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Structure of the O-specific polysaccharide of *Proteus vulgaris* O4 containing a new component of bacterial polysaccharides, 4,6-dideoxy-4-{*N*-[(*R*)-3-hydroxybutyryl]-L-alanyl}amino-D-glucose

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Abstract

A high-molecular-mass O-specific polysaccharide was obtained by mild acid degradation of *Proteus vulgaris* O4 lipopolysaccharide followed by GPC. The polysaccharide was studied by chemical methods along with ^{1}H and ^{13}C NMR spectroscopy, including two-dimensional COSY, TOCSY, NOESY, H-detected $^{1}H,^{13}C$ HMQC, and $^{1}H,^{13}C$ HMBC experiments. Solvolysis of the polysaccharide with trifluoromethanesulfonic (triflic) acid resulted in a Glc*p*A-(1→3)-GlcNAc disaccharide and a novel amino sugar derivative, 4,6-dideoxy-4-{*N*-[(*R*)-3-hydroxybutyryl]-L-alanyl}amino-D-glucose [Qui4N(HbAla)]. On the basis of the data obtained, the following structure of the tetrasaccharide repeating unit of the O-specific polysaccharide was established:

→4)-β-D-Glc*p*A-(1→3)-β-D-Glc*p*NAc-(1→2)-β-D-Qui*p*4N(HbAla)-(1→3)-α-D-Gal*p*-(1→

This structure is unique among the O-specific polysaccharides, which is in accordance with classification of the strain studied in a separate *Proteus* serogroup.

Keywords: *Proteus vulgaris*; Lipopolysaccharide; O-Antigen structure; Bacterial polysaccharide; 4,6-Dideoxy-4-{*N*-[(*R*)-3-hydroxybutyryl]-L-alanyl}amino-D-glucose; Triflic acid solvolysis; Selective cleavage

1. Introduction

Bacteria of the genus *Proteus* are a common cause of urinary tract infections, which can lead to severe complications, such as acute or chronic pyelonephritis and formation of bladder and kidney stones. Potential virulence factors of *Proteus* mediating the infectious processes are fimbriae, flagella, urease, proteases, hemolysins, invasiveness, capsular polysaccharide, and lipopolysaccharide (LPS).[1,2] The polysaccharide chain (O-antigen)

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of the LPS defines the serological O-specificity of the bacteria. Based on the immunospecificity of the O-antigens, two species, *Proteus mirabilis* and *Proteus vulgaris*, were classified into 60 O-serogroups.[3,4]

In most *P. mirabilis* and *P. vulgaris* O-serogroups studied so far, the O-specific polysaccharides contain acidic or both acidic and basic components, such as uronic acids, their amides with amino acids, and other non-sugar groups.[5] We now report the structure of a new acidic O-specific polysaccharide of *P. vulgaris* O4 containing D-glucuronic acid and an unusual *N*-acyl derivative of a 6-deoxy amino sugar.

2. Results and discussion

The O-specific polysaccharide was obtained by mild acid degradation of LPS isolated from bacterial cells of *P. vulgaris* O4 by the phenol–water procedure[6] followed by GPC on Sephadex G-50. After acid hydrolysis of the polysaccharide, chemical analyses using sugar and amino acid analyzers revealed the presence of galactose, 2-amino-2-deoxyglucose, another amino sugar that was identified as 4-amino-4,6-dideoxyglucose (Qui4N, see below), and alanine. After methanolysis of the polysaccharide and acetylation, glucuronic acid was identified by GLC. Analysis by GLC of the acetylated glycosides with (*S*)-2-butanol (for GlcN and GlcA) and (*R*)-2-octanol (for Gal) derived from the polysaccharide hydrolysate showed that all sugars have the D configuration. The absolute configuration of Qui4N was determined as D by analysis of the glycosylation effects in the ^{13}C NMR spectrum (see below). The L configuration of alanine was demonstrated by GLC of the acetylated (+)-2-octyl ester. GLC of the trifluoroacetylated (+)-2-octyl ester revealed the presence of (*R*)-3-hydroxybutyric acid in the polysaccharide hydrolysate.

The composition of the polysaccharide was further studied by GLC–MS of the N,O-methylated alditols[7] derived by acid hydrolysis, N-acetylation, borohydride reduction, and methylation (Fig. 1). Together with the products from Gal, GlcN and GlcA, three products from Qui4N were detected. Their molecular masses 277, 362, and 420 Da, determined by chemical ionization MS, corresponded to *N*-acetyl, *N*-(*N*-acetylalanyl), and *N*-[*N*-(3-hydroxybutyryl)alanyl] derivatives [Qui4NAc, Qui4N(AcAla), and Qui4N(HbAla), respectively]. In the electron impact mass spectrum, Qui4NAc showed a characteristic fragmentation giving the C-1,2,3,4 and C-4,5,6 primary ions at m/z 218 and 144, respectively. In contrast, two other derivatives containing alanine afforded no fragments of this kind, but the amino acyl cations $AcN(Me)CH(Me)CO^+$ and $Me\text{-}CH(OMe)CH_2CON(Me)CH(Me)CO^+$ at m/z

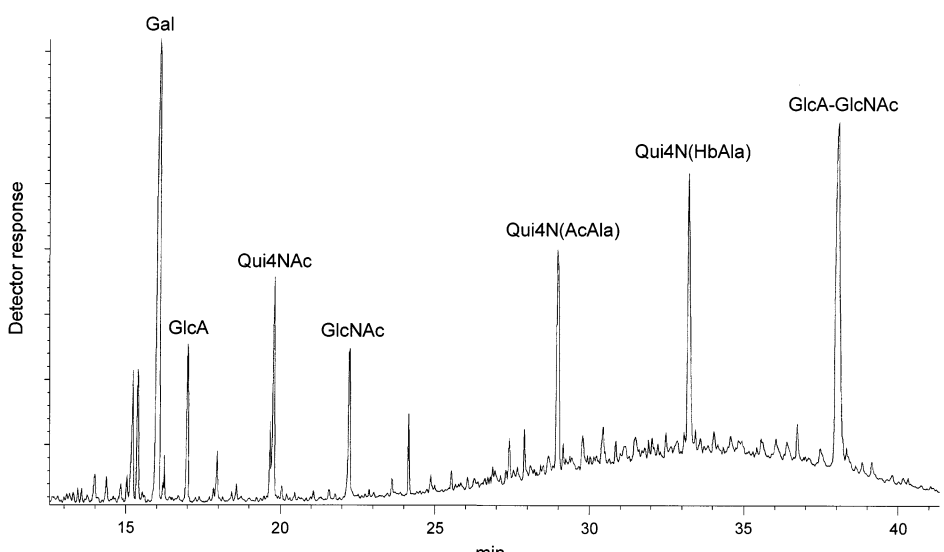
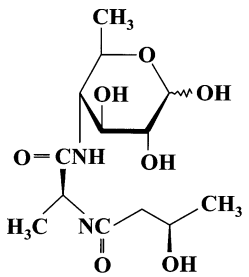


Fig. 1. GLC profile of the *N,O*-methylated alditols derived from the O-specific polysaccharide of *P. vulgaris* O4.



Scheme 1. Structure of the novel amino sugar derivative isolated from the O-specific polysaccharide of *P. vulgaris* O4 by triflic acid solvolysis.

128 and 186, respectively. These data suggested that the polysaccharide contains 4,6-dideoxy - 4 - [*N* - (3 - hydroxybutyryl)alanyl]-aminoglucose (Scheme 1), which partially lost the 3-hydroxybutyryl and *N*-(3-hydroxybutyryl)alanyl groups in the course of acid hydrolysis. In addition to the monosaccharides, a derivative of a GlcA → GlcNAc disaccharide was present, which was identified by a pseudomolecular ion $[M+H]^+$ at m/z 526 in the chemical ionization mass spectrum and fragment ions for the GlcA and reduced GlcNAc (GlcNAc-ol) moieties at m/z 233 and 276, respectively, in the electron impact mass spectrum.[8]

To confirm the attachment of alanine and the 3-hydroxybutyryl group to Qui4N, the polysaccharide was cleaved using anhydrous triflic acid. Separation of solvolysis products by GPC resulted in a monosaccharide and an oligosaccharide. Negative ion mode ESI MS of the monosaccharide revealed an $[M-H]^-$ pseudomolecular ion at m/z 319.26 corresponding to a Qui4N derivative containing one alanyl and one 3-hydroxybutyryl group (calculated molecular mass 320.16 Da). The ^{1}H and ^{13}C NMR spectra of the monosaccharide, assigned using 2D ^{1}H,^{1}H COSY and ^{1}H,^{13}C HMQC experiments (Tables 1 and 2), contained all expected signals for this compound, including signals for anomeric atoms (α-Qui4N and β-Qui4N at δ_H 5.39 and 4.79, δ_C 93.3 and 97.0, respectively), methyl groups of QuiN, Ala and Hb [δ_H 1.30–1.59, δ_C 18.1 (2C) and 23.2], carbons bearing nitrogen at δ 55.3, 58.1 and 58.0 (C-2 of Ala and C-4 of α-Qui4N and β-Qui4N), C-2 and C-3 of Hb at δ 45.6 and 66.3, respectively. Both ESI mass spectrum and NMR spectra showed a GlcNAc contamination, which could not be removed by GPC.

ESI MS of the oligosaccharide obtained by solvolysis of the polysaccharide with triflic acid, followed by borohydride reduction

Table 1
^{1}H NMR data (δ, ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6	CH_3CO
O-specific polysaccharide							
→4)-β-D-Glc*p*A-(1→	4.54	3.41	3.81	3.83	3.93		
→3)-β-D-Glc*p*NAc-(1→	4.91	3.85	3.77	3.51	3.52	3.76 [a]	2.04
→2)-β-D-Qui*p*4N-(1→	4.71	3.62	3.58	3.60	3.62	1.27	
→3)-α-D-Gal*p*-(1→	5.53	4.06	3.95	4.17	3.95	3.69	
(*R*)-3-Hydroxybutyryl		2.45	4.20	1.25			
L-Alanyl		4.33	1.40				
Disaccharide (reduced)							
β-D-Glc*p*A-(1→	4.55	3.38	3.54	3.52	3.75		
→3)-D-GlcNAc-ol	3.63 [b]	4.26	4.15	3.56	3.87	3.67 [c]	2.05
Monosaccharide							
α-D-Qui4N	5.39	3.60	3.74	3.58	3.97	1.30	
β-D-Qui4N	4.79	3.48	3.54	3.58	3.58	1.34	
(*R*)-3-Hydroxybutyryl			2.63	4.21	1.41		
L-Alanyl			4.29	1.59			

[a] H-6a; H-6b at δ 3.94.
[b] H-1a; H-1b at δ 3.84.
[c] H-6a; H-6b at δ 3.74.

Table 2
^{13}C NMR data (δ, ppm)

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	CH_3CO	CH_3CO
O-specific polysaccharide								
→4)-β-D-Glc*p*A-(1→	104.4	74.4	77.6	78.4	77.3	175.3		
→3)-β-D-Glc*p*NAc-(1→	103.1	56.7	84.0	70.4	77.3	62.7	23.7	175.9
→2)-β-D-Qui*p*4N-(1→	103.6	83.3	72.3	58.6	75.3	18.5		
→3)-α-D-Gal*p*-(1→	99.9	69.2	81.0	70.3	72.3	62.4		
(*R*)-3-Hydroxybutyryl	174.9	45.5	65.5	24.1				
L-Alanyl	176.8	51.5	18.5					
Disaccharide (reduced)								
β-D-Glc*p*A-(1→	103.5	74.2	76.6	72.9	75.9			
→3)-D-GlcNAc-ol	64.2	54.5	76.1	71.8	71.4	61.8	23.4	175.6
Monosaccharide								
α-D-Qui4N	93.3	73.3	71.4	58.1	67.7	18.1		
β-D-Qui4N	97.0	76.0	74.5	58.0	72.2	18.1		
(*R*)-(3-Hydroxybutyryl)	175.0	45.6	66.3	23.2				
L-Alanyl	176.4	55.3	18.1					

demonstrated a reduced disaccharide of GlcA and GlcNAc (the experimental and calculated molecular masses are 399.15 and 399.14 Da, respectively). Assignment of the ^{1}H NMR signals for this compound was performed using COSY and TOCSY experiments, tracing connectivities starting from H-1 of a β-linked sugar residue at δ 4.55 (d, $J_{1,2} \sim 8$ Hz) and H-1a,1b of an alditol residue at δ 3.63 and 3.84 (Table 1). The ^{13}C NMR spectrum that was assigned using a ^{1}H,^{13}C HMQC experiment (Table 2), showed that the sugar is GlcA (C-1 at δ 103.5, C-2 at δ 74.2, C-6 at δ 175.6) and the alditol is GlcNAc-ol (C-1 and C-6 at δ 64.2 and 61.8, C-2 at δ 54.5). A low-field position of the signal for C-3 (δ 76.1) indicated substitution of the alditol at position 3 and, hence, the reduced disaccharide has the structure of β-D-Glc*p*A-(1→3)-D-GlcNAc-ol.

Formation, from the polysaccharide, of the GlcA→GlcNAc disaccharide as the only oligosaccharide product showed that triflic acid cleaved completely the glycosidic linkages of Gal, GlcNAc and Qui4N(HbAla), whereas the linkage of GlcA was essentially stable. The latter was partially stable also during acid hydrolysis, which resulted in retention of GlcA and GlcNAc as a disaccharide (see above). Unlike hydrolysis, solvolysis with triflic acid did not affect amidic linkages which allowed isolation and concomitant identification of the complex *N*-acyl derivative of Qui4N. Earlier, solvolysis with triflic acid was applied to the isolation of an amide of D-GalA with N^{ε}-[(*R*)-1-carboxyethyl]-L-lysine from the O-specific polysaccharide of *Proteus mirabilis* O13[9] and to the selective cleavage of glycosidic linkages in various bacterial polysaccharides.[7,10]

Methylation analysis of the polysaccharide revealed the presence of 2,4,6-tri-*O*-methylgalactose, 4,6-dideoxy-3-*O*-methyl-4-(*N*-methyl)acetamidoglucose, and 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methyl)acetamidoglucose. When the methylated polysaccharide was carboxyl-reduced prior to hydrolysis, 2,3-di-*O*-methylglucose was identified in addition to the sugars mentioned above, which was evidently derived from GlcA. Therefore, the polysaccharide is linear and contains a 2-substituted residue of Qui4N, 3-substituted residues of GlcN and Gal, and a 4-substituted GlcA residue.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 2) was typical of a regular polymer. It contained signals for four anomeric carbons at δ 99.9–104.4, two carbons bearing nitrogen (C-2 of GlcN and C-4 of Qui4N) at δ 56.7 and 58.6, two $HOCH_2$–C groups (C-6 of GlcN and Gal) at δ 62.7 and 62.4, respectively, four CH_3–C groups (C-6 of Qui4N, C-3 of Ala, C-2 of Ac, and C-4 of Hb) at δ 18.5 (2C), 23.6, and 24.0, four CO groups (C-6 of GlcA, C-1 of Ala, Ac and Hb) at δ 175.3–176.8, and

15 other carbons linked to oxygen (sugar ring carbons and C-3 of Hb) at δ 65.5–84.0.

Accordingly, the ^{1}H NMR spectrum of the polysaccharide contained signals for four anomeric protons (δ 4.54–5.53), three CH_3–CH groups (δ 1.27–1.40, all d, $J_{1,2}$ 6–7 Hz; H-6 of Qui4N, H-3 of Ala, and H-4 of Hb), one *N*-acetyl group (δ 2.04, s), and other protons (δ 3.40–4.33). Therefore, the polysaccharide has a tetrasaccharide repeating unit containing one residue each of D-galactose, D-glucuronic acid, 2-acetamido-2-deoxy-D-glucose, and 4,6-dideoxy-4-{*N*-[(*R*)-3-hydroxybutyryl]-L-alanyl}amino-D-glucose.

The ^{1}H and ^{13}C NMR spectra of the polysaccharide were assigned using two-dimensional COSY, TOCSY, NOESY, and H-detected ^{1}H,^{13}C HMQC experiments (Tables 1 and 2), and spin systems of GlcA, GlcN, Qui4N, and Gal were identified. The coupling constant value of $J_{1,2} \sim 3$ Hz indicated that Gal is α-linked, whereas the values of $J_{1,2}$ 7–8 Hz for three other sugars showed that they are β-linked. The TOCSY spectrum contained cross-peaks of the corresponding H-1 signals with H-2,3,4 for Gal, H-2,3,4,5 for GlcA, and H-2,3,4,5,6 for GlcN. Signals for H-5,6 Gal were assigned by C-6/H-6 and H-6/H-5 correlations in the ^{1}H,^{13}C HMQC and COSY spectra, respectively. Signals of H-2,3,4,5 for Qui4N were not clearly resolved in the TOSCY spectrum and were assigned by correlations H-1/H-2 and H-5/H-6 in the COSY spectrum, C-4/H-4 and C-4/H-5 in the ^{1}H,^{13}C HMQC and HMBC spectra, respectively, and H-3/C-3 as the last remaining non-assigned cross-peak in the ^{1}H,^{13}C HMQC spectrum (Fig. 2).

A downfield displacement of the signals for C-2 of β-Qui4N, C-3 of GlcNAc and Gal, and C-4 of β-GlcA to δ 83.3, 84.0, 81.0, and 78.4 (Table 2), as compared with their position in the corresponding nonsubstituted monosaccharides at δ 74.4, 75.1, 74.0, and 71.74,[11,12] revealed the glycosylation pattern in the repeating unit. A NOESY experiment showed

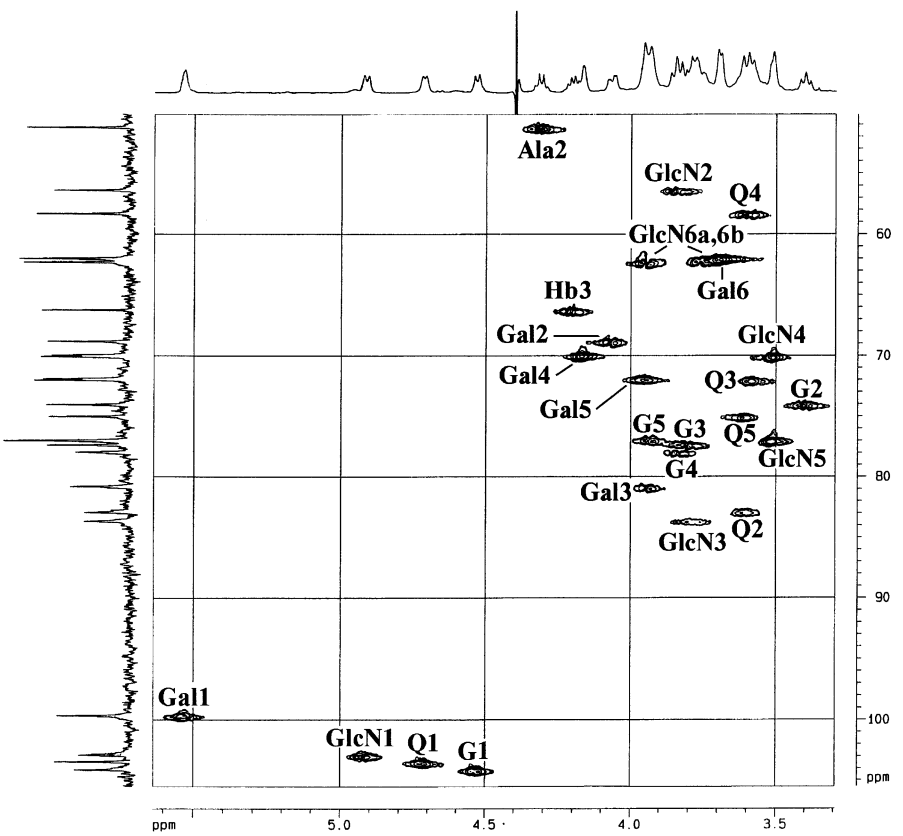


Fig. 2. Part of an H-detected ^{1}H,^{13}C HMQC spectrum of the O-specific polysaccharide of *P. vulgaris* O4. The corresponding parts of the ^{13}C and ^{1}H NMR spectra are displayed along the vertical and horizontal axes, respectively. Arabic numerals refer to atoms in sugar residues. G, GlcA; Q, Qui4N; Hb, 3-hydroxybutyryl.

Table 3
Serological reactivity of *Proteus* LPS with anti-*P. vulgaris* O4 serum (reciprocal titers)

Antigen	Enzyme immunosorbent assay	Passive hemolysis test
P. vulgaris O4	1,024,000	102,400
P. penneri 16	6,400	1,600
P. penneri 18	3,200	3,200
P. vulgaris O17	200	400
P. vulgaris O37	200	200
P. vulgaris O46	400	400

strong interresidue cross-peaks between the following transglycosidic protons: GlcA H-1, GlcNAc H-3, GlcNAc H-1,Qui4N H-2, Qui4N H-1,Gal H-3 and Gal H-1,GlcA H-4 at δ 4.53/3.77, 4.91/3.62, 4.71/3.95, and 5.53/3.83, respectively. Accordingly, the HMBC spectrum displayed the following expected cross-peaks between the anomeric protons and the linkage carbon atoms: GlcA H-1,GlcN C-3, GlcN H-1,Qui4N C-2, Qui4N H-1,Gal C-3, and Gal H-1,GlcA C-4 at δ 4.53/84.0, 4.91/83.3, 4.71/81.0, and 5.53/78.4, respectively. These data confirmed the modes of substitution of the monosaccharide residues and demonstrated their sequence in the repeating unit. A relatively low (by the absolute value) glycosylation effect on C-4 of D-Gal (-0.3 ppm) showed that the glycosylating β-Qui4N residue has the same, i.e. D configuration.[11]

Therefore, the O-specific polysaccharide of *P. vulgaris* O4 has the following structure:

→4)-β-D-Glc*p*A-(1→3)-β-D-Glc*p*NAc-(1→2)-β-D-Qui*p*4N(HbAla)-(1→3)-α-D-Gal*p*-(1→

To our knowledge, one of the polysaccharide components, namely 4,6-dideoxy-4-{*N*-[(*R*)-3-hydroxybutyryl]-L-alanyl}amino-D-glucose, has not been hitherto found in Nature. Interestingly, in bacterial polysaccharides Qui4N often carries unusual *N*-acyl groups, such as formyl, *N*-acetylglycyl, and 2,4-dihydroxy-3,3,4-trimethyl-5-oxoprolyl.[13] No Qui4N derivative has been found previously in *Proteus* polysaccharides, whereas L-alanyl, D-alanyl, and (*R*)-3-hydroxybutyryl groups have been reported as *N*-acyl substituents of D-GlcN, D-Qui3N, and D-Fuc3N, respectively.[5]

In serological studies, LPS from various *Proteus* O-serogroups were tested with rabbit polyclonal anti-*P. vulgaris* O4 serum. None of the LPS tested reacted, to a comparable extent with the homologous LPS, in enzyme immunosorbent assay and passive hemolysis (Table 3). However, a marked reactivity was observed for LPS of *P. penneri* strains 16 and 18, and a weak reactivity of *P. vulgaris* O17, O37, and O46. Accordingly, in Western blot the anti-*P. vulgaris* O4 serum recognized high- and low-molecular-mass species of the homologous LPS and high-molecular-mass LPS species from *P. penneri* 16 and 18 (marked reactivity), as well as those from *P. vulgaris* O17, O37, and O47 — which showed a weak reactivity (data not shown). These data suggested that the cross-reactive LPS shared a common epitope in the O-specific polysaccharide with LPS of *P. vulgaris* O4.

Comparison of the polysaccharide structures[14,15] (authors' unpublished data) indicated that they all contain a β-D-Glc*p*A-(1→3)-D-Glc*p*NAc disaccharide fragment which, most likely, is responsible for the serological cross-reactivity. However, data of Table 3 showed that only a minor fraction of anti-*P. vulgaris* O4 antibodies is specific to the common epitope, whereas the major fraction recognizes a unique epitope(s) on *P. vulgaris* O4 LPS. An attempt to confirm that the major epitope is associated with the bulky D-Qui4N(HbAla) residue failed since the isolated D-Qui4N(HbAla) monosaccharide did not inhibit the reaction of anti-*P. vulgaris* O4 with the homologous LPS in ELISA.

The unique structure of the O-specific polysaccharide and the present serological data substantiated classification of *P. vulgaris* strain PrK 9/57 into a separate *Proteus* serogroup.

3. Experimental

Bacterial strain, isolation and degradation of LPS.—*P. vulgaris* O4 (strain PrK 9/57) came from the Czech National Collection of Type

Cultures (Institute of Epidemiology and Microbiology, Prague). It was cultivated under aerobic conditions in nutrient broth (BTL, Łodz, Poland), and bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with water, and lyophilized. LPS was isolated from dried bacterial cells by hot phenol–water extraction[6] and purified by treatment with DNAse and RNAse and ultracentrifugation as described.[16]

Degradation of LPS (100 mg) was performed with aq 2% HOAc at 100 °C until lipid precipitated. The precipitate was removed by centrifugation (13,000*g*, 20 min), and the supernatant was fractionated by GPC on a column of Sephadex G-50 in 0.05 M pyridinium acetate buffer pH 4.5, monitored with a Knauer differential refractometer. The yield of the high-molecular-mass polysaccharide was 24% of the LPS weight.

Composition of the O-specific polysaccharide.—The polysaccharide was hydrolyzed with 2 M CF_3COOH (120 °C, 2 h). Amino sugars were identified using a Biotronik LC-2000 amino acid analyzer equipped with a column (0.4×22 cm) of Ostion LG AN B cation-exchange resin. Neutral sugars were identified using a Biotronik LC-2000 sugar analyzer on a column (0.4×15 cm) of Dionex A × 8 anion-exchange resin using 0.5 M sodium borate buffer pH 8.0 at 65 °C. Uronic acids were analyzed by GLC as acetylated methyl glycosides prepared by methanolysis of the polysaccharide with 1 M HCl in MeOH (80 °C, 16 h) followed by acetylation with 1:1 pyridine–Ac_2O (80 °C, 2 h). *N*,*O*-Methylated alditols for GLC–MS analysis were prepared by hydrolysis of the polysaccharide with 4 M HCl (65 °C, 4 h) followed by N-acetylation with acetic anhydride in satd aq $NaHCO_3$, conventional reduction with $NaBH_4$, and methylation.[17] The absolute configurations of the monosaccharides,[18–20] alanine,[21] and 3-hydroxybutyric acid[22] were determined as described.

Methylation analysis.—Methylation of the polysaccharide was performed with CH_3I in Me_2SO in the presence of sodium methylsulfinylmethanide.[17] A portion of the methylated polysaccharide was reduced with $LiBH_4$ in aq 70% 2-propanol (20 °C, 2 h). Partially methylated monosaccharides were derived by hydrolysis with 2 M CF_3COOH, converted into the alditol acetates, and analyzed by GLC–MS as in sugar analysis.

Triflic acid solvolysis.—The polysaccharide (15 mg) was treated with triflic acid (0.5 mL) at −4 °C for 1 h in anhydrous conditions. After neutralization with aq 25% ammonia at −4 °C, the reaction products were fractionated by GPC on TSK HW-40 (S) to give a disaccharide (2.8 mg) and a monosaccharide (1.1 mg).

GLC, GLC–MS and ESI MS.—GLC was performed with a Hewlett–Packard model 5890 chromatograph equipped with a DB-5 capillary column (Hewlett–Packard) using a temperature gradient of 100 (for Ala and Hb) or 160 °C (for sugars) (1 min) to 290 °C at 3 °C/min. GLC–MS was performed in the electron impact and chemical ionization (ammonia) modes on a Hewlett–Packard 5989A instrument on a HP-5 column or a Hewlett Packard 5890 chromatograph equipped with a NERMAG R10-10L mass spectrometer using the same chromatographic conditions as in GLC.

ESI MS was performed in the negative ion mode using a VG Quattro triple quadrupole mass spectrometer (Micromass, Altrincham) with MeCN as the mobile phase at a flow rate of 10 μL/min. Samples were dissolved in aq 50% MeCN at a concentration about 50 pmol/μL, and 10 μL was injected via a syringe pump into the electrospray source.

NMR spectroscopy.—NMR spectra were recorded with a Bruker DRX-500 spectrometer for a solution in D_2O at 60, 30, and 27 °C for the polysaccharide, disaccharide and monosaccharide, respectively. Internal acetone (δ_H 2.225, δ_C 31.45) served as reference. Standard Bruker software (XWINNMR 1.2) was used to acquire and process the NMR data. A mixing time of 200 and 100 ms was used in two-dimensional TOCSY and NOESY experiments. A delay of 60 ms was used in the HMBC experiment.

Serological techniques.—Rabbit polyclonal anti-*P. vulgaris* O4 serum was obtained as described.[23] Serological tests, inhibition, and Western blot were performed as described previously.[23] LPS and alkali-treated LPS were

used as antigen in enzyme immunosorbent assay and passive hemolysis test, respectively.

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