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ACCELERATED PUBLICATION

New Structures of the O-Specific Polysaccharides of *Proteus*. 2*. Polysaccharides Containing O-Acetyl Groups

A. N. Kondakova[1], F. V. Toukach[1], S. N. Senchenkova[1], N. P. Arbatsky[1], A. S. Shashkov[1], Y. A. Knirel[1], K. Zych[2], A. Torzewska[2], K. Kolodziejska[2], A. Rozalski[2], and Z. Sidorczyk[2]**

[1]Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninskii pr. 47, Moscow, 119991 Russia; fax: (095) 135-5328; E-mail: knirel@ioc.ac.ru
[2]Institute of Microbiology and Immunology, University of Lodz, Banacha 12/16, 90-237 Lodz, Poland; fax: (48) 42-6784932; E-mail: zsidor@biol.uni.lodz.pl

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Abstract—Structures of five new O-specific polysaccharides of *Proteus* bacteria were established. Four of them, *Proteus penneri* 4 (O72), *Proteus vulgaris* 63/57 (O37), *Proteus mirabilis* TG 277 (O69), and *Proteus penneri* 20 (O17), contain O-acetyl groups in non-stoichiometric quantities, and the polysaccharide of *P. penneri* 1 is structurally related to that of *P. penneri* 4. The structures were elucidated using NMR spectroscopy, including one-dimensional ^{1}H- and ^{13}C-NMR spectroscopy, two-dimensional ^{1}H,^{1}H correlation (COSY, TOCSY), H-detected ^{1}H,^{13}C heteronuclear multiple-quantum coherence (HMQC), heteronuclear multiple-bond correlation (HMBC), and nuclear Overhauser effect spectroscopy (NOESY or ROESY), along with chemical methods. The structural data obtained are useful as the chemical basis for the creation of the classification scheme for *Proteus* strains.

Key words: lipopolysaccharide, O-antigen, bacterial polysaccharide, structure, O-acetyl groups, *Proteus*

Although enterobacteria of the genus *Proteus* make up a part of the normal flora of the human gastrointestinal tract, under favorable conditions they cause urinary tract infections that can lead to severe complications, such as acute and chronic pyelonephritis and formation of kidney stones. The bacteria frequently cause diseases in patients with urinary tract abnormalities, with urinary catheter in place or after surgical intervention in the urogenital system [2, 3]. Recently, it has been suggested that *Proteus mirabilis* plays an etiopathogenic role in rheumatoid arthritis [4].

Abbreviations: COSY) correlation spectroscopy; Etn-P) ethanolamine phosphate; FucNAc) 2-acetamido-2,6-dideoxygalactose; Fuc3NHb) 3,6-dideoxy-3-[(R)-3-hydroxybutyramido]galactose; GlcA) glucuronic acid; Hb) 3-hydroxybutyryl group; HMBC) heteronuclear multiple-bond correlation; HMQC) heteronuclear multiple-quantum coherence; NOESY) nuclear Overhauser effect spectroscopy; ROESY) rotating-frame nuclear Overhauser effect spectroscopy; TOCSY) total correlation spectroscopy.

* Part 1 see ref. [1].

** To whom correspondence should be addressed.

Currently, *Proteus* rods are subdivided into four species, three of which, *P. mirabilis*, *P. vulgaris*, and *P. penneri*, are of clinical importance. The species *P. vulgaris* is heterogeneous with two biogroups, 2 and 3, the latter being further subdivided into genomospecies 3 (*Proteus hauseri*), 4, 5 and 6 (all unnamed) [5, 6]. Based on the immunospecificity of the cell-surface lipopolysaccharides, two species, *P. mirabilis* and *P. vulgaris*, are classified into 60 O-serogroups [7, 8], and recently new O-serogroups have been proposed for *P. penneri* strains [9, 10].

Aiming at creation of the molecular basis for classification of *Proteus* strain, we have been undertaking immunochemical studies of the polysaccharide chains of the lipopolysaccharides, which define the O-specificity of the bacteria [9]. Most polysaccharides studied contain acidic or both acidic and basic components, such as amino acids and phosphate and ethanolamine phosphate groups [1]. Although all of the polysaccharides have regular structures, the regularity of some polysaccharides is masked by the presence of ethanolamine phosphate or O-acetyl groups in non-stoichiometric quantities. We

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now report the determination of new structures of four O-specific polysaccharides containing O-acetyl groups, which were isolated from *P. vulgaris* 63/57 (O37), *P. mirabilis* TG 277 (O69), *P. penneri* 4 (O72) and 20 (O17), as well as that of *P. penneri* 1 (O72), which is structurally related to the polysaccharide of *P. penneri* 4.

MATERIALS AND METHODS

P. vulgaris 63/57 (O37) was from the Czech National Collection of Type Cultures (CNCTC, Institute of Epidemiology and Microbiology, Prague, Czech Republic). *P. mirabilis* TG 277 (O69) was kindly provided by Prof. J. L. Penner (Department of Medical Genetics, Toronto University, Ontario, Canada). Strains of *P. penneri* were from the collection of the Institute of Microbiology and Immunology (University of Lodz, Poland). 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 aqueous phenol [11] and purified by treatment with ribonuclease and deoxyribonuclease and by ultracentrifugation as described [12]. The lipopolysaccharides were degraded with 2% acetic acid at 100°C until the precipitation of lipid. High-molecular-mass O-specific polysaccharide was isolated from the water-soluble portion by gel-permeation chromatography on a Sephadex G-50 column in pyridine-acetate buffer pH 4.5 as described [12] using a Knauer differential refractometer (Germany) for monitoring.

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, or 85% aqueous H_3PO_4, δ_P 0 ppm). NMR spectra were assigned as described previously [1].

O-Deacetylation of the polysaccharides was performed with 12% aqueous ammonia at 60°C for 2 h, and the modified polysaccharides were isolated by gel-permeation chromatography on a Sephadex G-50 column.

The polysaccharides were hydrolyzed with 2 M trifluoroacetic acid (120°C, 2 h). Methanolysis of the polysaccharides was performed with 1 M hydrogen chloride in methanol and followed by acetylation with acetic anhydride in pyridine (100°C, 1 h). A portion of the acetylated methanolysis products was subjected to (+)-2-octanolysis in the presence of anhydrous trifluoroacetic acid (120°C, 16 h) followed by acetylation [13]. The acetylated derivatives were analyzed by GLC and GLC/mass spectrometry on a Hewlett Packard 5890 chromatograph (USA) with a DB-5 capillary column equipped with a Nermag R10-10L mass spectrometer (France), using a temperature gradient of 160°C (1 min) to 290°C at 3°C/min.

RESULTS

***Proteus penneri* strains 4 and 1 (serogroup O72).** The ^{13}C- (Fig. 1) and ^{1}H-NMR spectra of the polysaccharide of *P. penneri* 4 demonstrated structural heterogeneity, most likely owing to non-stoichiometric O-acetylation (there were signals for CH_3 of O-acetyl groups at δ_H 2.14 and 2.15 ppm, δ_C 21.7 (major), 21.4 and 21.5 ppm (both

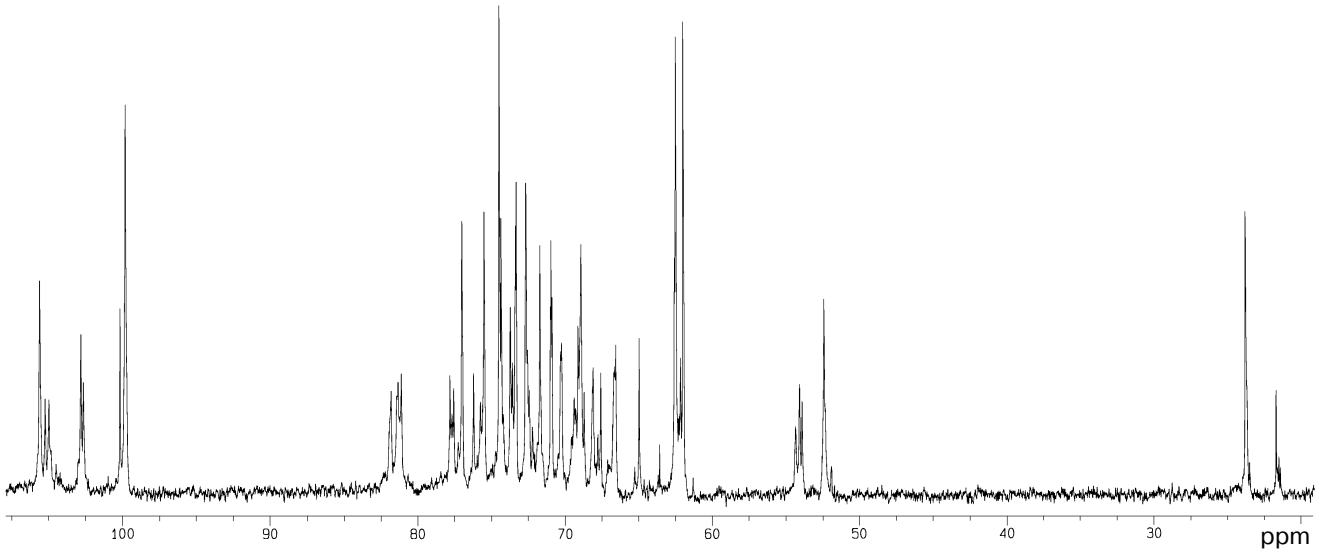


Fig. 1. ^{13}C-NMR spectrum of the polysaccharide of *P. penneri* 4 (O72) (the resonance region of CO groups is not shown).

minor)). After O-deacetylation with aqueous ammonia, the spectra showed a higher degree of regularity but a number of minor signals were still present, which were due to non-stoichiometric glucosylation (see below).

The ^{13}C-NMR spectrum of the modified polysaccharide showed a pentasaccharide repeating unit containing two amino sugars. There were signals for five anomeric carbons at δ 99.8-105.6 ppm, two nitrogen-bearing carbons at δ 52.4 and 54.1 ppm, three unsubstituted $HOCH_2$ groups at δ 62.0, 62.5, 62.6 ppm, and two substituted $HOCH_2$ groups at δ 66.6 and 68.1 ppm (data of an attached-proton test experiment [14]), other sugar ring carbons in the region δ 68.9-81.4 ppm, and two N-acetyl groups at δ 23.7 (CH_3) and 176.0 ppm (CO). Accordingly, the ^{1}H-NMR spectrum contained, *inter alia*, signals for five anomeric protons at δ 4.61-4.99 ppm and two N-acetyl groups at δ 2.05 and 2.06 ppm.

The major series in the two-dimensional homonuclear and heteronuclear shift-correlated spectra (COSY, TOCSY, ROESY, ^{1}H,^{13}C HMQC) of the O-deacetylated polysaccharide of *P. penneri* 4 was similar to those of the polysaccharide of *P. penneri* 1. Assignment of the spectra (Tables 1 and 2) revealed the presence in both polysaccharides of the same sugar residues, which were identified as one residue each of Gal*p* and Glc*p* and two residues of Gal*p*NAc. The spin system for Glc*p* was distinguished from those for Gal*p* and Gal*p*NAc on the basis of the $^3J_{H,H}$ coupling constants values [15], and the Gal*p*NAc spin systems were identified by lower field positions of the signals for H2 (δ 4.02-4.07 versus δ 3.76 ppm, respectively) as well as by their correlation to the corresponding nitrogen-bearing carbons at δ 52.5 and 54.1 ppm revealed by a ^{1}H,^{13}C HMQC experiment.

In addition to these four monosaccharides, the repeating unit of the *P. penneri* 4 polysaccharide contained another Glc*p* residue. Sugar analysis confirmed the composition of the *P. penneri* 1 and 4 polysaccharides and demonstrated the D-configuration of all monosaccharides. Therefore, the repeating unit of the *P. penneri* 1 polysaccharide is a tetrasaccharide containing two residues of D-GalNAc and one residue each of D-Glc and D-Gal, and that of the *P. penneri* 4 is a pentasaccharide containing two residues each of D-Glc (I and II) and D-GalNAc (I and II), one residue of D-Gal, and O-acetyl groups.

The structure of the simpler polysaccharide from *P. penneri* 1 was elucidated first. The $J_{H1,H2}$ coupling constant values of 7-8 Hz for Glc*p*, Gal*p*NAc^I and Gal*p*NAc^{II} were characteristic of β-linked sugars, whereas the $J_{H1,H2}$ value of 4 Hz showed that Gal*p* is α-linked. The $^1J_{C1,H1}$ coupling constant values of 162.5-165.4 Hz, which were determined from the non-decoupled HMQC spectrum, confirmed the β-configuration of Glc*p* and both Gal*p*NAc residues, and the value of 171.1 Hz was consistent with the α-configuration of Gal*p* [16].

In the ^{13}C-NMR spectrum of the polysaccharide, significant low-field displacements of the signals for C6 of Glc*p*, C3 and C4 of Gal*p*, and C3 of Gal*p*NAc^I to δ 66.8, 81.3, 77.1, and 81.6 ppm, as compared with their positions in the spectra of the corresponding unsubstituted monosaccharides at δ 61.9, 70.4, 70.6, and 72.4 ppm [17], respectively, were due to the known α-effects of glycosylation. These data showed that the polysaccharide is branched, Glc*p* is 6-substituted, Gal*p* 3,4-disubstituted, and Gal*p*NAc^I 3-substituted. No significant displacements were observed for the C2-C6 signals of Gal*p*NAc^{II} and, hence, it occupies the non-reducing end of the side chain.

A ROESY experiment with the *P. penneri* 1 polysaccharide revealed the following correlations between the transglycosidic protons: Gal*p* H1/Glc*p* H6b at δ 4.95/3.75, Glc*p* H1/Gal*p*NAc^I H3 at δ 4.59/3.89, Gal*p*NAc^I H1/Gal*p* H4 at δ 4.96/4.39, and Gal*p*NAc^{II} H1/Gal*p* H3 at δ 4.61/3.96 ppm. These data were in accordance with the substitution pattern as determined by the ^{13}C-NMR chemical shift data and revealed the full sequence of the monosaccharide residues in the repeating unit.

Therefore, the O-specific polysaccharide of *P. penneri* 1 has the structure shown in Table 3.

The major series of the signals in the ^{13}C-NMR spectrum of the O-deacetylated polysaccharide of *P. penneri* 4 was close to that of the *P. penneri* 1 polysaccharide (Table 2), except for the signals for C5 and C6 of Gal*p*NAc^I, which were shifted from δ 75.7 and 61.9 to δ 73.7 and 68.1 ppm, respectively. Such displacements are typical of the effects of glycosylation by α-linked monosaccharides at position 6 [17] and were in accordance with the presence of an additional α-Glc*p*^{II} residue. The terminal position and the α-configuration of Glc*p*^{II} were additionally confirmed by the chemical shifts for C2-C5, which were close to those for α-glucopyranose [17]. In the ^{1}H-NMR spectrum, marked displacements were also observed for the signals of Gal*p*NAc^I (in particular, the H5 signal shifted from δ 3.67 to 3.87 ppm), whereas the chemical shifts of the signals for the other sugar residues common for both polysaccharides were essentially the same. Therefore, the major repeating unit of the O-deacetylated polysaccharide of *P. penneri* 4 differs from the repeating unit of the polysaccharide of *P. penneri* 1 only in the presence of one additional α-glucopyranose residue attached at position 6 of GalNAc^I.

A minor series of the signals in the NMR spectra of the *P. penneri* 4 polysaccharide belonged to a tetrasaccharide repeating unit lacking the terminal Glc*p*^{II} residue and, thus, having the same structure as the polysaccharide of *P. penneri* 1. Hence, Glc*p*^{II} is present in the *P. penneri* 4 polysaccharide in a non-stoichiometric amount. As judged by the ratio of the integral intensities of the signals in the major and minor series, the degree of α-glucosylation is ~75-80%.

Comparison of the ^{13}C-NMR spectra of the initial and O-deacetylated polysaccharides of *P. penneri* 4 showed that in the former the signals for C5 and C6 of

Table 1. ^{1}H-NMR data (δ, ppm)

Residue	H1	H2	H3	H4	H5	H6a, H6b
O-Deacetylated polysaccharide of *Proteus penneri* 4 (O72)[a]						
→6)-β-Glc*p*^{I}-(1→	4.62	3.32	3.51	3.62	3.60	4.05, 3.75
→3)-β-Gal*p*NAc^{I}-(1→ 6 ↑	4.99	4.09	3.92	4.16	3.87	3.95, 3.73
→4)-α-Gal*p*-(1→ 3 ↑	4.97	3.76	3.95	4.36	3.97	
β-Gal*p*NAc^{II}-(1→	4.61	4.03	3.74	4.00	3.69	
α-Glc*p*^{II}-(1→	4.96	3.58	3.70	3.44	3.66	3.87, 3.77
Polysaccharide of *Proteus penneri* 1 (O72)						
→6)-β-Glc*p*-(1→	4.59	3.31	3.51	3.55	3.59	4.05, 3.75
→3)-β-Gal*p*NAc^{I}-(1→	4.96	4.07	3.89	4.12	3.66	
→4)-α-Gal*p*-(1→ 3 ↑	4.95	3.76	3.96	4.39	3.96	
β-Gal*p*NAc^{II}-(1→	4.61	4.02	3.74	3.99	3.69	
O-Deacetylated polysaccharide of *Proteus vulgaris* 63/57 (O37)						
→3)-β-Glc*p*A^{I}-(1→	4.60	3.49	3.70	3.80	4.02	
→3)-α-Glc*p*NAc-(1→	5.26	4.12	3.93	3.64	4.04	3.84
→3)-β-Glc*p*A^{II}-(1→	4.61	3.49	3.70	3.83	4.03	
→4)-α-Glc*p*-(1→	5.33	3.62	3.86	3.66	4.12	3.86
Polysaccharide of *Proteus vulgaris* 63/57 (O37)[b]						
→3)-β-Glc*p*A^{I}-(1→	4.61[c]	3.46	3.65[d]	3.79[e]	3.95	
→3)-α-Glc*p*NAc6Ac-(1→	5.23	4.12	3.91	3.66	4.27	4.34, 4.41
→3)-β-Glc*p*A^{II}-(1→	4.60[c]	3.46	3.70[d]	3.81[e]	3.95	
→4)-α-Glc*p*-(1→	5.33	3.58	3.86	3.66	4.12	3.85, 3.91

Note: Additional chemical shifts for the N-acetyl groups are δ 2.01-2.07 ppm.

[a] Data for the major repeating unit containing the terminal glucose residue.

[b] Data for the O-acetylated repeating unit.

[c-e] Assignment could be interchanged.

Table 2. ^{13}C-NMR data (δ, ppm)

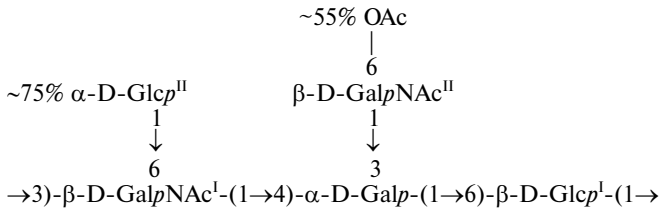
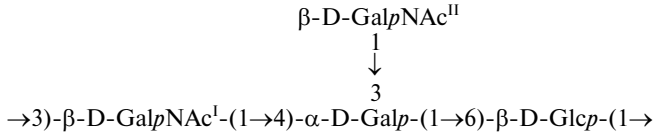
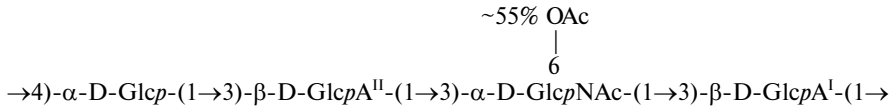
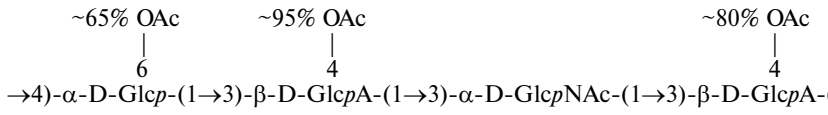
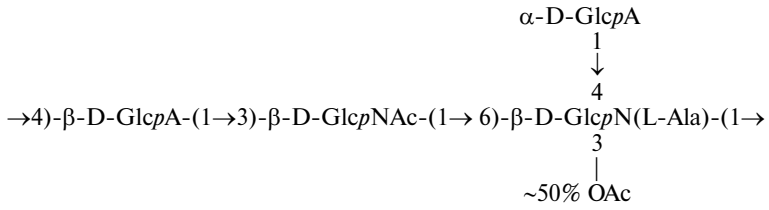
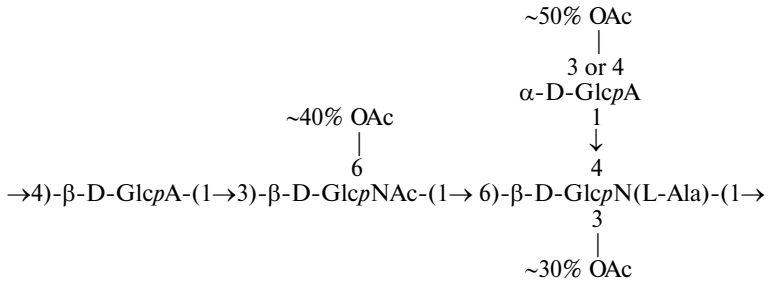
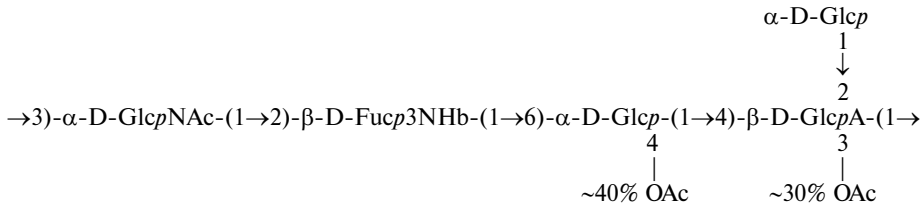
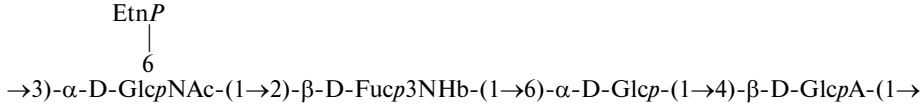
Residue	C1	C2	C3	C4	C5	C6
P. penneri 4 (O72) O-deacetylated polysaccharide[a]						
→6)-β-Glc*p*^{I}-(1→	105.6	74.3	77.0	70.3	75.5	66.6
→3)-β-Gal*p*NAc^{I}-(1→ 6 ↑	102.6	52.4	81.4	69.4	73.7	68.1
→4)-α-Gal*p*-(1→ 3 ↑	99.8	68.9	81.1	77.6	71.7	62.5
β-Gal*p*NAc^{II}-(1→	105.0	54.1	72.5	69.1	76.2	62.6
α-Glc*p*^{II}-(1→	99.8	72.6	74.5	71.0	73.3	62.0
P. penneri 1 (O72) polysaccharide						
→6)-β-Glc*p*-(1→	105.6	74.3	76.9	70.4	75.4	66.8
→3)-β-Gal*p*NAc^{I}-(1→	102.6	52.5	81.6	69.2	75.7	61.9
→4)-α-Gal*p*-(1→ 3 ↑	99.7	68.8	81.3	77.1	71.5	62.2
β-Gal*p*NAc^{II}-(1→	105.1	54.1	72.5	69.1	76.2	62.5
P. vulgaris 63/57 (O37) O-deacetylated polysaccharide						
→3)-β-Glc*p*A^{I}-(1→	103.9	73.0	82.8	72.9	75.6	173.6
→3)-α-Glc*p*NAc-(1→	99.3	53.6	82.2	69.4	73.0	61.4
→3)-β-Glc*p*A^{II}-(1→	103.6	73.0	82.8	72.9	75.4	173.6
→4)-α-Glc*p*-(1→	100.0	72.9	72.8	80.0	71.6	60.8
P. vulgaris 63/57 (O37) polysaccharide[b]						
→3)-β-Glc*p*A^{I}-(1→	104.0	72.9	83.5	73.1	75.9	
→3)-α-Glc*p*NAc6Ac-(1→	99.5	53.7	82.0	69.8	70.8	64.7
→3)-β-Glc*p*A^{II}-(1→	103.7	73.0	83.0	73.1	75.7	
→4)-α-Glc*p*-(1→	100.0	72.9	72.9	80.1	71.7	60.8

Note: Additional chemical shifts for NAc groups are δ 23.3-23.7 (CH_3) and 175.1-176.3 ppm (CO).

[a] Data for the major repeating unit, bearing terminal Glc*p* residue.

[b] Data for the O-acetylated repeating unit.

Table 3. Structures of the O-specific polysaccharides of *Proteus* determined in this work and structurally related polysaccharides

Proteus strain	Structure of the O-specific polysaccharide
Proteus penneri 4 (O72)	
Proteus penneri 1 (O72)	
Proteus vulgaris 63/57 (O37)	
Proteus vulgaris 72/57 (O46) [20]	
Proteus mirabilis TG 277 (O69)	
Proteus penneri 25 (O69) [21]	
Proteus penneri 20 (= *Proteus penneri* 16 [22])	
Proteus vulgaris 32/57 (O17) [1]	

GalNAc^{II} are partially shifted from δ 76.2 and 62.6 to δ 73.6 and 65.0 ppm, respectively. These displacements indicated O-acetylation of this monosaccharide at position 6 [18]. This conclusion was confirmed by a downfield displacement in the ^{1}H-NMR spectrum of the signals for H6a and H6b of GalNAc^{II} from δ 3.70-3.85 to δ 4.70 and 4.62 ppm due to a deshielding effect of the O-acetyl group. The degree of O-acetylation at position 6 of GalNAc^{II} was estimated as ~60%, and, as judged by the ratio of the integral intensities of the signals for the O-acetyl and N-acetyl groups in the ^{1}H-NMR spectrum, the total degree of O-acetylation in the repeating unit is ~75%. The position of minor O-acetyl groups (~15%) was not determined.

The data obtained showed that the major repeating unit of the O-specific polysaccharide of *P. penneri* 4 has the structure shown in Table 3.

***Proteus vulgaris* 63/57 (serogroup O37).** Sugar analysis after full acid hydrolysis of the polysaccharide revealed the presence of Glc, GlcN, and GlcA, all having the D-configuration.

The ^{13}C-NMR spectrum of the polysaccharide (Fig. 2) contained signals of different intensities, most likely, owing to non-stoichiometric O-acetylation (there was a signal for CH_3 of an O-acetyl group at 21.5 ppm). The ^{13}C-NMR spectrum of the O-deacetylated polysaccharide contained a major series of signals consisting of signals for four anomeric carbons at δ 99.3-103.9 ppm, one nitrogen-bearing carbon (C2 of Glc*p*N) at δ 53.6 ppm, two $HOCH_2$ groups (C6 of Glc*p* and Glc*p*N) at δ 61.4 and 60.8 ppm, two COOH groups (C6 of Glc*p*A) at δ 173.6 ppm, 15 other sugar ring carbons at δ 69.4-82.8 ppm, and one N-acetyl group (CH_3 at δ 23.2 ppm, CO at δ 175.5 ppm). The absence from the ^{13}C-NMR spectrum of signals in the region of δ 83-88 ppm characteristic for furanosides [19] showed that all monosaccharides are in the pyranose form.

The ^{1}H-NMR spectrum of the initial polysaccharide contained a major series of signals for four anomeric protons at δ 4.60-5.33 ppm, one N-acetyl group at δ 2.03 ppm, and other protons at δ 3.46-4.41 ppm. Both ^{13}C- and ^{1}H-NMR spectra of the O-deacetylated polysaccharide contained also a minor series of signals, which was absent from the spectra of the initial polysaccharide and, therefore, reflected a structural heterogeneity, which resulted from degradation in the course of alkaline O-deacetylation.

The ^{1}H-NMR spectrum of the O-deacetylated polysaccharide was assigned using two-dimensional COSY, TOCSY, NOESY, and ^{1}H,^{13}C HMQC experiments (Table 1). The spin system of Glc*p*NAc was identified by a correlation of the H2 proton at δ 4.12 ppm with a nitrogen-bearing carbon at δ 53.6 ppm observed in the HMQC spectrum. The $J_{1,2}$ coupling constant value of ~3 Hz indicated that Glc*p* and Glc*p*NAc are α-linked, whereas the $J_{1,2}$ value of ~8 Hz showed that both Glc*p*A residues (I and II) are β-linked.

The ^{1}H,^{13}C HMQC experiment enabled full assignment of the ^{13}C-NMR spectrum of the O-deacetylated polysaccharide (Table 2). Relatively low-field positions at δ 80-84 ppm of the signals for C3 of Glc*p*NAc, Glc*p*A^I and Glc*p*A^{II} and C4 of Glc*p*, as compared with their positions in the spectra of the corresponding nonsubstituted monosaccharides [6], revealed the glycosylation pattern in the repeating unit.

In the NOESY spectrum of the O-deacetylated polysaccharide, the direct assignment of cross-peaks between the transglycosidic protons was complicated by a coinci-

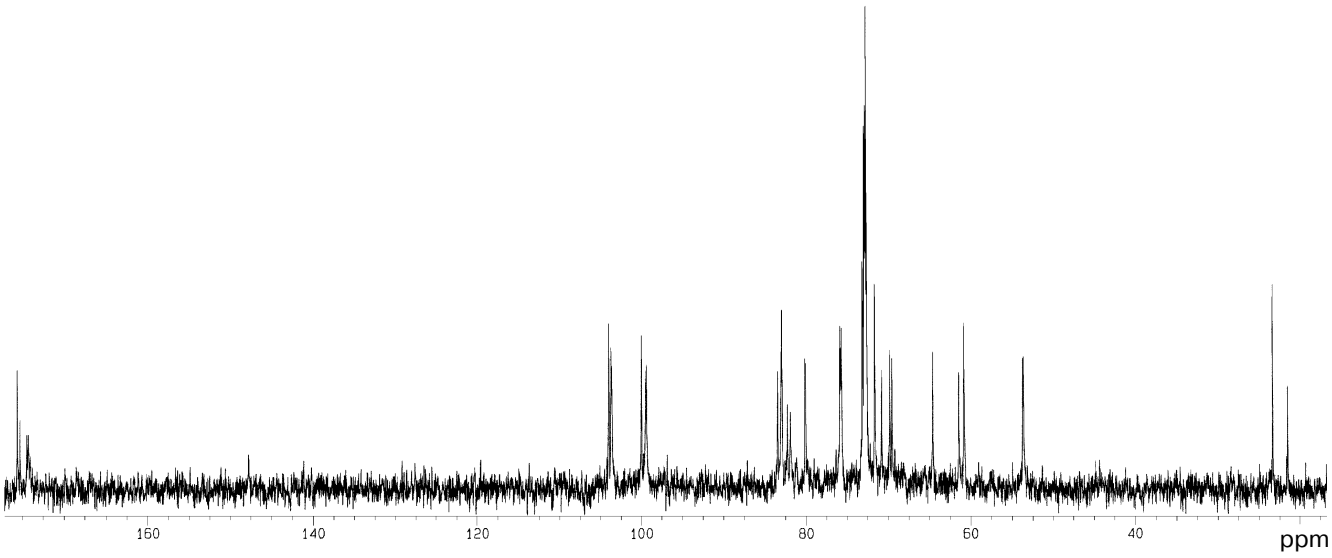


Fig. 2. ^{13}C-NMR spectrum of the polysaccharide of *P. vulgaris* 63/57 (O37).

dence of the H1 and H3 signals of $GlcA^{I}$ and $GlcA^{II}$ at δ ~4.60 and 3.70 ppm, respectively. This problem could be overcome by taking into account the modes of substitution of the monosaccharide residues, which were determined independently by the ^{13}C-NMR chemical shift data (see above). Therefore, the cross-peaks at δ 4.60/3.66, 5.33/3.70, 4.61/3.93, and 5.26/3.70 ppm could be assigned to $GlcA^{I}$ H1/Glc H4, Glc H1/$GlcA^{II}$ H3, $GlcA^{II}$ H1/GlcNAc H3, and GlcNAc H1/$GlcA^{I}$ H3 correlations, respectively. These data confirmed the structure of the repeating unit of the polysaccharide.

The position of the O-acetyl group was determined by comparison of the ^{13}C-NMR spectra of the initial and O-deacetylated polysaccharides. A lower field position of the H6a,6b signals of GlcNAc at δ 4.34, 4.41 ppm in the former as compared to that at δ 3.84, 3.84 ppm in the latter could only be caused by O-acetylation of GlcNAc at O6. Differences in the chemical shifts of Glc*p*NAc C5 and C6 (δ 70.8 and 64.7 versus 73.0 and 61.9 ppm; β- and α-effects of O-acetylation [18], respectively) confirmed the location of the O-acetyl group at position 6. Relative integral intensities of the signals for the O-acetylated and non-O-acetylated GlcNAc residues showed that the degree of O-acetylation is ~55%.

The structure of the repeating units of the O-specific polysaccharide of *P. vulgaris* 63/57 (O37) and a related polysaccharide of *P. vulgaris* O46 [20] are shown in Table 3.

***Proteus mirabilis* TG 277 (serogroup O69).** Sugar analysis of the polysaccharide showed the presence of GlcA and GlcN.

The ^{13}C-NMR spectrum (Fig. 3) suggested that the polysaccharide has a tetrasaccharide repeating unit that contains an O-acetyl group in a non-stoichiometric quantity (there was a signal at δ 21.7 ppm). The spectrum of the O-deacetylated polysaccharide was typical of a regular polymer. It contained signals for four anomeric carbons (δ 101.9-104.0 ppm), three nitrogen-bearing carbons (δ 50.8-56.0 ppm), one substituted (δ 68.8 ppm) and one unsubstituted $HOCH_2$ group (δ 61.9 ppm, data of an INEPT experiment), two methyl groups at δ 17.6 and 23.7 ppm, and four carbonyl groups at δ 172.5-176.0 ppm.

The ^{13}C-NMR spectrum of the initial polysaccharide resembled that of *Proteus penneri* 25 from serogroup O69, which was studied by us earlier [21]. For instance, regions for the anomeric carbons (δ 102-104 ppm) and nitrogen-bearing carbons (δ 55-56 ppm) were similar. Moreover, the spectra of the O-deacetylated polysaccharides from the two strains were practically identical. Therefore, the two polysaccharides differed in the position of the O-acetyl groups only. The structure of the repeating unit and the O-acetylation pattern of the polysaccharide of *P. penneri* 25 is shown in Table 3. A peculiar feature of this polysaccharide is the presence of a glucosamine residue N-substituted by L-alanine (GlcNAla) and three sites of non-stoichiometric O-acetylation.

Comparison of the ^{13}C-NMR spectra of the initial and O-deacetylated polysaccharides of *P. mirabilis* TG 277 showed that the O-acetyl group is located at position 3 of about half GlcNAla residues. This followed from a splitting of the C2 signal of GlcN (δ 56.1 ppm in the non-acetylated residue and δ 55.1 ppm in the O-acetylated residue; β-effect of O-acetylation [18]). The non-stoichiometric O-acetylation of GlcNAla was confirmed by a splitting of the signals for C2 and C3 of alanine (δ 50.5/50.8 and 17.1/17.6 ppm, respectively) owing to its attachment to N2 of GlcN located near the O-acetylation

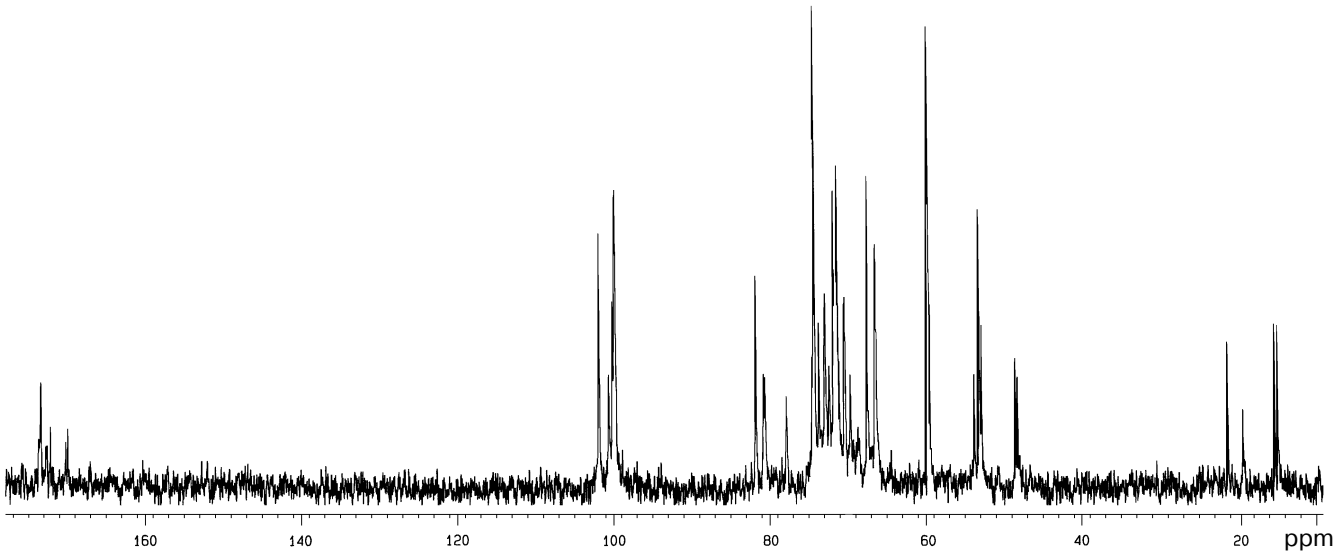


Fig. 3. ^{13}C-NMR spectrum of the polysaccharide of *P. mirabilis* TG 277 (O69).

site. The signals of the other sugar residues, including those of GlcNAc and GlcA, which are partially O-acetylated in the polysaccharide of *P. penneri* 25 [21], were not split and their chemical shifts were the same in the initial and O-deacetylated polysaccharides of *P. mirabilis* TG 277. Therefore, whereas there are three O-acetylation sites in the polysaccharide of *P. penneri* 25, the polysaccharide of *P. mirabilis* TG 277 has only one of them. The full structure of the *P. mirabilis* TG 277 polysaccharide is shown in Table 3.

***Proteus penneri* 20 (serogroup O17).** The O-antigen of *P. penneri* 20 was found to be serologically identical to that of *P. penneri* 16, whose structure has been established earlier [22]. The *P. penneri* 16 polysaccharide includes a rarely occurring component, 3,6-dideoxy-3-[(R)-3-hydroxybutyramido]-D-galactose [D-Fuc3NHb] (Table 3). It also contains O-acetyl groups, but their positions have not been determined. The ^{13}C-NMR spectra of the polysaccharides of *P. penneri* 16 and 20 were identical and, hence, the polysaccharides have the same structure. This was additionally confirmed by the identity of the ^{1}H- and ^{13}C-NMR spectra of the O-deacetylated polysaccharides from both strains.

In order to determine the location of the O-acetyl groups, the polysaccharide of *P. penneri* 20 was studied in detail using two-dimensional NMR spectroscopy. Comparison of the ^{13}C-NMR spectra of the initial (Fig. 4) and O-deacetylated polysaccharides showed no significant difference in chemical shifts of GlcNAc, Fuc3NHb and the lateral Glc residue, whereas those of GlcA and 6-substituted Glc were markedly shifted. As compared with the spectrum of the O-deacetylated polysaccharide, in the spectrum of the initial polysaccharide the intensities of the signals of GlcA and 6-substituted Glc decreased and new signals for the corresponding O-acetylated residues appeared.

Position 3 is the only possible site of O-acetylation in GlcA since positions 2 and 4 are glycosylated. Acetylation of this residue at O3 was confirmed by characteristic upfield displacements, due to β-effects of O-acetylation [18], of the signals for C2 and C4 from δ 78.6 and 77.3 to δ 77.5 and 76.6 ppm, respectively. Determination of the effects of O-acetylation in 6-substituted Glc was complicated by a coincidence of many signals for the O-acetylated residue with signals of other monosaccharides. However, the location of acetyl groups at O2 and O3 could be excluded since neither β-effect on C1 and C4 nor α-effect on C3 was observed. Hence, the 6-substituted Glc residue is O-acetylated at position 4. As judged by the relative integral intensities of the signals in the O-acetylated and non-acetylated residues, the degree of O-acetylation of GlcA and Glc is ~30 and ~40%, respectively. The full structure of the repeating unit of the polysaccharide of *P. penneri* 20 is shown in Table 3.

DISCUSSION

O-Acetylation in bacterial glycopolymers plays an important biological role. For instance, 3-O-acetylation of 2-acetamido-2-deoxy-D-mannuronic acid in the capsular polysaccharide of *Staphylococcus aureus* type 5 increased significantly the resistance of the bacterium to opsonophagocytic killing and the ability to colonize the kidneys in a mouse model [23]. 6-O-Acetylation of N-acetylmuramic acid increased the resistance of peptidoglycan of various human pathogens (*Neisseria gonorrhoeae*, *Proteus mirabilis*, *Staphylococcus aureus*) to hydrolytic degradation by lysozyme and muramidases [24]. A biosynthetic modification of extracellular bacterial polysaccharides that contributes to virulence (e.g., alginate of *Pseudomonas* species [25, 26] and Vi antigen of *Salmonella typhi* and *Citrobacter freundii* [27]) by O-acetylation of D-mannuronic acid and 2-acetamido-2-deoxy-D-galacturonic acid, respectively, has important implications, e.g., affects the binding properties of car-

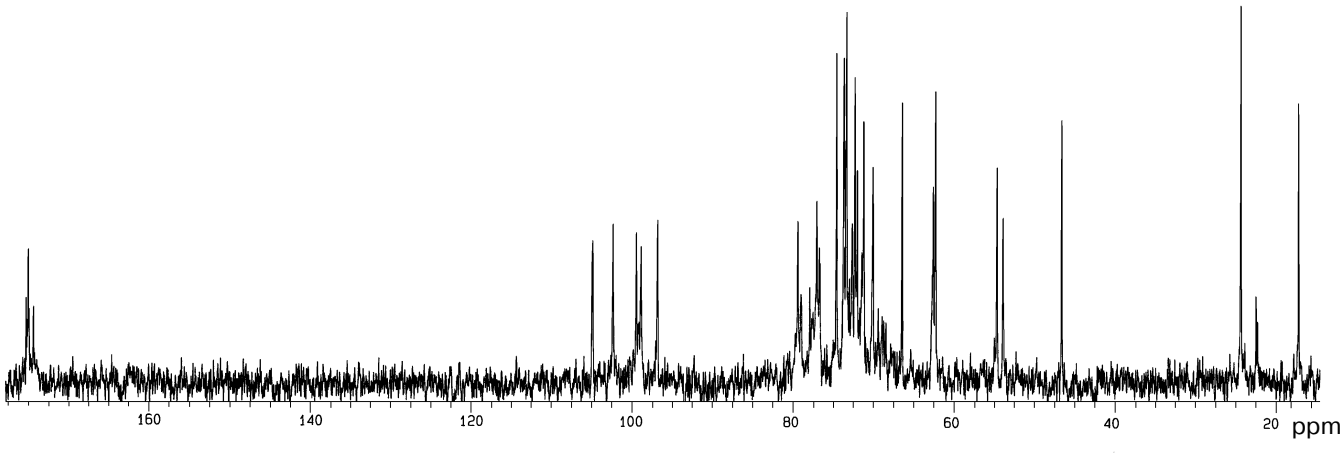


Fig. 4. ^{13}C-NMR spectrum of the polysaccharide of *P. penneri* 20 (O17).

boxyl groups. 8-O-Acetylation of legionaminic acid in the O-specific polysaccharide chain of the lipopolysaccharide increases the hydrophobicity of *Legionella pneumophila* cell surface and thus enhances the ability of the microorganism to form stable aerosols, which may promote the spread of the bacterium during outbreaks [28].

O-Acetylation in specific bacterial polysaccharides creates the antigenic diversity, which enhances the survival of the bacteria by promotion of immune avoidance. For instance, O-acetyl groups significantly contribute to the epitope specificity of *L. pneumophila* lipopolysaccharide, particularly to its recognition by monoclonal antibodies [29, 30]. O-Acetylation is in part responsible for serotype-conversion by modification of the O-specific polysaccharide of *Shigella flexneri* [31]. O-Acetyl transferase of this and some other bacteria has a bacteriophage origin. Lysogenic conversion in *Salmonella enterica* sv. *typhimurium* by specific bacteriophages A3 and A4 caused O-acetylation of the O-polysaccharide, which altered the binding behavior of the bacteriophages and prevented subsequent infection [32].

In the O-specific polysaccharides of the bacteria of the genus *Proteus*, the O-acetyl groups are rather common and also contribute to the immunospecificty of strains. For instance, the O-acetylation pattern of the O-polysaccharide served as the basis for the classification of *P. vulgaris* strains 72/57 and 63/57 into two different serogroups, O37 and O46, respectively [7]. Their O-specific polysaccharides are built up of tetrasaccharide repeating units having the same structure except that the former has three O-acetylation sites (at Glc and two GlcA residues) and the latter only one O-acetylation site at another sugar residue (GlcNAc) (Table 3). Studies with O-antisera against *P. vulgaris* 63/57 (O37) and 72/57 (O46) revealed close serological relatedness of the lipopolysaccharides of these strains [33]. The O-acetyl groups themselves were not found to form particular epitopes but rather to interfere with binding of anti-*P. vulgaris* 63/57 (O37) serum to *P. vulgaris* 72/57 (O46) antigen. Based on these data, we propose to exclude *P. vulgaris* 72/57 from serogroup O46 and to classify it into *Proteus* serogroup O37 as the second subgroup.

In several other *Proteus* serogroups there are pairs of structurally similar O-specific polysaccharides that differ in the presence or the absence of O-acetyl groups either alone or along with other structural features. For instance, the O-specific polysaccharide of *P. mirabilis* TG 277 studied in this work is a lower O-acetylated variant of the polysaccharide of *P. penneri* 25 [21] from serogroup O69 [10], which lacks two of three sites of O-acetylation present in the *P. penneri* 25 polysaccharide (Table 3). These data show that *P. mirabilis* TG 277 should be classified into the same *Proteus* serogroup O69 as *P. penneri* 25.

The trisaccharide repeating units of the polysaccharides of *P. penneri* strains 19 and 62 have the same topology and sugar composition and differ only in the O-acetylation of a GlcNAc residue in strain 19 but not in strain 62, as well as in the mode of substitution of N-acetylisomuramic acid (at position 4 or 6, respectively) [9, 10]. Hence, these strains, along with the *P. penneri* 71 strain, which contains N-acetylglucosamine instead of N-acetylisomuramic acid and lacks O-acetyl groups, were classified intro serogroup O64 as three subgroups [34].

The O-specific polysaccharides of *P. penneri* strains 1 and 4 studied in this work differ in glucosylation of one of the GalNAc residues and O-acetylation of the other residue in *P. penneri* 4 (Table 3). Based on these data, we propose to combine *P. penneri* strains 1 and 4 in one *Proteus* serogroup, O72, as two subgroups.

Similarly, the polysaccharide of *P. penneri* 16 and 20 on one hand and *P. vulgaris* 32/57 from serogroup O17 [1] on the other hand have the same structure of the main carbohydrate chain but differ in the presence of a terminal glucose residue and an O-acetyl group in *P. penneri* and the presence of ethanolamine phosphate in *P. vulgaris*. These data and the data of serological studies, which will be published elsewhere, showed that it is reasonable to include strains *P. penneri* 16 and 20, as well as serologically identical strains *P. penneri* 10, 18, 32, 45, 108 and 111, into *Proteus* serogroup O17 as the second subgroup.

Therefore, differences in the O-acetylation pattern contribute to the differentiation between *Proteus* strains having structurally and serologically related O-antigens. On the other hand, the structural data of this work, along with serological data [10], showed that it is reasonable to combine such strains in one serogroup, which improves the existing classification of *Proteus* strains.

It should be noted that in the most *Proteus* O-specific polysaccharides O-acetylation is non-stoichiometric and thus masks the regularity of the polymers. For instance, the degree of O-acetylation at each site in the O-specific polysaccharides studied in this work varies from 30 to 95% (Table 3). This peculiar feature enabled the suggestion that O-acetylation is a post-polymerization modification of the O-specific polysaccharides, but the exact mechanisms of the *Proteus* O-antigen biosynthesis have not been clarified yet.

In some polysaccharides the degree of O-acetylation is so low that the exact location of the O-acetyl groups could be determined only tentatively, if at all. For instance, in previous studies of the polysaccharide of *P. penneri* 16 (O17) the positions of O-acetyl groups, which are present in a minority of the repeating units, were not determined [22]. In this work, the polysaccharide of *P. penneri* 20 was found to have the same structure as that of *P. penneri* 16, including the O-acetylation pattern, and further studies showed that O-acetyl groups in this polysaccharide substitute ~30% GlcA residues and ~40% Glc residues (Table 3). In the O-specific polysaccharides of *P. mirabilis* O23 [35] and *P. vulgaris* O32 [36], the location

of the O-acetyl groups was determined only tentatively. The position of only one, major O-acetyl group was determined in the polysaccharide of *P. penneri* 4 (this work), whereas the attachment sites of the other, minor O-acetyl groups remain unknown.

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