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Structure and serological characterization of the O-antigen of *Proteus mirabilis* O18 with a phosphocholine-containing oligosaccharide phosphate repeating unit

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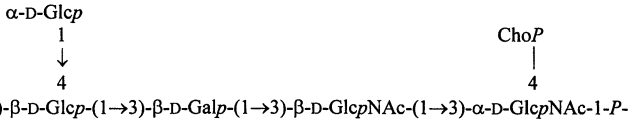
Abstract

A phosphorylated, choline-containing polysaccharide was obtained by O-deacylation of the lipopolysaccharide (LPS) of *Proteus mirabilis* O18 by treatment with aqueous 12% ammonia, whereas hydrolysis with dilute acetic acid resulted in depolymerisation of the polysaccharide chain by the glycosyl phosphate linkage. Treatment of the O-deacylated LPS with aqueous 48% hydrofluoric acid cleaved the glycosyl phosphate group but, unexpectedly, did not affect the choline phosphate group. The polysaccharide and the derived oligosaccharides were studied by NMR spectroscopy, including 2D ^{1}H,^{1}H COSY, TOCSY, ROESY, ^{1}H,^{13}C HMQC and HMQC–TOSCY experiments, along with chemical methods, and the following structure of the pentasaccharide phosphate repeating unit was established:



Where ChoP = Phosphocoline Immunochemical studies of the LPS, O-deacylated LPS and partially dephosphorylated pentasaccharide using rabbit polyclonal anti-*P. mirabilis* O18 serum showed the importance of the glycosyl phosphate group in manifesting the serological specificity of the O18-antigen.

Keywords: Lipopolysaccharide; O-Polysaccharide; Glycosyl phosphate; Phosphocholine; *Proteus mirabilis*

1. Introduction

Proteus mirabilis is a one of the important human facultative pathogens that frequently cause urinary tract and wound infections,[1] which can lead to acute and chronic pyelonephritis, bacteriemia, and formation of bladder and kidney stones.[2–4] Surface antigens of *P. mirabilis* have been suggested to play a role in rheumatoid arthritis.[5–7] Lipopolysaccharide (LPS) is the major component of the outer membrane of the cell envelope and is considered as one of the virulent factors of *Proteus*.[1,2] The O-polysaccharide chain of the LPS (O-antigen) defines the immunospecificity of the bacterium.

Aiming at establishing the molecular basis for serological classification and cross-reactivity of *Proteus* strains, we have determined structures of the O-polysaccharides of various *Proteus* O-serogroups.[8] Many of them are phosphorylated and contain phosphate-linked polyalcohols or amino alcohols.[8,9] In this paper, we

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report on the unique structure of the O-polysaccharide of *P. mirabilis* O18, which has a phosphocholine-containing oligosaccharide phosphate repeating unit, and on a possible role of phosphorylated groups in manifesting the serological specificity of the O18-antigen.

2. Results and discussion

2.1. Chemical studies

The LPS was obtained by phenol–water extraction of dry bacterial cells of *P. mirabilis* O18. Mild acid degradation of the LPS with dilute acetic acid at 100 °C gave only oligosaccharides and no polysaccharide. Therefore, for structural studies the LPS was treated with aqueous 12% ammonia to give a high-molecular-mass product (LPS-OH), which represents an O-polysaccharide linked to the core-O-deacylated lipid A moiety. In addition, in order to ensure that that no alkali-labile substituent was present but lost upon O-deacylation, the LPS was delipidated by deamination with nitrous acid, which cleaved the glycosidic linkage of a 2-amino-2-deoxyhexose present in the outer region of the LPS core to release the polysaccharide portion.[10] The ^{13}C NMR spectra of both polymers were essentially identical in respect to sugar signals, and therefore only the LPS-OH was studied further.

The ^{13}C NMR spectrum of the LPS-OH (Fig. 1) contained signals for five anomeric carbons δ 94.8–104.8, three nitrogen-bearing carbons at δ 54.6–56.0, seven signals for CH_2 groups at δ 61.0–62.1 and 66.0 and 19 signals for sugar-ring oxygen-bearing carbons in the region δ 67.5–84.2. Signals at δ 23.6 (2 C, CH_3), 174.9 and 175.5 (both CO) belonged to two *N*-acetyl groups, and an intense signal at δ 55.3 (3 C, CH_3), together with two additional signals for CH_2 groups at δ 61.0 and 67.5 that were split due to coupling to phosphorus, indicated the presence of choline (compare published data[11]). The ^{1}H NMR spectrum of the LPS-OH contained, inter alia, signals for five anomeric protons at δ 4.51–5.39, two *N*-acetyl groups at δ 2.05 and 2.05 (each 3 H, s), and methyl groups of choline at δ 3.23 (9 H, s).[11] In the ^{31}P NMR spectrum of the LPS-OH, there were two signals for phosphate groups at δ −1.7 and −2.0. Therefore, the O-polysaccharide has a pentasaccharide repeating unit containing three hexose residues, two *N*-acetylhexosamine residues, a choline residue and two phosphate groups Fig. 2.

The ^{1}H and ^{13}C NMR spectra of the LPS-OH were assigned using 2D ^{1}H,^{1}H COSY, TOCSY, ROESY, H-detected ^{1}H,^{13}C HMQC and ^{1}H,^{13}C HMQC–TOCSY experiments (Tables 1 and 2), and spin systems for one Gal*p* residue and two residues each of Glc*p* and Glc*p*NAc were revealed. The TOCSY spectrum showed correlations of H-1 with H-2–H-4 for Gal and H-2–H-5 for the other sugar residues. Spin systems of Glc*p* and Glc*p*NAc were identified by relatively large $J_{2,3}$, $J_{3,4}$ and $J_{4,5}$ coupling constants of 8–10 Hz estimated from the 2D NMR spectra, and that of Gal*p* was distinguished by significantly lower $J_{3,4}$ and $J_{4,5}$ values (<3 Hz). The GlcNAc residues were demonstrated by H-2/C-2 correlations at δ 4.10/56.6 and 3.75/56.0 observed in the ^{1}H,^{13}C HMQC spectrum. The identity of the monosaccharides was confirmed by sugar analysis (see below).

The residues of Glc and GlcNAc were designated as Glc^{I}, Glc^{II}, Glc*p*NAc^{I} and Glc*p*NAc^{II} according to their positions in the repeating unit determined in the course of further structural studies (see structure **1** in Fig. 3). Low-field positions of the H-1 signals at δ 5.39 and 5.37, together with $J_{1,2}$ coupling constant values of $\sim$3 Hz,

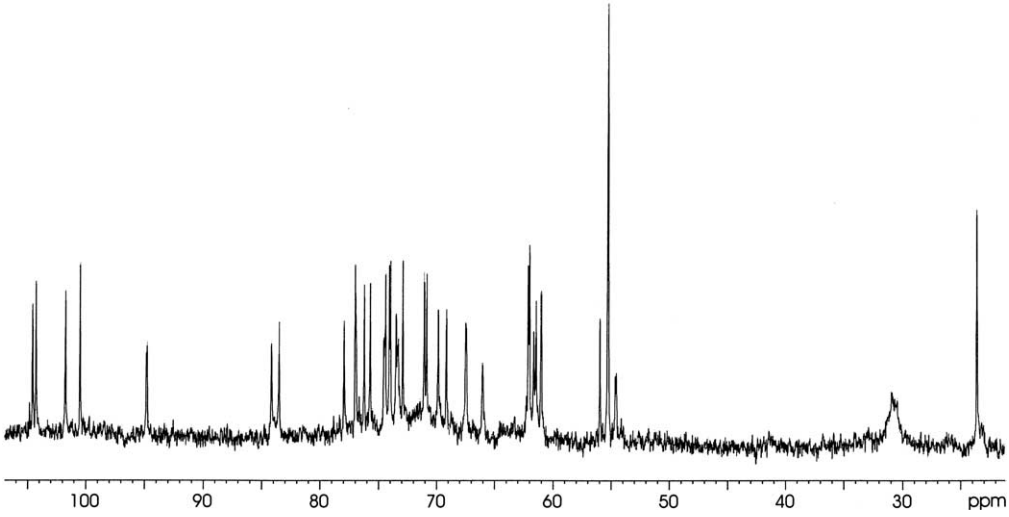


Fig. 1. ^{13}C NMR (90 MHz) spectrum of the LPS-OH obtained by alkaline treatment of the LPS of *P. mirabilis* O18. The region of signals for CO groups is not shown.

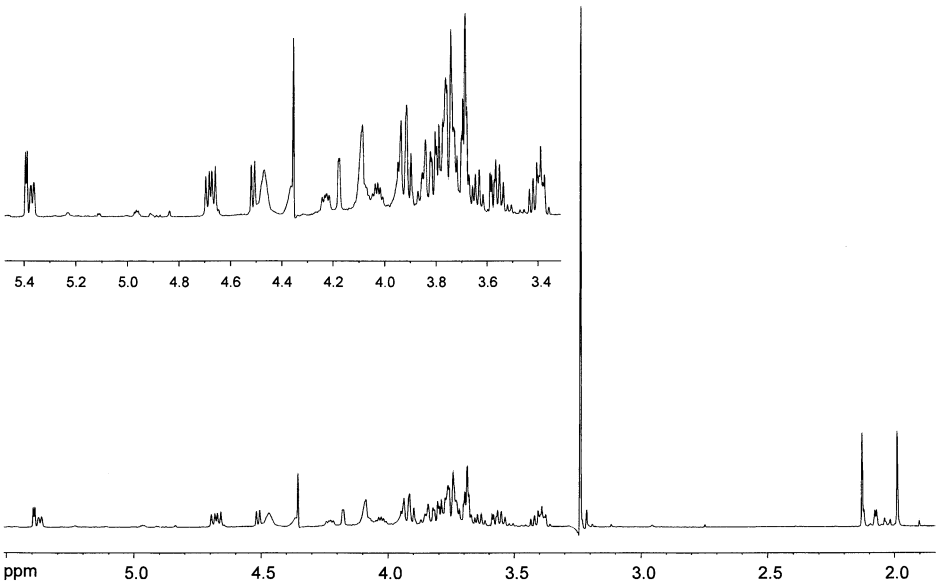


Fig. 2. ^{1}H NMR (600 MHz) spectrum of the LPS-OH obtained by alkaline treatment of the LPS of *P. mirabilis* O18.

indicated that Glc*p*^{II} and Glc*p*NAc^{I} are α-linked, whereas high-field positions of the H-1 signals at δ 4.51–4.69 and larger $J_{1,2}$ coupling constant values of ~ 7 Hz showed that the three other monosaccharides are β-linked. The anomeric configurations were confirmed by a ROESY experiment, which revealed characteristic H-1/H-2 correlations for α-Glc*p*^{II} and α-Glc*p*NAc^{II} and H-1/H-3 and H-1/H-5 correlations for β-Gal*p*, β-Glc*p*^{I} and β-Glc*p*NAc^{I}.

Low-field displacements in the ^{13}C NMR spectrum of the signals for C-3 of Gal and GlcNAc^{II} to δ 83.5 (+ 9.7) and δ 84.2 (+9.2), C-3 and C-4 of GlcNAc^{I} to 76.6 and 73.4 (+4.9 and +2.3), C-4 and C-6 of Glc^{I} to δ 78.0 (+7.3) and 66.0 (+4.2), respectively, as compared with

Table 1
^{1}H NMR chemical shifts (δ)

Sugar residue	H-1 (H-1a,1b)	H-2	H-3	H-4	H-5	H-6a,6b	CH_3CO
Polysaccharide **1**							
→3,4)-α-Glc*p*NAc^{I}-1-*P*-	5.37	4.10	4.08	4.08	3.95	3.84, 3.93	2.05
→3)-β-Glc*p*NAc^{II}-(1→	4.69	3.75	3.85	3.55	3.38	3.76, 3.92	2.05
→3)-β-Gal*p*-(1→	4.51	3.67	3.75	4.07	3.73	3.75, 3.81	
→4,6)-β-Glc*p*^{I}-(1→	4.67	3.42	3.79	3.64	3.70	4.02, 4.22	
α-Glc*p*^{II}-(1→	5.39	3.58	3.70	3.38	3.74	3.75, 3.89	
Cho*P*	4.48	3.69	3.23 [a]				
Oligosaccharide **2**							
→3,4)-GlcNAc-ol	3.61, 3.66	4.25	4.26	4.31	3.97	3.63, 3.87	2.01
→3)-β-Glc*p*NAc-(1→	4.65	3.85	3.84	3.54	3.50	3.72, 3.92	2.06
→3)-β-Gal*p*-(1→	4.51	3.65	3.78	4.17	3.70	3.70, 3.73	
→4)-β-Glc*p*^{I}-(1→	4.67	3.38	3.76	3.65	3.55	3.77, 3.89	
α-Glc*p*^{II}-(1→	5.39	3.55	3.66	3.39	3.68	3.72, 3.83	
Cho*P*	4.34	3.64	3.23 [a]				
Oligosaccharide **3**							
→3,4)-α-Glc*p*NAc^{I}	5.03	4.11	4.12	4.10	4.03	3.83, 3.92	2.04
→3)-β-Glc*p*NAc^{II}-(1→	4.70	3.75	3.82	3.55	3.42		2.02
→3)-β-Gal*p*-(1→	4.52	3.70	3.82	4.22	3.75		
→4,6)-β-Glc*p*^{I}-(1→	4.70	3.43	3.82	3.71	3.75	4.09, 4.21	
α-Glc*p*^{II}-(1→	5.42	3.60	3.72	3.44	3.76	3.79, 3.92	
Cho*P*	4.43	3.70	3.23 [a]				

[a] The signal for the *N*-methyl groups (9H).

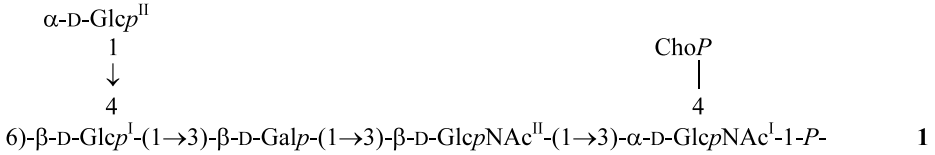
Table 2
^{13}C NMR chemical shifts (δ)

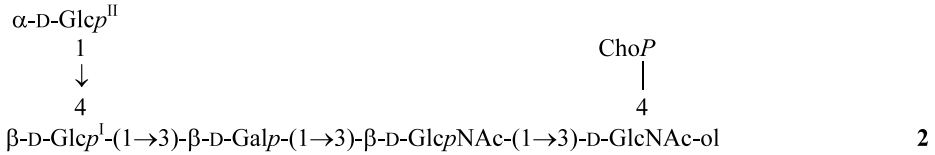
Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	CH_3CO	CH_3CO
Polysaccharide **1**								
→3,4)-α-Glc*p*NAc^{I}-1-*P*-	94.8 [a]	55.6 [a]	76.6	73.4 [a]	73.6	61.7	174.9 [b]	23.6
→3)-β-GlcpNAc^{II}-(1→	101.8	56.0	84.2	69.9	75.7	61.5	175.5 [b]	23.6
→3)-β-Gal*p*-(1→	104.5	71.1	83.5	69.2	77.0	62.1		
→4,6)-β-Glc*p*^{I}-(1→	104.8	74.4	76.3	78.0	74.0 [a]	66.0 [a]		
α-Glc*p*^{II}-(1→	100.5	72.9	74.1	70.9	74.0	62.0		
Cho*P*	67.5 [a]	61.0 [a]	55.3 [b]					
Oligosaccharide **2**								
→3,4)-GlcNAc-ol	61.9	52.8	77.0	76.3 [a]	71.9 [a]	63.9	175.1 [b]	23.5 [b]
→3)-β-Glc*p*NAc-(1→	104.8	55.9	83.5	70.1	76.1	62.1	175.7 [b]	23.6 [b]
→3)-β-Gal*p*-(1→	104.3	71.0	83.4	69.4	76.1	62.1		
→4)-β-Glc*p*^{I}-(1→	102.9	74.4	77.2	77.8	75.6	61.7		
α-Glc*p*^{II}-(1→	100.8	72.9	74.1	70.6	73.9	61.7		
Cho*P*	67.2 [a]	60.9 [a]	55.2 [c]					
Oligosaccharide **3**								
→3,4)-α-Glc*p*NAc^{I}	91.7	54.7	76.6	72.9	72.4	61.4		
→3)-β-Glc*p*NAc^{II}-(1→	104.3	56.0	83.8	69.8	75.7			
→3)-β-Gal*p*-(1→	104.7	71.0	83.7	69.2	76.3			
→4,6)-β-Glc*p*^{I}-(1→	104.9	74.4	77.0	77.2	74.4	65.7		
α-Glc*p*^{II}-(1→	100.3	72.5	74.0	70.7	73.8	61.5		
Cho*P*	67.4	61.2	55.3 [c]					

[a] The signal is split due to coupling to phosphorus.
[b] Assignment could be interchanged.
[c] The signal for the *N*-methyl groups (3C).

their positions in the spectra of the corresponding non-substituted monosaccharides,[12] showed the substitution pattern of the sugar residues. The chemical shifts for Glc^{II} were close to those of the unsubstituted monosaccharide, thus indicating its terminal position in a side chain.

The $^{1}H,^{31}P$ HMQC spectrum showed correlations between the phosphorus signal at δ −1.7 and signals for H-1 of GlcNAc^{I} at δ 5.37 and H-6a,6b of Glc^{I} at δ 4.02 and 4.22. The other phosphorus signal at δ −2.0 gave a cross-peak with H-1 of choline at δ 4.48 and either H-3 or H-4 of GlcNAc^{I} at δ 4.08. These findings indicate





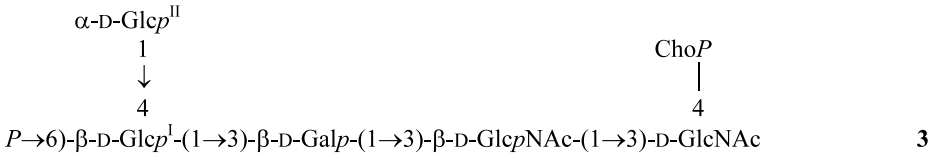


Fig. 3. Structures of the O-polysaccharide of *P. mirabilis* O18 (1) and the oligosaccharides obtained by dephosphorylation, followed by borohydride reduction (2) and mild acid hydrolysis (3). Cho*P* stands for phosphocholine and GlcNAc-ol for 2-acetamido-2-deoxyglucitol.

that one phosphate group 1 →6 interlinks the residues of GlcNAc^{I} and Glc^{I} and the other links choline residues to either O-3 or O-4 of GlcNAc^{I}.

In the ROESY spectrum, the following correlations were observed between the anomeric protons and protons at the linkage carbons: Glc^{II} H-1/Glc H-4 at δ 5.39/3.64, Glc^{I} H-1/Gal H-3 at δ 4.67/3.75, Gal H-1/GlcNAc^{II} H-3 at δ 4.51/3.85; GlcNAc^{II} H-1/GlcNAc^{I} H-3 or H4 at δ 4.69/4.08. These data defined the monosaccharide sequence and the glycosylation pattern in the O-polysaccharide repeating unit, except for that of GlcNAc^{I}, which can be either glycosylated at position 3 and phosphorylated at position 4 or vice versa. Although the character of splitting of the cross-peaks in the 2D NMR spectra and the ^{13}C NMR chemical shifts counted in favour of the former choice (structure **1** in Fig. 3), the unambiguous conclusion was prevented by too close proximity of the H-3 and H-4 signals of GlcNAc^{I} in the ^{1}H NMR spectrum.

To resolve this difficulty the LPS-OH was dephosphorylated by treatment with 48% aqueous HF to give an oligosaccharide, which was subsequently borohydride-reduced. The resultant oligosaccharide **2** was studied by ESIMS and NMR spectroscopy, and it was found that, unexpectely, phosphocholine was not eliminated upon dephosphorylation. The positive-ion mode ESI mass spectrum showed peaks for M^+ (minor) and $[M-H+Na]^+$ (major) molecular ions at *m/z* 1078.42 and 1100.41 (the calculated molecular mass for **2** is 1078.41 Da). In the capillary skimmer dissociation mass spectrum, there were peaks for fragment ions at *m/z* 1041.32 and 917.32 that correspond to the loss from the major molecular ion of the $(CH_3)_3N$ and Cho*P* groups, respectively. The resistance of phosphocholine towards HF treatment could be accounted for by a steric hindrance or influence of the positive charge of the choline group.

The ^{1}H and ^{13}C NMR spectra of the oligosaccharide **2** were assigned as described above for the LPS-OH, and the chemical shifts are tabulated in Tables 1 and 2. In contrast to the spectrum of the LPS-OH, the H-3 and H-4 signals of the reduced GlcNAc^{I} residue (GlcNAc-ol) were well resolved in the ^{1}H NMR spectrum of the oligosaccharide **2**, and the substitution pattern of this residue could be unambiguously determined. Particularly, the position of phosphocholine at O-4 of GlcNAc^{I} was demonstrated by correlation of the signal for H-4 of GlcNAc-ol to the phosphate signal at δ 4.31/−0.2 in the ^{1}H,^{31}P HMQC spectrum. Therefore, it can be concluded that the oligosaccharide **2** has the structure shown in Fig. 3.

Further evidence for the O-polysaccharide structure was obtained by studies of the oligosaccharides that formed upon mild acid hydrolysis of the LPS as a result of cleavage of the acid-labile glycosyl phosphate linkage in the main chain of the O-polysaccharide. Subsequent fractionation of the products by GPC on Sephadex G-50 and TSK HW-40 gave three oligosaccharides. The oligosaccharide eluted from the column second and third were found to be a dimer and a monomer of the O-polysaccharide repeating unit, respectively (data of the ^{1}H NMR spectra).

Sugar analysis of the monomer **3** showed that it contains all components of the polysaccharide, including glucose, galactose and GlcN. GLC of the acetylated (*S*)-2-octyl glycosides showed the D configuration of all constituent monosaccharides. Methylation analysis of the oligosaccharide **3** revealed derivatives from a terminal hexose, a 3-substituted hexose and a 3-substituted hexosamine, which were derived from non-phosphorylated sugar residues. Finally, a 2D NMR spectroscopic study was performed as described above for the O-polysaccharide (for the ^{1}H and ^{13}C NMR chemical shifts see Tables 1 and 2), which enabled determination of the structure of the oligosaccharide **3** shown in Fig. 3. This structure is in full agreement with the O-polysaccharide structure **1**.

The O-polysaccharide studied is distinguished by the presence of phosphocholine, which to our knowledge has not been hitherto reported as a component of an O-polysaccharide chain of LPS. Earlier, this compound was found in teichoic acid (C-polysaccharide) and lipoteichoic acid of the mitis group of streptococci[11,13–15] and in the LPS core region of *Haemophilus* and *Neisseria*.[16–20] Phosphocholine is present also in other biologically important glycoconjugates, including a glyceroglycolipid[21] and platelet-activating factor-like ether glycerolipids[22] of *Mycoplasma fermentas*. It appears likely that phosphocholine is involved in cellular adhesion of bacteria to host cells and stimulation of innate immune system, thus playing a key role in potentiating the microorganism–host interaction. Another peculiar feature of the O-polysaccharide of *P. mirabilis* O18 is the presence of a glycosyl phosphate group. Glucosyl and galactosyl phosphates have been earlier reported in a number of *Proteus* O-polysaccharides, but 2-acetamido-2-deoxyglycosyl phosphate was found for the first time in this work.

2.2. Serological studies

Charged substituents, including phosphate groups, are known to play an important role in manifesting the immunospecificity of bacterial polysaccharides, including *Proteus* O-antigens.[8] In connection with this, the LPS from *Proteus vulgaris* O1 (OX19),[23] *P. vulgaris* O21,[24] and *P. mirabilis* O40 (structural elucidation is in progress) with phosphorylated O-polysaccharides have been chosen for serological studies. Anti-*P. mirabilis* O18 serum reacted strongly with the homologous O18 LPS in the enzyme immunosorbent assay (EIA) (titre 1:1,024,000) and in the Western blot, whereas none of

the heterologous LPSs tested cross-reacted to a comparable extent. A weak cross-reactivity was observed for the LPS of *P. mirabilis* O40 and *P. vulgaris* OX19 in EIA (titre 1:2000 for both). In the Western blot, a weak reaction of anti-O serum *P. mirabilis* O18 with the LPS of *P. mirabilis* O40 and *P. vulgaris* OX19 was limited to fast-migrating bands, which correspond to the core-lipid A moiety (data not shown). Therefore, the unique structure of the O-polysaccharide of *P. mirabilis* O18 is in accordance with the classification of the strain studied in a separate *Proteus* serogroup.

For epitope analysis of the *P. mirabilis* O18-antigen, LPS, LPS-OH and the oligosaccharide **2** (Fig. 3) were tested as inhibitors. The LPS and the LPS-OH inhibited reaction in EIA in the homologous system *P. mirabilis* O18 LPS/anti-*P. mirabilis* O18 serum at doses of 78 and 312 ng, respectively, whereas **2** was inactive even at a dose up to 5000 ng. The lack of inhibition by **2** may suggest that the bridging phosphate is important in serological specificity on *P. mirabilis* O18. Similar importance of the bridging phosphate was found in the serological specificity of *P. vulgaris* O1 (OX19).[23] In addition the lack of inhibition of oligosaccharide **2** (containing phosphocholine) may suggest that this component is not crucial for antibody binding. To confirm this finding the inhibition of the reaction in EIA was performed using as the inhibitor phosphocholine chloride calcium salt ($C_5H_{13}CaClNO_4P$). Phosphocholine salt was inactive even at a dose up to 50,000 ng, constituting a proof of insignificance this group as a serological epitope.

To estimate the importance of *P. mirabilis* strains that belong to O18 serogroup, 98 clinical isolated of *P. mirabilis* from cases of urinary tract infections were tested with rabbit polyclonal anti-*P. mirabilis* O18 serum. From them, seven isolates reacted strongly in EIA (Table 3). Western blot of proteinase K-treated bacterial cells confirmed the cross-reactivity of two clinical strains, which showed different binding patterns. Anti-*P. mirabilis* O18 serum recognized slow migrating bands of the LPS of *P. mirabilis* 1784 and fast-migrating bands of the LPS of *P. mirabilis* 49, which correspond to high- and low-molecular-mass species with and without O-polysaccharide chain, respectively (Fig. 4, lanes 2 and 4). These data indicate that cross-reactive epitopes are

Table 3
Serological reactivity of proteinase K-treated bacterial cells of *P. mirabilis* O18 and *P. mirabilis* clinical isolates with anti-*P. mirabilis* O18 serum in EIA (reciprocal titre)

Proteus mirabilis strain						
O18	46	49	59	70	82	1784
512,000	8000	16,000	8000	4000	16,000	256,000

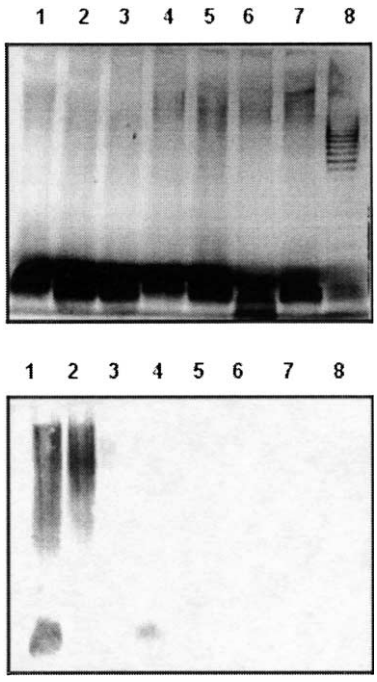


Fig. 4. Silver-stained SDS-PAGE (A) and Western blot with anti-*P. mirabilis* O18 serum diluted 1:500 (B) of whole cells of the following strains: 1, *P. mirabilis* O18; 2, *P. mirabilis* 1784; 3, *P. mirabilis* 46; 4, *P. mirabilis* 49; 5, *P. mirabilis* 59; 6, *P. mirabilis* 70; 7, *P. mirabilis* 82; 8, *P. mirabilis* 91.

located on the O-antigen of *P. mirabilis* 1784 and on the LPS core of *P. mirabilis* 49 and suggest that strains of *P. mirabilis* O18 may play a role of a pathological agent in humans.

3. Experimental

3.1. Bacteria, growth, and isolation of the lipopolysaccharide

Proteus mirabilis O18 (PrK 34/57), O40 (PrK 66/57), *P. vulgaris* O1 (OX19) and O21 (PrK 39/57) were from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague). A total of 82 clinical isolates of *P. mirabilis* came from the Department of Clinical Microbiology, Karolinska Hospital (Stockholm, Sweden), and 16 strains came from the Swietokrzyskie Oncology Center (Kielce, Poland). *P. mirabilis* O18 was cultivated under aerobic conditions in a fermenter (Chemap AG, Switzerland) in nutrient broth (BTL, Poland) under controlled conditions (37 °C, pH 7.4–7.6, pO_2 75–85%). Cells were harvested at the end of the logarithmic growth phase, centrifuged (5000*g*, 30 min), washed with distilled water and lyophilized. The LPS was isolated by the phenol–water procedure[25] and purified by treatment with DNAse and RNAse (Boehringer Mannheim, Germany) as de-

scribed.[26] The LPS preparations thus obtained were free of nucleic acid and contained $<2.5\%$ proteins.

3.2. O-Deacylation and deamination of the lipopolysaccharide

The LPS (100 mg) was treated with aq 12% NH_3 (6 mL) at 37 °C for 24 h, a precipitate was removed by centrifugation, and the supernatant was fractionated by GPC on Sephadex G-50 (S) (Amersham Biosciences, Sweden) using 0.05 M pyridinium acetate buffer as eluent (10 mL HOAc and 4 mL pyridine in 1 L water) and monitoring with a differential refractometer (Knauer, Germany) to give a high-molecular-mass product (18 mg).

The LPS (100 mg) was treated with a mixture of 1% aq $NaNO_2$ (5 mL) and 10% aq HOAc (5 mL) at 25 °C for 3 h. Fractionation of the supernatant by GPC on Sephadex G-50 (S) as described above resulted in a high-molecular-mass polysaccharide (20 mg).

3.3. Dephosphorylation

The polysaccharide (18 mg) that was obtained by O-deacylation of the LPS was treated with aq 48% HF (3 mL) at 4 °C for 16 h. After removal of the reagent by lyophilisation using a NaOH cartridge to absorb HF, the product was purified by GPC on TSK HW-40 (S) and reduced with $NaBH_4$ in water to give oligosaccharide **2** (10 mg).

3.4. Mild acid degradation of the lipopolysaccharide

The LPS (100 mg) was hydrolysed with 2% aq HOAc at 100 °C for 2.5 h, and a lipid precipitate was removed by centrifugation (13,000*g*, 15 min). The carbohydrate portion was fractionated by GPC on Sephadex G-50 (S) as described above. A resultant oligosaccharide fraction (35 mg) was further fractionated on a column (90×2.5 cm) of TSK HW-40 (S) (Supelco, USA) use 1% aq HOAc to give three oligosaccharides in yields 16.4, 5.4, and 10.3 (compound **3**) mg (in order of their elution from the column).

3.5. Sugar analysis

Oligosaccharide **3** was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h), and the sugars were identified as the alditol acetates[27] by GLC using a Hewlett–Packard 5989A instrument equipped with an HP-5 column and a temperature gradient of 150 (3 min) to 320 °C at 5 °C min^{-1}. The absolute configurations of the monosaccharides were determined by GLC of the acetylated (*S*)-2-octyl glycosides[28,29] using a Hewlett–Packard 5880 instrument with a DB-5 column and a temperature gradient of 160 (1 min) to 250 °C at 3 °C min^{-1}.

3.6. Methylation analysis

Methylation of the oligosaccharide **3** was performed with CH_3I in dimethyl sulfoxide in the presence of sodium methylsulfinylmethanide.[30] Partially methylated monosaccharides were derived by hydrolysis under the same conditions as in the sugar analysis, conventionally reduced with $NaBH_4$, acetylated, and analyzed by GLC–MS on a Hewlett–Packard 5890 chromatograph equipped with a NERMAG R10-10L mass spectrometer (France) using a temperature gradient of 160 (1 min) to 250 °C at 3 °C min^{-1}.

3.7. NMR spectroscopy

The samples were deuterium-exchanged by freeze-drying from 99.9% D_2O and then examined as solutions in 99.96% D_2O at 60 °C. Spectra were recorded on a Bruker DRX-600 spectrometer (Germany). Chemical shifts refer to internal acetone (δ_H 2.225; δ_C 31.45) and external 85% aq H_3PO_4 (δ_P 0). 2D NMR spectra were obtained using standard Bruker software, and XWINNMR 2.6 program (Bruker) was used to acquire and process the NMR data. The parameters used for 2D experiments were essentially the same as described previously.[31] A mixing time of 100 ms was used in TOCSY and HMQC–TOCSY and 225 ms in ROESY experiments.

3.8. Mass spectrometry

Ion cyclotron resonance Fourier transform ESIMS was performed in the positive-ion mode using an APEX II Instrument (Bruker Daltonics, Billerica, USA) equipped with a 7 T actively shielded magnet and an Apollo ion source. Mass spectra were acquired using standard experimental sequences as provided by the manufacturer. Samples were dissolved at a concentration of ~ 10 ng μL^{-1} in a 50:50:0.001 (v/v/v) mixture of 2-PrOH–water–Et_3N and sprayed at a flow rate of 2 $\mu L\ min^{-1}$. Capillary entrance voltage was set to 3.8 kV, and dry gas temperature to 150 °C. Capillary skimmer dissociation was induced by increasing the capillary exit voltage from -100 to -350 V.

3.9. Serological techniques

Rabbit polyclonal anti-*P. mirabilis* O18 serum was obtained as described.[32] EIA and inhibition of the reaction in EIA were performed as described previously.[32]

3.10. SDS-PAGE and Western blot

Proteinase K (Sigma)-treated whole bacterial cells or isolated LPS were separated by SDS-PAGE according

to Laemmli.[33] The gels were silver-stained according to Tsai and Frash[34] or electroblotted onto nitrocellulose plates (Schleicher & Schüll, Germany), which were incubated with diluted (1:250) immune rabbit sera as primary antibodies and then with horseradish peroxidase-conjugated goat *anti*-rabbit IgG (Sigma) as the secondary antibodies.

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References

1. Rozalski, A.; Sidorczyk, Z.; Kotelko, K. *Microb. Mol. Biol. Rev.* **1997**, *61*, 65–89.
2. Holmgren, K.; Danielson, B. G.; Fellström, B. *Scand. J. Urol. Nephrol.* **1987**, *21*, 219–223.
3. Warren, J. W. In *Urinary Tract Infections. Molecular Pathogenesis and Clinical Management*; Mobley, H. T. L.; Warren, J. W., Eds.; ASM Press: Washington, DC, 1996; pp 3–28.
4. Melekos, M. D.; Naber, K. G. *Int. J. Antimicrob. Agents* **2000**, *15*, 247–256.
5. Blankenberg-Spronkels, S. H.; Fielder, M.; Feltkamp, T. E.; Tiwana, H.; Wilson, C.; Ebringen, A. *J. Rheumatol.* **1998**, *25*, 743–747.
6. Senior, B. W.; Anderson, G. A.; Morley, K. D.; Kerr, M. A. *J. Infect.* **1999**, *38*, 99–106.
7. Tiwana, H.; Wilson, C.; Alvarez, A.; Abuknesha, R.; Bansal, S.; Ebringen, A. *Infect. Immun.* **1999**, *67*, 2769–2775.
8. Knirel, Y. A.; Kaca, W.; Rozalski, A.; Sidorczyk, Z. *Pol. J. Chem.* **1999**, *73*, 895–907.
9. Toukach, F. V.; Kondakova, A. N.; Arbatsky, N. P.; Senchenkova, S. N.; Shashkov, A. S.; Knirel, Y. A.; Zych, K.; Rózalski, A.; Sidorczyk, Z. *Biochemistry (Moscow)* **2002**, *67*, 265–276.
10. Vinogradov, E. V.; Sidorczyk, Z.; Knirel, Y. A. *Aust. J. Chem.* **2002**, *55*, 61–67.
11. Karlsson, C.; Jansson, P.-E.; Sorensen, U. B. S. *Eur. J. Biochem.* **1999**, *265*, 1091–1097.
12. Bock, K.; Pedersen, C. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 27–65.
13. Kulakowska, M.; Brisson, J.-R.; Griffith, D. W.; Young, N. M.; Jennings, H. J. *Can. J. Chem.* **1993**, *71*, 644–648.
14. Fischer, W.; Behr, T.; Hartmann, R.; Peter-Katalinic, J.; Egge, H. *Eur. J. Biochem.* **1993**, *215*, 851–857.
15. Bergström, N.; Jansson, P. E.; Kilian, M.; Sõrensen, U. B. S. *Eur. J. Biochem.* **2000**, *267*, 7147–7157.
16. Weiser, J. N.; Shchepetov, M.; Chong, S. T. H. *Infect. Immun.* **1997**, *65*, 943–950.
17. Howard, M. D.; Cox, A. D.; Weiser, J. N.; Schurig, G. G.; Inzana, T. J. *J. Clin. Microbiol.* **2000**, *38*, 4412–4419.
18. Lysenko, E.; Richards, J. C.; Cox, A. D.; Stewart, A.; Martin, A.; Kapoor, M.; Weiser, J. N. *Mol. Microbiol.* **2000**, *35*, 234–245.
19. Schweda, E. K. H.; Brisson, J. R.; Alvelius, G.; Martin, A.; Weiser, J. N.; Hood, D. W.; Moxon, E. R.; Richards, J. C. *Eur. J. Biochem.* **2000**, *267*, 3902–3913.
20. Serino, L.; Virji, M. *Mol. Microbiol.* **2000**, *35*, 1550–1559.
21. Zähringer, U.; Wagner, F.; Rietschel, E. T.; Ben-Menachem, G.; Deutsch, J.; Rottem, S. *J. Biol. Chem.* **1997**, *272*, 26262–26270.
22. Wagner, F.; Rottem, S.; Held, H.-D.; Uhlig, S.; Zähringer, U. *Eur. J. Biochem.* **2000**, *267*, 6276–6286.
23. Kaca, W.; Swierzko, A. S.; Ziolkowski, A.; Amano, K.-I.; Senchenkova, S. N.; Knirel, Y. A. *Microbiol. Immunol.* **1998**, *42*, 669–675.
24. Bartodziejska, B.; Toukach, F. V.; Vinogradov, E. V.; Senchenkova, S. N.; Shashkov, A. S.; Ziolkowski, A.; Czaja, J.; Perry, M. B.; Knirel, Y. A.; Rozalski, A. *Eur. J. Biochem.* **2000**, *267*, 6888–6896.
25. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.
26. Shashov, A. S.; Toukach, F. V.; Paramonov, N. A.; Ziolkowski, A.; Senchenkova, S. N.; Kaca, W.; Knirel, Y. A. *FEBS Lett.* **1996**, *386*, 247–251.
27. Sawardeker, J. S.; Sloneker, J. H.; Jeanes, A. *Anal. Chem.* **1965**, *37*, 1602–1603.
28. Leontein, K.; Lindberg, B.; Lönngren, J. *Carbohydr. Res.* **1978**, *62*, 359–362.
29. Shashkov, A. S.; Senchenkova, S. N.; Nazarenko, E. L.; Zubkov, V. A.; Gorshkova, N. M.; Knirel, Y. A.; Gorshkova, R. P. *Carbohydr. Res.* **1997**, *303*, 333–338.
30. Hakomori, S.-I. *J. Biochem. (Tokyo)* **1964**, *55*, 205–208.
31. Hanniffy, O.; Shashkov, A. S.; Senchenkova, S. N.; Tomshich, S. V.; Komandrova, N. A.; Romanenko, L. A.; Knirel, Y. A.; Savage, A. V. *Carbohydr. Res.* **1999**, *321*, 132–138.
32. Kondakova, A. N.; Fudala, R.; Senchenkova, S. N.; Shashkov, A. S.; Knirel, Y. A.; Kaca, W. *Carbohydr. Res.* **2003**, *338*, 1191–1196.
33. Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
34. Tsai, C.; Frasch, C. E. *Anal. Biochem.* **1982**, *119*, 115–119.