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Structure of the O-polysaccharide of *Proteus mirabilis* O19 and reclassification of certain *Proteus* strains that were formerly classified in serogroup O19

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: Bacteria of the genus *Proteus* are a common cause of urinary tract infections. The O-polysaccharide chain of their LPS (O-antigen) defines the serological specificity of these bacteria. Based on the immunospecificity of the O-antigens, two species, *P. mirabilis* and *P. vulgaris*, were classified into 49 O-serogroups, and more O-serogroups for strains of these species and *P. penneri* have been subsequently proposed.

Materials and Methods: The lipopolysaccharide of *P. mirabilis* CCUG 19011 from serogroup O19 was degraded under mildly acidic and mildly alkaline conditions. Polysaccharides thus obtained were studied by chemical methods, including *O*-deacetylation, sugar and methylation analyses, and ^{1}H- and ^{13}C-NMR spectroscopy. Antisera were obtained by immunization of New Zealand white rabbits with heat-killed bacteria. In serological studies, enzyme immunosorbent assay, passive hemolysis test, and inhibition of passive hemolysis were used.

Results: The following structure of the O-polysaccharide repeating unit was established:
→3)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*NAc4,6(*R*-Pyr)-(1→4)-α-D-Gal*p*A-(1→3)-α-L-Rha*p*2Ac-(1→
where *R*-Pyr is (*R*)-1-carboxyethylidene (an acetal-linked pyruvic acid). This structure is significantly different from the O-polysaccharide structures of *P. vulgaris, P. hauseri* and *P. penneri* strains from the same *Proteus* serogroup O19.

Conclusions: Based on immunochemical studies of the lipopolysaccharides, it is suggested 1) to keep *P. vulgaris* CCUG 4654 and *P. penneri* 31 in serogroup O19 as two subgroups, 2) to reclassify *P. mirabilis* CCUG 19011 into a new *Proteus* serogroup, O51, and 3) to classify serologically related strains, including *P. vulgaris* ATCC 49990, *P. hauseri* 1732-80 and 1086-80, *P. penneri* 15, and some other *P. penneri* strains, in yet another *Proteus* serogroup, O52.

Key words: ***Proteus mirabilis*** **• lipopolysaccharide • O-polysaccharide • structure • serological classification • pyruvic acid**

Abbreviations: **DOC-PAGE** – sodium deoxycholate polyacrylamide gel electrophoresis, **EIA** – enzyme immunosorbent assay, **GalA** – galacturonic acid, **LPS** – lipopolysaccharide, **LPSOH** – alkali-treated lipopolysaccharide, **NMR** – nuclear magnetic resonance, **OPS** – O-specific polysaccharide, **PHT** – passive hemolysis test, **PS** – polysaccharide, **Rha** – ramnose.

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INTRODUCTION

The genus *Proteus* belongs to the *Enterobacteriaceae* family and originally contained two species *P. vulgaris* and *P. mirabilis*. Strains that were identified as *P. vulgaris* appeared to be heterogenous and formed three biogroups, characterized by biochemical reactions[16]. Biogroup 1 is distinguished by a negative reaction for indole production, salicin fermentation, and aesculin hydrolysis, whereas biogroup 2 (= genospecies 2) is positive for salicin and aesculin and biogroup 3 negative for salicin and aesculin[9]. In 1982, biogroup 1 was transformed to the species *P. penneri*[9]. Genetic studies of O'Hara et al.[17] showed the existence of four genospecies within biogroup 3, which were named *Proteus* genomospecies 3, 4, 5 and 6. On the basis of DNA hybridization, Brenner et al.[4] recommended that biogroup 2 retain the name *P. vulgaris.* In 2000, O'Hara et al.[17] proposed that *Proteus* genomospecies 3 be named *Proteus hauseri* in honor to Gustaw Hauser, the German microbiologist who first described the genus *Proteus*[8]. The species *P. hauseri* is negative for salicin fermentation, aesculin hydrolysis and deoxyribonuclease. It can be distinguished from the *Proteus* genospecies 4, 5 and 6 because it is negative in utilization of tartrate.

Bacteria of the genus *Proteus* are a common cause of urinary tract infections that can lead to severe complications, such as formation of bladder and kidney stones, as well as acute or chronic pyelonephritis. A number of virulence factors that mediate the infection process have been described in these bacteria, including its lipopolysaccharide (LPS, endotoxin)[20]. The O--polysaccharide chain of the LPS (O-antigen) defines the serological specificity of a bacterium. Based on the immunospecificity of the O-antigens, two species, *P. mirabilis* and *P. vulgaris*, have been classified into 49 O-serogroups, and more O-serogroups for strains of these species and *P. penneri* were proposed later[14, 18, 26].

Immunochemical studies of the O-antigens help to improve the classification of *Proteus*[12, 23, 26]. In this study we investigated the O-antigens of *Proteus* serogroup O19 strains, including *P. mirabilis* CCUG 19011 and *P. vulgaris* CCUG 4654 (U349). Earlier, based on chemical and serological data, it was proposed to classify *P. penneri* strain 31 in serogroup O19 as a subgroup[13]. *P. vulgaris* ATCC 49990 was reported to cross-react with the reference *Proteus* O19 antiserum[19], and this strain as well as *P. penneri* 15 and two *P. hauseri* strains, which share the O-antigen with strain ATCC 49990, are also involved in the present serological study. The structures of the O-antigens of *P. vulgaris* CCUG 4654[24], ATCC 49990[19], *P. penneri* 15[27] and *P. hauseri* (authors' unpublished data) have been established earlier, and that of *P. mirabilis* CCUG 19011 was determined in this work.

MATERIALS AND METHODS

Bacterial strains

The *Proteus* strains came from the Culture Collection of the University of Goeteborg, Sweden (*P. mirabilis* CCUG 19011 and *P. vulgaris* CCUG 4654), the Czech National Collection of Type Culture, Institute of Epidemiology and Microbiology, Prague, Czech Republic (*P. vulgaris* PrK 37/57 and *P. vulgaris* U349), the American Type Culture Collection (*P. vulgaris* ATCC 49990), and the collection of the Institute of Microbiology and Immunology, University of Łódź, Poland (*P. penneri* 15). The *P. hauseri* strains were kindly provided by Dr. C. M. O'Hara from the Center for Disease Control and Prevention, Atlanta, USA. Both *P. vulgaris* CCUG 4654 and PrK 37/57 are described in the Kaufmann-Perch scheme as *P. vulgaris* strain U349.

Cultivation of bacteria and isolation of the LPS

Bacteria were cultivated under aerobic conditions in nutrient broth (BTL, Łódź, Poland). The LPS was isolated from the dried bacterial mass of all strains by hot phenol/water extraction[25] and purified by treatment with cold aqueous 50% trichloroacetic acid followed by separating the precipitate by centrifugation and dialysis of the supernatant.

Deacylation of the LPS

The LPS was saponified with 0.25 M NaOH (36°C, 2 h) for serological analyses or with aqueous 12.5% ammonia (37°C, 16 h) for nuclear magnetic resonance (NMR) spectroscopic studies to give LPS_{NaOH} and LPS_{NH_4OH}, respectively.

Mild acid degradation of the LPS

The LPS (150 mg) was hydrolyzed with 2% AcOH at 100°C until precipitation of lipid, and the water-soluble portion was fractionated by gel chromatography on a column (56 × 2.6 cm) of Sephadex G-50 to give the O-polysaccharide (PS, 28 mg).

Removal of pyruvic acid

The PS (28 mg) was treated with 2% AcOH (105°C, 6 h) and subsequent gel chromatography on a column (90 × 2.5 cm) of TSK HW-40 (S) (Merck, Germany) in water gave a polysaccharide devoid of pyruvic acid (PS_{AcOH}, 23 mg).

O-deacetylation

The PS_{AcOH} (23 mg) was treated with aqueous 12.5% ammonia at 37°C for 16 h, the solution was desalted on a column (90 × 2.5 cm) of TSK HW-40 (S) (Merck, Germany) in water and freeze-dried to give an *O*-deacetylated polysaccharide (PS_{NH_4OH}, 17 mg).

Sugar analyses

Hydrolysis was performed with 2 M CF_3COOH (120°C, 2 h). Amino sugars were identified using a Biotronik LC2000 amino acid analyzer (Germany) equipped with a column (0.4 × 22 cm) of Ostion LG AN B cation-exchange resin using 0.35 M sodium citrate buffer (pH 5.28) at 80°C. Neutral sugars and uronic acids were identified using a Biotronik LC2000 sugar analyzer (Germany) on a column (0.4 × 15 cm) of Dionex A × 8 anion-exchange resin using 0.5 M sodium borate buffer (pH 8.0) or 0.04 M sodium phosphate buffer (pH 2.4) at 70°C, respectively. The absolute configurations of the monosaccharides were determined by GLC of the acetylated (*R*)-2--octyl glycosides (for Rha, GalN and GlcN) according to the published method[7, 15] modified as described[21]. GLC was performed with a Hewlett-Packard Model 5890 chromatograph (USA) equipped with a DB5 fused-silica capillary column (25 m × 0.25 mm) and a temperature gradient of 160°C (1 min) to 250°C at 10°C/min.

Methylation analysis

Methylation was performed with CH_3I in dimethyl sulfoxide in the presence of sodium methylsulfinylmethanide[5]. A portion of the methylated PL was reduced with $LiBH_4$ in aqueous 70% 2-propanol (20°C, 2 h). Partially methylated monosaccharides were derived by hydrolysis under the same conditions as in sugar analysis, converted into the alditol acetates and analyzed by GLC-MS[10].

NMR spectroscopy

NMR spectra were recorded with a Bruker DRX-500 spectrometer (Germany) for solutions in D_2O at 30°C, using internal acetone (δ_H 2.225, δ_C 31.45) as reference. Standard Bruker software (XWINNMR 2.1) was used to acquire and process the NMR data. A mixing time of 300 ms was used in 2D TOCSY and ROESY experiments.

Serological techniques

Antisera were obtained by immunization of New Zealand white rabbits with heat-killed bacteria (100°C, 2.5 h) as described previously[1]. Enzyme immunosorbent assay (EIA), passive hemolysis test (PHT) and inhibition of passive hemolysis were performed as reported earlier[22]. The LPS and LPS_{NaOH} were used as antigens in EIA and PHT, respectively. Absorption of antisera (1 ml, 1:50 diluted with phosphate-buffered saline) was carried out at 4°C for 1 h with 100 µl packed sheep red blood cells coated with the respective LPS_{NaOH} as antigen (200 µg LPS in 200 µl sheep red blood cells). The level of antibodies after absorption was determined using PHT. DOC--PAGE and Western blot were performed as described earlier[2].

RESULTS AND DISCUSSION

Chemical studies

The PS was obtained by mild acid degradation of the LPS isolated from *P. mirabilis* CCUG 19011 by the phenol/water procedure[25]. Sugar analyses after full acid hydrolysis of the PS revealed Rha, GlcN, GalN and galacturonic acid (GalA). GLC analysis of the acetylated glycosides with (*R*)-2-octanol showed that Rha has the L configuration and the other monosaccharides have the D configuration.

The ^{13}C-NMR spectrum of the PS indicated the presence of an *O*-acetyl group and a pyruvic acid acetal (signals for CH_3 at δ 21.6 and 26.4, respectively). The spectrum showed an irregular structure due to non-stoichiometric substitution with pyruvic acid. Therefore, the PS was hydrolyzed with 2% AcOH for a longer time to remove the acetal group. The ^{13}C--NMR spectrum (Fig. 1, top) demonstrated that the deacetalated polysaccharide (PS_{AcOH}, Fig. 2) has a regular structure. The ^{1}H-NMR spectrum (not shown) of the PS_{AcOH} contained signals for one *O*--acetyl and two *N*-acetyl groups at δ 2.15, 2.07 and 2.09 in the ratio ~1:2, respectively.

The PS_{AcOH} was *O*-deacetylated by treatment with aqueous ammonia. The ^{13}C-NMR spectrum (Fig. 1, bottom) of the resultant polysaccharide (PS_{NH_4OH}, Fig. 2) contained signals for four anomeric carbons at δ 97.8-102.9, two nitrogen-bearing carbons (C2 of GlcN and GalN) at δ 56.8 and 49.5, two $HOCH_2$-C groups (C6 of GalN and GlcN) at δ 61.7 and 61.8, one COOH group (C6 of GalA) at δ 173.7, 14 other sugar ring carbons at δ 69.3-82.9, and two *N*-acetyl groups (CH_3 at δ 23.7 and 23.8, CO at δ 175.2 and 175.4). The absence of signals in the ^{13}C-NMR spectrum in the region δ 83-88 that are characteristic for furanosides[3] showed that all monosaccharides are in the pyranose form. Accordingly, the ^{1}H-NMR spectrum of the PS_{NH_4OH} contained 5 signals in the region at δ

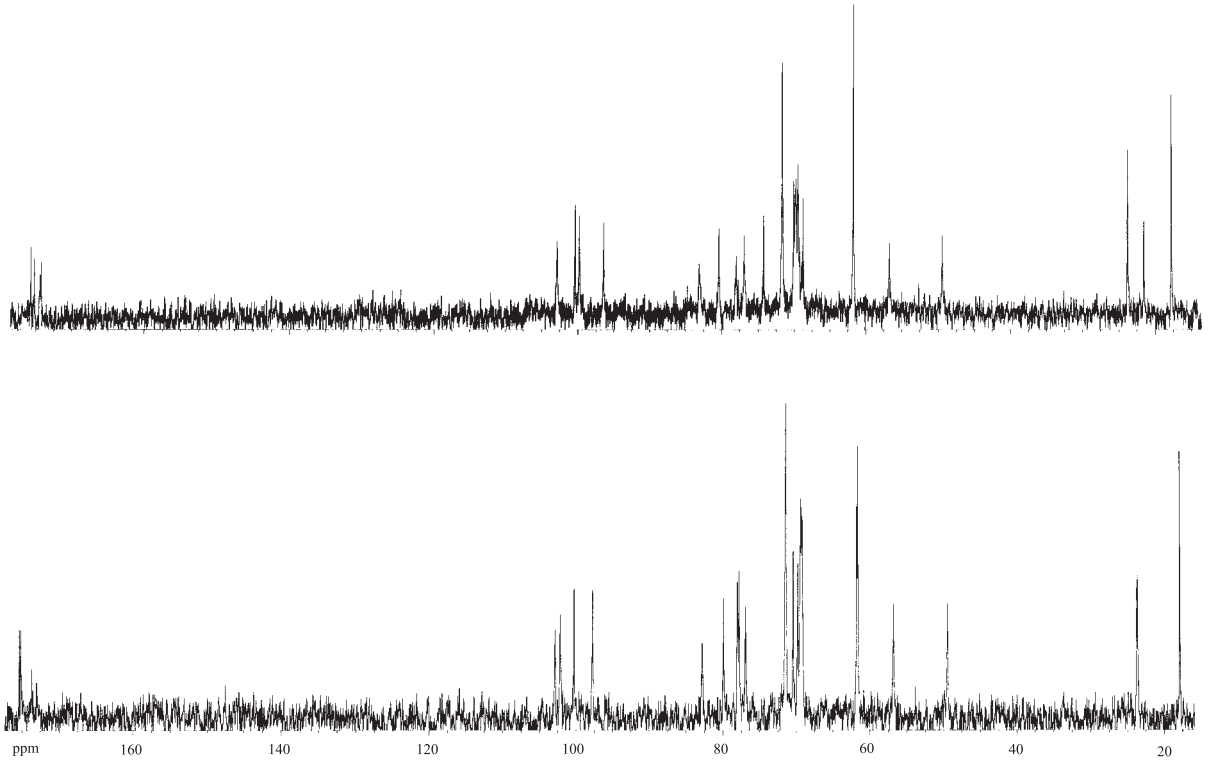

Figure 1. ^{13}C-NMR spectra of the *O*-deacetalated polysaccharide (PS_{AcOH}, top) and the *O*-deacetylated polysaccharide (PS_{NH_4OH}, bottom) from *P. mirabilis* CCUG 19011.

PS_{AcOH}

→3)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*A-(1→3)-α-L-Rha*p*2Ac-(1→

PS_{NH_4OH}

→3)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*A-(1→3)-α-L-Rha*p*-(1→

LPS_{NH_4OH}

→3)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*NAc4,6(*R*-Pyr)-(1→4)-α-D-Gal*p*A-(1→3)-α-L-Rha*p*-(1→

O-polysaccharide

→3)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*NAc4,6(*R*-Pyr)-(1→4)-α-D-Gal*p*A-(1→3)-α-L-Rha*p*2Ac-(1→

Figure 2. Structures of the repeating units of the O-polysaccharide from *P. mirabilis* CCUG 19011 and modified polysaccharides. Rha*p*2Ac stands for 2-*O*-acetylrhamnopyranose and Gal*p*NAc4,6(*R*-Pyr) for 2-acetamido-4,6-*O*-[*R*-1-carboxyethylidene]-2 deoxygalactopyranose.

4.64-5.17 (four anomeric protons and H5 of GalA, see below), one signal for a CH_3-*C* group (H6 of Rha) at δ 1.24, other sugar signals at δ 3.45-4.40, and two *N*-acetyl groups at δ 2.07 and 2.09.

Therefore, the O-polysaccharide has a tetrasaccharide repeating unit containing one residue each of L-rhamnose, D-galacturonic acid, 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-galactose, along with the *O*-acetyl group and the acetal-linked pyruvic acid.

Methylation analysis of the PS_{NH_4OH} resulted in identification of 2,4-di-*O*-methylrhamnose, 2-deoxy-4,6-di-*O*-methyl-2-(*N*methyl)acetamidogalactose and 2-deoxy-4,6-di-*O*-methyl-2-(*N*methyl)acetamidoglucose derivatives. When the methylated polysaccharide was carboxyl-reduced prior to hydrolysis, 2,3-di-*O*-methylgalactose was identified in addition to the sugars mentioned above, which was evidently derived from GalA. Therefore, the O-polysaccharide is linear and contains 3-substituted residues of Rha, GlcN and GalN and a 4-substituted residue of GalA.

The ^{1}H- and ^{13}C-NMR spectra of the PS_{NH_4OH} were assigned using 2D COSY, TOCSY, NOESY, and H-detected ^{1}H,^{13}C heteronuclear multiple-quantum coherence (HMQC) measurements (Table 1). The TOCSY spectrum showed cross-peaks of H1 with H2-

Table 1. ^{1}H- and ^{13}C-NMR data of the *O*-deacetylated polysaccharide (PS_{NH_4OH}) from *P. mirabilis* CCUG 19011[a]

Sugar residue	Chemical shift (δ, ppm)					
	H1	H2	H3	H4	H5	H6
→4)-α-D-Gal*p*A-(1→	5.17	3.94	4.10	4.45	4.90	
→3)-α-L-Rha*p*-(1→	4.91	3.95	3.85	3.53	4.05	1.24
→3)-β-D-Glc*p*NAc-(1→	4.64	3.84	3.64	3.56	3.45	3.78, 3.92
→3)-α-D-Gal*p*NAc-(1→	4.93	4.30	3.96	4.30	4.40	3.72
	C1	C2	C3	C4	C5	C6
→4)-α-D-Gal*p*A-(1→	97.8	69.3	69.8	80.0	71.5	173.7
→3)-α-L-Rha*p*-(1→	102.2	69.3	77.9	71.5	70.5	17.8
→3)-β-D-Glc*p*NAc-(1→	102.9	56.8	82.9	69.5	77.0	61.8
→3)-α-D-Gal*p*NAc-(1→	100.3	49.5	78.1	69.5	71.5	61.7

[a] The chemical shifts for N-acetyl groups are δ_H 2.07 and 2.09; δ_C 23.7, 23.8 (both Me), 175.2, 175.4 (both CO).

-H6 of GlcNAc and with H2-H4 of GalNAc and GalA residues. For GalA there was also an H4/H5 cross--peak. The spin system of Rha was identified by H1/H2 and H1/H3 correlations in the TOCSY spectrum and H3/H4 and H4/H5 correlations in the COSY spectrum. The spin systems of GlcNAc and GalNAc were confirmed by correlations of signals for the protons at nitrogen-bearing carbons at δ 3.84 and 4.30 to the corresponding carbon signals at δ 56.8 and 49.5, respectively. A $J_{1,2}$ coupling constant value of 3 Hz indicated that GalNAc is α-linked, whereas $J_{1,2}$ values of 8 Hz showed that GalA and GlcNAc are β-linked. The position of the signals for H5 at δ 4.05 and C5 at δ 70.5 indicated that Rha is α-linked (compare published data[11]).

Relatively low-field positions of the signals for C3 of Rha, GlcNAc and GalNAc and C4 of GalA at δ 77.9, 82.9, 78.1 and 80.0, as compared with their positions in the spectra of the corresponding non-substituted monosaccharides[11], demonstrated the glycosylation pattern in the repeating unit. The ROESY spectrum showed interresidue cross-peaks at δ 5.17/3.85, 4.91/3.64, 4.64/3.96 and 4.93/4.45, which were assigned to GalA H1/Rha H3, Rha H1/GlcNAc H3, GlcNAc H1/GalNAc H3 and GalNAc H1/GalA H4 correlations, respectively. These data defined the full sequence of the monosaccharide residues in the O-polysaccharide.

The position of the *O*-acetyl group was determined using ^{1}H,^{13}C HMQC with the PS_{AcOH}, which showed a displacement of a Rha H2/C2 cross-peak to 5.19/70.2, as compared with its position at δ 3.95/69.3 in the ^{1}H,^{13}C HMQC spectrum of the PS_{NH_4OH}. This displacement was due to a deshielding effect of the *O*-acetyl group and indicated *O*-acetylation of Rha at position 2.

In order to determine the site of acylation with pyruvic acid, the LPS was *O*-deacylated by treatment with aqueous ammonia. In contrast to the spectrum of the original PS, the ^{13}C-NMR spectrum of the resultant LPS_{NH_4OH} (of the structural formula presented on Fig. 2) showed a regular structure. Signals for C4 and C6 of GalNAc in the ^{13}C-NMR spectrum of the LPS_{NH_4OH} shifted downfield to δ 73.1 and 64.1 and signals for C3 and C5 upfield to δ 76.9 and 66.5, compared with their positions at δ 69.5, 61.7, 78.1 and 71.5, respectively, in the ^{13}C-NMR spectrum of PS_{NH_4OH}. Other signals in the spectrum shifted insignificantly; hence, pyruvic acid is attached at positions 4 and 6 of GalNAc. The ^{13}C-NMR chemical shifts of δ_C 26.3 for the pyruvic acid acetal methyl group indicated the *R* configuration of the pyruvic acid acetal carbon (compare published data[6]).

On the basis of the data obtained, it was concluded that the repeating unit of the O-polysaccharide of *P. mirabilis* CCUG 19011 has the structure shown in Fig. 2.

Serological studies

The LPS of *Proteus* O19 and related strains were tested with polyclonal rabbit O-antisera against *P. mirabilis* CCUG 19011, *P. vulgaris* CCUG 4654, *P. vulgaris* PrK 37/57 and ATCC 49990, *P. penneri* 15, and *P. hauseri* 1086-80 and 1732-80 in Western blot (Fig. 3 and 4), EIA and PHT (Table 2). In addition, absorption and inhibition techniques were used (Tables 3 and 4). Data obtained for the LPS of two *P. vulgaris* strains, CCUG 4654 and PrK 37/57 (CNCTC U349), as well as those of two *P. hauseri* strains, 1086-80 and 1732-80, were pairwise essentially identical, and data of only the first strain from each pair are shown. No cross-reactivity in EIA was observed when anti-*P. vulgaris* ATCC 49990 and anti-*P. hauseri* sera were tested with the LPS of a number of other *P. mirabilis*, *P. vulgaris* and *P. penneri* strains that contain sugar residues in common with the O-polysaccharides studied (data not shown).

In Western blot (Figs. 3 and 4), all O-antisera recognized slow migrating bands of both homologous and

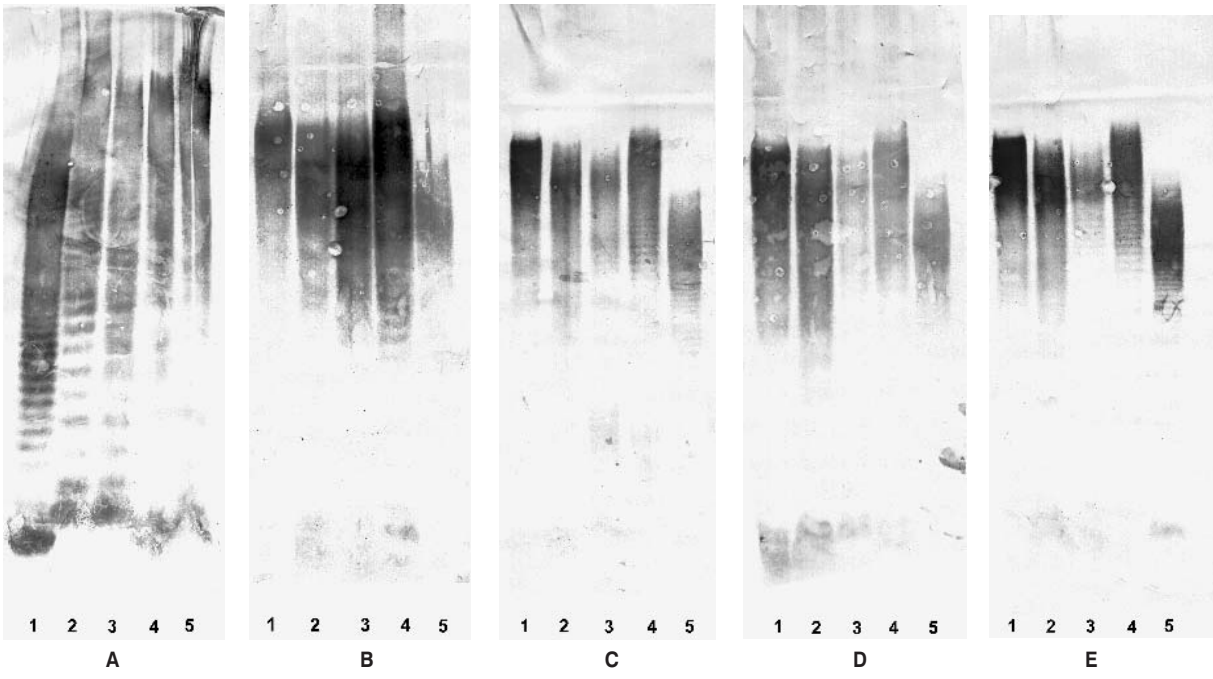

Figure 3. Western blot with antisera against *P. mirabilis* CCUG 19011 (**A**), *P. vulgaris* CCUG 4654 (**B**), *P. vulgaris* PrK 37/57 (**C**), *P. vulgaris* ATCC 49990 (**D**) and *P. penneri* 15 (**E**) of the LPS from the following *Proteus* strains: 1 – *P. mirabilis* CCUG 19011, 2 – *P. vulgaris* CCUG 4654, 3 – *P. vulgaris* U349, 4 – *P. vulgaris* ATCC 49990, 5 – *P. penneri* 15.

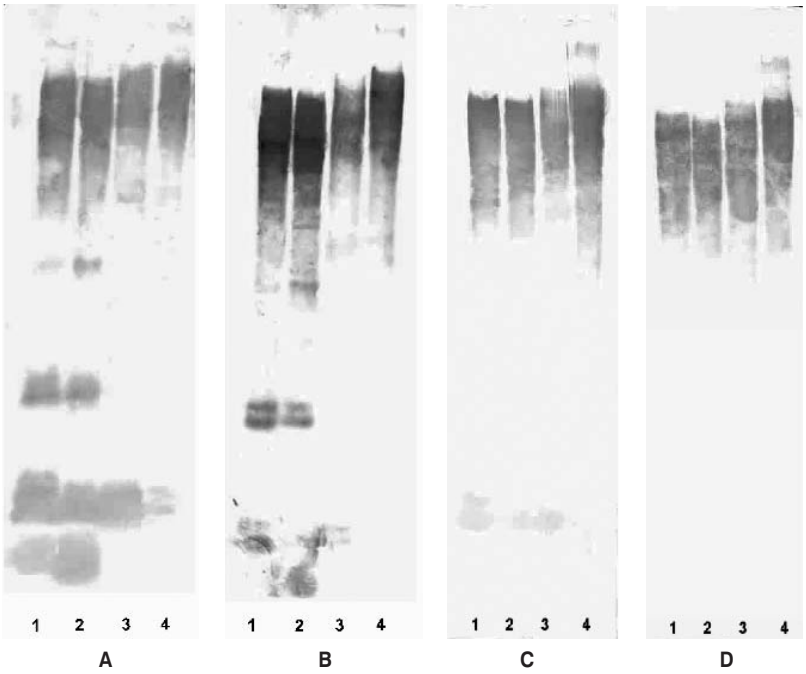

Figure 4. Western blot with antisera against *P. hauser*i 1732-80 (**A**)*, P. hauser*i 1086-80 (**B**),*P. penner*i 15 (**C**) and *P. vulgaris* ATCC 49990 (**D**) of the LPS from the following *Proteus* strains: 1 – *P. hauser*i 1732-80, 2 – *P. hauser*i 1086-80, 3 – *P. penner*i 15, 4 – *P. vulgaris* ATCC 49990.

heterologous LPS that correspond to high-molecular-mass species with O-polysaccharides consisting of large numbers of O-repeating units. Therefore, the LPS studied share epitopes on the O-polysaccharide part. In addition, anti-*P. mirabilis* CCUG 19011 serum reacted weakly with fast migrating bands of the heterologous LPS, which indicated sharing an epitope(s) also on the LPS core region (Fig. 3). The strongest cross-reactivity was observed between anti-*P. hauseri* sera and the LPS of *P. vulgaris* ATCC 49990 and *P. penneri* 15 and *vice versa* (Fig. 4). This finding was expected taking into account the structural identity of the O-antigens of these strains (Table 5).

Table 2. Reactivity of *Proteus* O-antisera with the LPS of *Proteus* O19 and related strains

Antigen from *Proteus* strain	Reciprocal titer with O-antiserum against				
	P. vulgaris CCUG 4654	*P. mirabilis* CCUG 19011	*P. hauseri* 1732-80	*P. vulgaris* ATCC 49990	*P. penneri* 15
EIA					
P. vulgaris CCUG 4654	**128,000**	4,000	16,000	1,000	nt
P. mirabilis CCUG 19011	1,600	**256,000**	64,000	2,000	nt
P. hauseri 1732-80	16,000	32,000	**1,024,000**	256,000	nt
P. vulgaris ATTC 49990	1,000	2,000	256,000	**512,000**	nt
P. penneri 15	8,000	8,000	512,000	256,000	nt
PHT					
P. vulgaris CCUG 4654	**51,200**	25,600	nt	12,800	12,800
P. mirabilis CCUG 19011	3,200	**25,600**	nt	1,600	3,200
P. hauseri 1732-80	nt	nt	**102,400**	25,600	nt
P. vulgaris ATTC 49990	12,800	12,800	102,400	**25,600**	25,600
P. penneri 15	25,600	25,600	3,200	25,600	**51,200**

LPS and alkali-treated LPS were used as antigen in EIA and PHT, respectively. Data of the homologous LPS are shown in bold type; nt – not tested.

Table 3. Reactivity with the homologous alkali-treated LPS in PHT of *Proteus* O-antisera absorbed with alkali-treated LPS of *Proteus* O19 and related strains

O-antiserum against *Proteus* strain	Reciprocal titer with the homologous alkali-treated LPS of O-antisera absorbed with the alkali-treated LPS from *Proteus* strain				
	Control	*P. vulgaris* CCUG 4654	*P. mirabilis* CCUG 19011	*P. vulgaris* ATCC 49990	*P. penneri* 15
P. vulgaris CCUG 4654	40,960	**10**	40,960	20,480	20,480
P. mirabilis CCUG 19011	10,240	5,120	**10**	5,120	5,120
P. vulgaris ATCC 49990	20,480	5,120	5,120	**10**	320
P. penneri 15	40,960	2,560	40,960	320	**10**

Sheep red blood cells were used as control. Data of the O-antiserum absorbed with the homologous alkali-treated LPS are shown in bold type.

Table 4. Inhibition with alkali-treated LPS from *Proteus* O19 and related strains of the reaction in PHT of *Proteus* O-antisera with the homologous alkali-treated LPS

Homologous test-system O-antiserum/alkali-treated LPS of *Proteus* strain	Minimal inhibiting dose (ng) of the alkali-treated LPS from strain			
	P. vulgaris CCUG 4654	*P. mirabilis* CCUG 19011	*P. vulgaris* ATCC 49990	*P. penneri* 15
P. vulgaris CCUG 4654	**2.4**	5,000	5,000	5,000
P. mirabilis CCUG 19011	5,000	**2.4**	5,000	5,000
P. vulgaris ATCC 49990	5,000	5,000	**4.8**	**4.8**

Data of the homologous LPS are shown in bold type.

Table 5. Structures of the O-polysaccharides of *Proteus* strains formerly classified to serogroup O19 and related strains and a suggestion for their reclassification

Serogroup	*Proteus* strain	O-polysaccharide structure	Reference
O19a	*P. vulgaris* CCUG 4654, PrK 37/57, CNCTC U349	→4)-α-D-Gal*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*-(1→	24
O19a,19b	*P. penneri* 31	→4)-α-D-Gal*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*-(1→ 6 | Etn*P*	13
O51	*P. mirabilis* CCUG 19011	→3)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*NAc4,6(*R*-Pyr)-(1→4)-α-D-Gal*p*A-(1→3)-α-L-Rha*p*2Ac-(1→	a
O52	*P. vulgaris* ATCC 49990	→3)-β-D-Glc*p*NAc-(1→2)-α-D-Gal*p*4,6(*R*-Pyr)-(1→4)-β-D-Gal*p*-(1→	19
	P. hauseri 1732-80, 1086-80		b
	P. penneri 15, 49, 76, 91, 110		27

[a] This work.
[b] Authors' unpublished data.

The close serological relatedness of *P. hauseri* 1732--80, *P. vulgaris* ATTCC 49990, and *P. penneri* 15 was confirmed by EIA and PHT data (Table 2). Anti-*P. hauseri* 1732-80 serum markedly cross-reacted in EIA with the LPS of *P. mirabilis* CCUG 19011 and *P. vulgaris* CCUG 4654, whereas anti-*P. vulgaris* ATTCC 49990 serum cross-reacted much more weakly. *Vice versa*, anti-*P. mirabilis* CCUG 19011 and anti-*P. vulgaris* CCUG 4654 sera markedly cross-reacted with the LPS of *P. hauseri,* whereas the other heterologous LPSs showed a weak cross-reactivity. In PHT, cross--reactions in both ways with all LPSs were more pronounced (Table 3), which could be accounted for by a different exposure of the LPS in PHT and EIA.

While the homologous alkali-treated LPS removed practically all antibodies from the corresponding O--antisera, the heterologous LPS reduced antibody titers only insignificantly (data of PHT, Table 3). Similar results were obtained in inhibition of the reaction in PHT (Table 4). Strong inhibitors were only the homologous LPS and that with the structurally identical O-antigen (*P. penneri* 15 LPS in the *P. vulgaris* ATCC 49990 test system; Table 4). These data suggest that the cross-reactivity of the LPS from *P. vulgaris* CCUG 4654, *P. mirabilis* CCUG 19011 and *P. vulgaris* ATCC 49990 (*P. hauseri*, *P. penneri* 15) that was observed in direct tests is due to the presence of minor antigen epitopes, either on the O-antigen or on the LPS core, whereas the major epitopes on the O-antigens are different.

The serological data are in agreement with the structures of the O-polysaccharides (Table 5). Indeed, the O-polysaccharide structure of *P. vulgaris* CCUG 4654 (PrK 37/57, CNCTC U349) is quite different from those of the other strains studied. The O-polysaccharides of both *P. mirabilis* CCUG 19011 and *P. vulgaris* ATCC 49990 (*P. hauseri*, *P. penneri* 15) contain a pyruvic acid acetal that is attached to positions 4 and 6 of α-D-Gal*p*NAc and α-D-Gal*p*, respectively, but the size of the repeating units and the other components are different. Since it is not reasonable to classify strains with structurally and serologically different O-antigens in one O-serogroup, these data show the expediency of reclassifying *Proteus* strains that have been formerly classified in serogroup O19. We suggest keeping *P. vulgaris* CCUG 4654 (PrK 37/57, CNCTC U349) and *P. penneri* 31 in serogroup O19 as two subgroups O19a and O19a,19b as proposed earlier[24] and transferring *P. mirabilis* CCUG 19011 to another *Proteus* serogroup, O51 (Table 5). *P. vulgaris* ATCC 49990, *P. hauseri, P. penneri* 15 and some other serologically identical *P. penneri* strains should be classified in a new *Proteus* serogroup, O52, as well (Table 5).

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REFERENCES

1. Bartodziejska B., Shashkov A. S., Babicka D., Grachev A. A., Torzewska A., Paramonov N. A., Chernyak A. Y., Rozalski A. and Knirel Y. A. (1998): Structural and serological studies on a new acidic O-specific polysaccharide of *Proteus vulgaris* O32. Eur. J. Biochem., 256, 488–493.

2. Bartodziejska B., Shashkov A. S., Torzewska A., Grachev A. A., Ziółkowski A., Paramonov N. A., Rozalski A. and Knirel Y. A. (1999): Structure and serological specificity of a new acidic O-specific polysaccharide of *Proteus vulgaris* O45. Eur. J. Biochem., 259, 212–217.

3. Bock K. and Pedersen C. (1983): Carbon-13 nuclear magnetic resonance spectroscopy of monosaccharides. Adv. Carbohydr. Chem. Biochem., 41, 27–66.

4. Brenner D. J., Farmer III J. J., Fanning G. R., Steigerwalt A. G., Klykken P., Wathert H. G., Hickman F. W. and Ewing H. W. (1978): Deoxyribonucleic acid relatedness of *Proteus* and *Providencia* species. Int. J. Syst. Bacteriol., 28, 269–282.

5. Conrad H. E. (1972): Methylation of carbohydrates with methyl iodide in dimehyl sulfoxide in the presence of methylsulfinyl-anion. Methods Carbohydr. Chem., 6, 361–364.

6. Garegg P. J., Jansson P.-E., Lindberg B., Lindh F., Lunngren J., Kvarnstrum I. and Nimmich W. (1980): Configurations of the acetal carbon of pyruvic acid acetals in some bacterial polysaccharides. Carbohydr. Res., 78, 127–132.

7. Gerwig G. J., Kamerling J. P. and Vliegenthart J. F. G. (1978): Determination of the D and L configuration of neutral monosaccharides by high-resolution capillary g.l.c. Carbohydr. Res., 62, 349–357.

8. Hauser G. (1885): Über Fäulnisbakterien und deren Beziehung zur Septicämia. Vogel, Leipzig, p. 107.

9. Hickman F. W., Steigerwalt A. G., Farmer J. J. III and Brenner D. J. (1982): Identification of *Proteus penneri* sp. Now., formerly known as *Proteus vulgaris* indole negative or as *Proteus vulgaris* biogroup 1. J. Clin. Microbiol., 15, 1097–1102.

10. Jansson P.-E., Kenne L., Liedgren H., Lindberg B. and Lönngren J. (1976): A practical guide to the methylation analysis of carbohydrates. Chem. Commun. Univ. Stockholm, 8, 1–75.

11. Jansson P.-E., Kenne L. and Widmalm G. (1989): Computer-assisted structural analysis of polysaccharides with an extended version of CASPER using ^{1}H- and ^{13}C-N.M.R. data. Carbohydr. Res., 188, 169–191.

12. Knirel Y. A., Kaca W., Rozalski A. and Sidorczyk Z. (1999): Structure of O-antigenic polysaccharides of *Proteus* bacilli. Pol. J. Chem., 73, 859–907.

13. Kondakova A. N., Zych K., Senchenkova S. N., Zablotni A., Shashkov A. S., Knirel Y. A. and Sidorczyk Z. (2003): Structure of O-polysaccharide leads to classification of *Proteus penneri* 31 in *Proteus* serogroup O19. FEMS Immunol. Med Microbiol., 39, 73–79.

14. Larsson P. (1984): Serology of *Proteus mirabilis* and *Proteus vulgaris*. Methods Microbiol., 14, 187–214.

15. Leontein K., Lindberg B. and Lönngren J. (1978): Assignment of absolute configuration of sugars by g.l.c. of their acetylated glycosides formed from chiral alcohols. Carbohydr. Res., 62, 359–362.

16. O'Hara C. M., Brenner F. W. and Miller J. M. (2000): Classification, identification, and clinical significance of *Proteus*, *Providencia*, and *Morganella*. Clin. Microbiol. Rev., 13, 534–546.

17. O'Hara C. M., Brenner F. W., Steigerwalt A. G., Hill B. C., Holmes B., Grimont P. A. D., Hawkey P. M., Penner J. L., Miller J. M. and Brenner D. J. (2000): Classification of *Proteus vulgaris* biogroup 3 with the recognition of *Proteus hauseri* sp. Nov. nom. Re. and unnamed *Proteus* genomospecies. Int. J. Syst. Evol. Microbiol., 50, 1869–1875.

18. Penner J. L. and Hennessy J. N. (1980): Separate O-grouping schemes for serotyping clinical isolates of *Proteus vulgaris* and *Proteus mirabilis*. J. Clin. Microbiol., 12, 77–82.

19. Perry M. B. and MacLean L. L. (1994): The structure of the polysaccharide produced by *Proteus vulgaris* (ATCC 49990). Carbohydr. Res., 253, 257–263.

20. Rozalski A. (2002): Molecular basis of the pathogenicity of *Proteus* bacteria. Adv. Clin. Exp. Med., 11, 3–18.

21. Shashkov A. S., Senchenkova S. N., Nazarenko E. L., Zubkov V. A., Gorshkova N. M., Knirel Y. A. and Gorshkova R. P. (1997): Structure of a phosphorylated polysaccharide from *Shewanella putrefaciens* strain S29. Carbohydr.Res., 303, 333–338.

22. Torzewska, A., Kondakova A. N., Perepelov A. V., Senchenkova S. N., Shashkov A. S., Rozalski A. and Knirel Y. A. (2001): Structure of the O-specific polysaccharide from *Proteus vulgaris* O37 and close serological relatedness of the lipopolysaccharides of *P. vulgaris* O37 and *P. vulgaris* O46. FEMS Immunol. Med. Microbiol., 31, 227–234.

23. Toukach F. V., Kondakova A. N., Arbatsky N. P., Senchenkova S. N., Shashkov A. S., Knirel Y. A., Zych K., Rozalski A. and Sidorczyk Z. (2002): New structures of the O-specific polysaccharides of bacteria of the genus *Proteus.* Part 1. Phosphate-containing polysaccharides. Biochemistry, 67, 265–276.

24. Vinogradov E. V., Kaca W., Knirel, Y. A., Różalski A. and Kochetkov N. K. (1989): Structural studies on the fucosamine containing O-specific polysaccharide of *Proteus vulgaris* O19. Eur. J. Biochem., 180, 95–99.

25. Westphal O. and Jann K. (1965): Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem., 5, 83–91.

26. Zych K., Kowalczyk M., Knirel Y.A. and Sidorczyk Z. (2000): New serogroups of the genus *Proteus* consisting of *Proteus penneri* strains only. Adv. Exp. Med. Biol., 485, 339–344.

27. Zych K, Kowalczyk M., Toukach F. V., Paramonov N. A., Shashkov A. S., Knirel Y. A. and Sidorczyk Z. (1997): Structural and immunochemical studies on the O-specific polysaccharide of *Proteus penneri* strain 15. Arch. Immunol. Ther. Exp., 45, 435–441.