in manifesting the immunospecificity of the O-antigens of *P. mirabilis* [30, 41-43].

The polysaccharide of *P. mirabilis* G1 studied in this work is another polysaccharide that contains N$^{\alpha}$-(D-galacturonoyl)-L-lysine (*3*). It was found to be structurally related to the polysaccharides of *P. mirabilis* S1959 and OXK, which are identical to each other and classified

into *Proteus* serogroup O3 [22, 23]. Compared to the polysaccharide of *P. mirabilis* G1, they differ only in the presence of an additional side chain composed of the single D-glucose residues. As one could expect, these strains are serologically closely related and suggested to be classified into *Proteus* serogroup O3 as two subgroups [21]. The serogroup O3 strains showed a serological relatedness also to *P. mirabilis* O28. Their O-antigens have no structural similarity except for that they share the amide *3* [30], which is also a component of the lipopolysaccharide core of *P. mirabilis* O28 [40]. In Western blot, O-antiserum against *P. mirabilis* G1 reacted with both O-antigen and core of the lipopolysaccharide of *P. mirabilis* O28 [21]. The importance of the amino acid-associated core epitope was confirmed by a cross-reactivity of O-antisera against *P. mirabilis* G1 and O3 with the lipopolysaccharide of *P. mirabilis* R14 [34], which contains the amide *3* in the core region only.

Yet another amino acid-containing polysaccharide studied in this work is that of *P. vulgaris* O44, which includes N^{α}-(D-glucuronoyl)-L-alanine (*2*). This is for the first time that this component was found in *Proteus* O-antigens, whereas earlier only N^{α}-(D-galacturonoyl)-L-alanine has been identified [7]. Interestingly, alanine in both D and L enantiomeric forms is the only amino acid that occurs in *Proteus* O-antigens also as an N-acyl substituent of various amino sugars [7]. The structure of the polysaccharide of *P. vulgaris* O44 is unique among *Proteus* O-antigens, which is in accordance with classifying this strain into a separate *Proteus* O-serogroup. The amide *2* does not seem to play a role in manifesting the *P. vulgaris* O44 immunospecificity since O-antiserum against this strain did not react with a synthetic acrylamide-based glycoconjugate containing an amide of β-D-Glc*p*A with L-Ala (these data will be published elsewhere).

The polysaccharide of *P. myxofaciens* contains a unique amino acid derivative, N^{ε}-[(R)-1-carboxyethyl]-L-lysine ("alaninolysine"), whose α-amino group amidates a residue of D-galacturonic acid to form the amide *4*. The first bacterial polysaccharides found to include this unusual component were the O-antigens of *Providencia alcalifaciens* O23 [16, 24] and *P. mirabilis* O13 [26, 27], in which "alaninolysine" is linked to the carboxyl group of D-glucuronic acid and D-galacturonic acid, respectively. Later, an amide of D-galacturonic acid with an isomeric amino acid, N^{ε}-[(S)-1-carboxyethyl]-L-lysine, has been identified in the O-antigen of *Providencia rustigianii* O14 [25]. As aminoalkyl phosphate groups, both lysine and N^{ε}-(1-carboxyethyl)lysine endow the polysaccharides with zwitterionic character.

Like L-lysine (see above), "alaninolysine" has been shown to play an important role in manifesting the immunospecificity of the O-antigen of *P. mirabilis* O13 [44]. A preliminary serological investigation revealed multiple cross-reactivity of O-antigens that contain any of the isomers of N^{ε}-(1-carboxyethyl)-L-lysine (their structures are shown in Table 3), but further studies are necessary to define cross-reactive epitopes. Although different in sugar composition, the polysaccharide of *Providencia alcalifaciens* O23 shows a marked similarity to that of *P. myxofaciens* in the positions and configurations of the glycosidic linkages (Table 3). In spite of this, O-antiserum against *P. myxofaciens* cross-reacted with the lipopolysaccharide of *Providencia* only weakly. Based on this finding and on the unique structure of the polysaccharide of *P. myxofaciens* among *Proteus* O-antigens, we suggest classifying this bacterium into a new *Proteus* O-serogroup.

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