Structures of the O-specific polysaccharides and a serological cross-reactivity of the lipopolysaccharides of *Proteus mirabilis* O24 and O29

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Abstract **Strains of *Proteus mirabilis* belonging to serogroups O24 and O29 are frequent in clinical specimens. Anti-*P. mirabilis* O24 serum cross-reacted with the lipopolysaccharide (LPS) of *P. mirabilis* O29 and vice versa. The structures of the O-specific polysaccharides (OPSs, O-antigens) of both LPSs were established using sugar analysis and one- and two-dimensional ^{1}H- and ^{13}C-NMR spectroscopy and found to be different. SDS-PAGE and Western immunoblotting suggested that the serological cross-reactivity of the LPSs is due to a common epitope(s) on the core-lipid A moiety, rather than on the OPS. Therefore, the epitope specificity and the structures of the O-antigens studied are unique among *Proteus* serogroups.**

Key words: O-antigen; Lipopolysaccharide; Bacterial polysaccharide structure; Serological cross-reactivity; *Proteus mirabilis*

1. Introduction

Enterobacteria of the genus *Proteus* are a common cause of urinary tract infections in human, which often result in severe complications, such as pyelonephritis and formation of bladder and kidney stones [1]. Lipopolysaccharide (LPS, endotoxin) is considered as one of the virulence factors of *Proteus* which, after being released from bacterial cells, mediates the infectious processes [1,2]. The LPS consists of three moieties: a lipid part (lipid A) which anchors LPS into the outer membrane and is responsible for the endotoxicity of the LPS, an oligosaccharide core region, and an O-specific polysaccharide chain (OPS, O-antigen) which defines the immunospecificity of the bacterium. On the basis of the O-antigens, strains of *Proteus* are classified serologically into more than 60 serogroups [3–5]. A serological cross-reactivity was observed for strains of different serogroups [5], thus showing that the existing serogrouping scheme does not reflect all, but major, antigenic relationships between *Proteus* strains.

Immunochemical studies of LPSs are important for substantiation of the serological cross-reactivity of strains on the molecular level and for improvement of the classification of *Proteus* on the chemical basis. In the LPSs of most *Proteus* strains studied, the OPSs are acidic due to the presence of various acidic sugars and non-sugar components; a few strains of *Proteus vulgaris* and *Proteus penneri* have neutral OPSs [5]. Now, we report the structures of the OPSs of two serologically cross-reactive strains of *Proteus mirabilis* belonging to serogroups O24 and O29. Strains of these serogroups are frequent among isolates from clinical specimens [3] and were confirmed in this work to play a role in human pathology. Immunochemical studies of the OPSs and LPSs were performed in order to reveal the epitope specificity of the cross-reactive anti-LPS antibodies. Part of the data of *P. mirabilis* O24 has been reported [6].

2. Material and methods

2.1. Bacterial strains, isolation and degradation of the LPSs

P. mirabilis O24 and O29 (strains PrK 47/57 and 52/57, respectively) came from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague). The LPSs were isolated using the phenol-water procedure [7] and purified by treatment with DNAse and RNAse (Sigma, USA) and ultracentrifugation as described [7,8]. The isolated LPSs were essentially free of nucleic acids and contained $<2\%$ proteins.

Delipidation of the LPSs was performed with aqueous 2% acetic acid at 100°C, the precipitate was removed by centrifugation (13 000 × *g*, 20 min), and the supernatant was fractionated on a column (2.5 × 65 cm) of Sephadex G-50 (Pharmacia, Sweden) in 0.05 M pyridinium acetate buffer pH 4.5 with monitoring by a Knauer differential refractometer (Germany) to give the corresponding high molecular mass OPS and core oligosaccharide fractions.

Alkali-treated LPSs were prepared by saponification of the LPSs with aqueous 0.25 M NaOH (56°C, 2 h). Smith degradation of the alkali-treated LPS from *P. mirabilis* O24 was performed with 0.1 M $NaIO_4$ (20°C, 48 h, in the dark), the resultant product was reduced with $NaBH_4$ and desalted on Sephadex G-50.

2.2. NMR spectroscopy

NMR spectra were recorded with a Bruker DRX-500 spectrometer for a solution in D_2O at 30 or 60°C using internal acetone (δ_H 2.225, δ_C 31.45) as reference. Standard Bruker software (XWINNMR 1.2) was used to acquire and maintain the NMR data. A mixing time of 200 and 100 ms was used in two-dimensional TOCSY and NOESY experiments with *P. mirabilis* O29 OPS, respectively, or 300 ms in a NOESY experiment with *P. mirabilis* O24 OPS. The ^{1}H- and ^{13}C-NMR spectra were assigned using two-dimensional COSY, TOCSY, and H-detected ^{1}H,^{13}C heteronuclear multiple-quantum coherence (HMQC) experiments. The assignment of β-linked sugars was confirmed making use of intraresidue H1/H3, H5 correlations revealed by a NOESY experiment. Spin systems of monosaccharides were identified based on the typical $^{3}J_{H,H}$ coupling constant values. Amino sugars were distinguished by correlations of protons at carbons bearing nitrogen to the corresponding carbons at δ 50–57, which were revealed by a ^{1}H,^{13}C HMQC experiment.

2.3. Sugar analysis

The OPSs were hydrolyzed with 2 M CF_3COOH (120°C, 2 h) or

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methanolyzed with 1 M HCl/MeOH (80°C, 16 h). Amino sugars were identified on a Biotronik LC-2000 amino acid analyser (Germany) equipped with a column (0.4×22 cm) of a Ostion LG AN B cation-exchange resin using 0.35 M sodium citrate buffer pH 5.28 at 80°C. Neutral sugars were analysed with a Biotronik LC-2000 sugar analyser, using a column (0.4×15 cm) of a Dionex A×8–11 anion-exchange resin and 0.5 M sodium borate buffer pH 8.0 at 65°C. Uronic acids were analysed by GLC as acetylated methyl glycosides using a Hewlett-Packard Model 5890 chromatograph with an Ultra 2 capillary column and a temperature gradient of 160°C (1 min) to 290°C at 10°C/min. The absolute configurations of monosaccharides were determined by GLC of acetylated (*S*)-2-butyl glycosides according to the published method [9,10] modified as described [11].

2.4. Preparation of sera and serological assays

Rabbit antisera against surface heat-stabile antigens of *Proteus* were obtained as described [12]. Briefly, a New Zealand rabbit received i.v. increasing doses of heat killed bacteria on days 0, 4, 7 and 11. On day 15, 20 ml blood was taken by cardiac puncture and early serum was prepared. After a single booster injection on day 50, the rabbit was extinguished on day 58 to give late serum. Unless stated otherwise, the data of early serum are given. Human sera were collected from healthy individuals or hospitalised patients who gave informed consent.

ELISA and passive hemolysis (PH) were performed essentially as described [12]. In ELISA, human or rabbit sera were dispensed to plates containing antigens (LPS or *Proteus* whole cells), and fixed antibodies were quantified with horseradish peroxidase-conjugated goat anti-human or anti-rabbit IgG (Sigma).

Proteinase K- (Sigma) treated whole bacterial cells or LPSs were separated by SDS-PAGE according to Laemmli [13]. The gels were silver-stained according to Tsai and Frasch [14] or electroblotted onto nitrocellulose plates (Shleicher and Schull, Germany), which were incubated with diluted (1:100) immune rabbit sera as primary antibodies and then with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) as secondary antibodies [15].

3. Results and discussion

3.1. Elucidation of the structure of the OPS of P. mirabilis O24

Sugar analysis of the OPS after full acid hydrolysis, including determination of the absolute configurations of monosaccharides, revealed 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose in the ratio 2:1, as well as D-galactose. The ^{13}C-NMR spectrum of the OPS [6] (Table 1) contained signals for four anomeric carbons in the region δ 102.5–106.6, three carbons linked to nitrogen (C2 of hexosamines) in the region δ 53.0–56.2, four HO*C*H$_2$-C groups (C6 of galactose and hexosamines) at δ 61.4–62.4, 13 other sugar ring carbons in the region δ 69.9–82.8, and three *N*-acetyl groups at δ 23.6–23.9 (CH$_3$) and δ 175.4–175.7 (CO). Accordingly, the ^{1}H-NMR spectrum of the OPS (Table 1) contained, inter alia, signals for four anomeric protons in the region δ 4.44–4.91 and three *N*-acetyl groups at δ 2.04 (6H) and 2.09 (3H). Therefore, the OPS is composed of tetrasaccharide repeating units containing one residue each of D-galactose and 2-acetamido-2-deoxy-D-glucose, and two residues of 2-acetamido-2-deoxy-D-galactose.

Relatively large coupling constant values of $J_{1,2}$ 7–8 Hz, determined from the ^{1}H-NMR spectrum of the OPS, showed that all monosaccharide residues are in the pyranose form and β-linked. In the ^{13}C-NMR spectrum of the OPS, the positions of the signals for C2–C6 of Gal at δ 72.2, 74.2, 69.9, 76.1, and 62.4 (or 62.0), respectively, were close to those of non-substituted β-Gal*p* [16] and, hence, Gal occupies the terminal position in the side chain. As a result of the glycosylation, the signals for C4 of one of the GlcNAc residues (GlcNAc^{I}), C3 of the other residue (GlcNAc^{II}), C3 and C4 of GalNAc were shifted downfield to δ 81.0, 82.8, 81.8, and 75.6, respectively, compared to their positions in the spectra of the corresponding non-substituted monosaccharides [16]. These data showed that the OPS is branched and allowed determination of the positions of substitution of the monosaccharides.

The NOESY spectrum of the OPS contained interresidue cross-peaks between the following protons: Gal H1/GalNAc H3 (strong) and H4 (weak), GalNAc H1/GlcNAc^{I} H4, GlcNAc^{I} H1/GlcNAc^{II} H3, and GlcNAc^{II} H1/GalNAc H4 (strong), H3 and H5 (both weak). These data confirmed the positions of substitution of the monosaccharide residues,

Table 1
125-MHz ^{13}C-NMR and 500-MHz ^{1}H-NMR data of the OPSs

Sugar residue	Chemical shift (δ, ppm)					
	C1	C2	C3	C4	C5	C6
P. mirabilis O24						
β-D-Gal*p*-(1→	106.6	72.2	74.2	69.9	76.1	62.4[a]
→3,4)-β-D-Gal*p*NAc-(1→	103.0	53.0	81.8	75.6	75.6	62.0[a]
→4)-β-D-Glc*p*NAc^{I}-(1→	102.5	56.2	73.4	81.0	75.6	61.4
→3)-β-D-Glc*p*NAc^{II}-(1→	102.5	56.0	82.8	69.9	76.1	62.4
P. mirabilis O29						
α-D-Gal*p*NAc^{I}-(1→	96.2	50.5	68.6	69.4	72.1	61.9
→3,4)-β-D-Gal*p*NAc^{II}-(1→	102.3	51.5	77.3	69.0	75.7	61.9
4)-β-D-Glc*p*A-(1→	105.0	73.4	74.8	81.5	76.0	
→3)-β-D-Gal*p*NAc^{III}-(1→	100.0	53.8	79.2	69.0	76.2	61.9
	H1	H2	H3	H4	H5	H6a; H6b
P. mirabilis O24						
β-D-Gal*p*-(1→	4.44	3.58	3.63	3.96	3.66	3.77
→3,4)-β-D-Gal*p*NAc-(1→	4.58	3.90	3.90	4.31	3.69	3.74; 3.77
→4)-β-D-Glc*p*NAc^{I}-(1→	4.61	3.70	3.77	3.58	3.54	3.65; 3.85
→3)-β-D-Glc*p*NAc^{II}-(1→	4.91	3.77	3.83	3.50	3.39	3.74; 3.91
P. mirabilis O29						
α-D-Gal*p*NAc^{I}-(1→	5.08	4.34	3.70	4.05	3.88	3.78
→3,4)-β-D-Gal*p*NAc^{II}-(1→	4.60	4.22	3.84	4.44	3.71	3.78
→4)-β-D-Glc*p*A-(1→	4.52	3.41	3.64	3.78	3.84	
→3)-β-D-Gal*p*NAc^{III}-(1→	4.94	3.74	4.10	4.13	3.44	3.79

Chemical shifts for *C*H$_3$CON are δ_C 23.6–23.9 and δ_H 2.04–2.10, for CH$_3$*C*ON and COOH δ_C 175.2–176.2.
[a]Assignment could be interchanged.

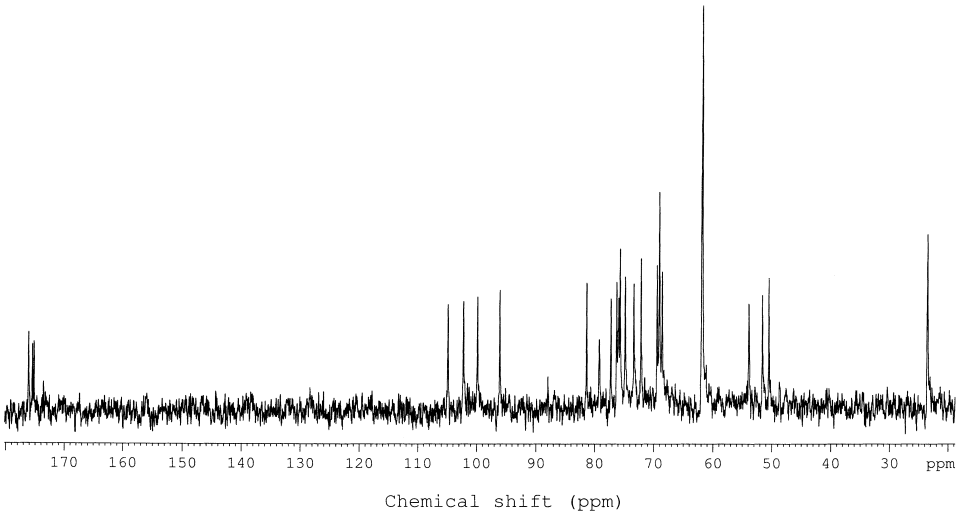

Fig. 1. 125-MHz ^{13}C-NMR spectrum of the OPS of *P. mirabilis* O29.

showed that GlcNAc^{II} and Gal are attached to GalNAc at positions 4 and 3, respectively, and allowed determination of the full monosaccharide sequence in the repeating unit. Therefore, the OPS of *P. mirabilis* O24, which is the first neutral OPS found in this species, has the following structure:

β-D-Gal*p*
1
↓
3
→3)-β-D-Glc*p*NAc^{II}-(1→4)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc^{I}-(1→

3.2. Elucidation of the structure of the OPS of P. mirabilis O29

Sugar analysis showed the presence of 2-amino-2-deoxy-D-galactose and D-glucuronic acid (D-GlcA) as components of the OPS. The ^{13}C-NMR spectrum of the OPS (Fig. 1, Table 1) was typical of a regular polymer. It contained signals for four anomeric carbons at δ 96.2–105.0, three HO*C*H$_2$-C groups (C6 of GalN) at δ 61.9, three carbons bearing nitrogen (C2 of GalN) at δ 50.5, 51.5 and 53.8, 13 other sugar ring carbons at δ 68.6–81.5, and *N*-acetyl groups (CH$_3$ at δ 23.6, CO at δ 175.2–176.2). Accordingly, the ^{1}H-NMR spectrum (Table 1) contained signals for four anomeric protons at δ 4.52–5.08, other signals of sugar ring protons at δ 3.41–4.44, and three *N*-acetyl groups at δ 2.06–2.10. Therefore, the OPS has a tetrasaccharide repeating unit containing three residues of 2-acetamido-2-deoxy-D-galactose and one residue of D-glucuronic acid.

The coupling constant value of $J_{1,2}$ 3 Hz indicated that one of the GalNAc residues (GalNAc^{I}) is α-linked, whereas the values of $J_{1,2}$ 8 Hz showed that GlcA and two other GalNAc residues (GalNAc^{II} and GalNAc^{III}) are β-linked. The NOESY spectrum showed GalNAc^{III} H1/GalNAc^{II} H4 and GalNAc^{II} H1/GlcA H4 interresidue cross-peaks, thus demonstrating a GalNAc^{III}(1→4)GalNAc^{II}(1→4)GlcA sequence. H1 of GalNAc^{I} gave two interresidue cross-peaks, with H3 and H4 of GalNAc^{II}, that is typical of α-(1→3)-linked disaccharides with the same absolute configuration of the constituent monosaccharides [17]. Likewise, H1 of GlcA gave two cross-peaks with H3 and H4 of GalNAc^{III}.

In the ^{13}C-NMR spectrum of the OPS, the positions of the signals for C2–C6 of GalNAc^{I} at δ 50.5, 68.6, 69.4, 72.1, and 61.9, respectively, were typical of the non-substituted α-Gal*p*-NAc [16]. Relatively low-field positions of the signals for C3 of GalNAc^{III} and C4 of GlcA at δ 79.2 and 81.5, as compared with those in non-substituted β-Gal*p*NAc and β-Glc*p*A [16], resulted from the substitution at O3 and O4, respectively. Taking into account the NOESY data, one could conclude that GlcA is attached to GalNAc^{III} at position 3. Of the signals for C3 and C4 of GalNAc^{II}, only that for C3 was shifted, as expected, downfield to δ 77.3, whereas the signal for C4 remained almost at the same position at δ 69.0 as in non-substituted β-Gal*p*NAc [16]. This could be accounted for by strong deviation from the expected regular conformation of the 1→4-glycosidic linkage in a sterically hindered trisaccharide fragment of three GalNAc residues with vicinal substitution at positions 3 and 4 of GalNAc^{II} (compare also published data [18]).

On the basis of the data obtained, it was concluded that the OPS of *P. mirabilis* O29 has the following structure:

α-D-Gal*p*NAc^{I}
1
↓
3
→3)-β-D-Gal*p*NAc^{III}-(1→4)-β-D-Gal*p*NAc^{II}-(1→4)-β-D-Glc*p*A-(1→

As the OPS of *P. mirabilis* O24 studied in this work and most other *Proteus* OPSs with known structure [5], the OPS of *P. mirabilis* O29 is enriched in *N*-acetylamino sugars. Also typical of *Proteus* OPSs, this is an acidic polysaccharide containing D-glucuronic acid, a component of OPSs of many other *Proteus* serogroups [5]. Although different in sugar composition, the main chains of the OPSs of *P. mirabilis* O24 and O29 exhibit some degree of structural similarity.

3.3. Serological studies

Sera of 75 healthy volunteers and 56 hospitalised patients were tested in ELISA for the presence of IgG class antibodies against LPSs of *P. mirabilis*. 48.0% and 49.3% sera of volunteers contain anti-*P. mirabilis* O24 and anti-*P. mirabilis* O29 antibodies, respectively. The level of patients' sera reacting with *P. mirabilis* O24 and O29 LPSs was significantly higher

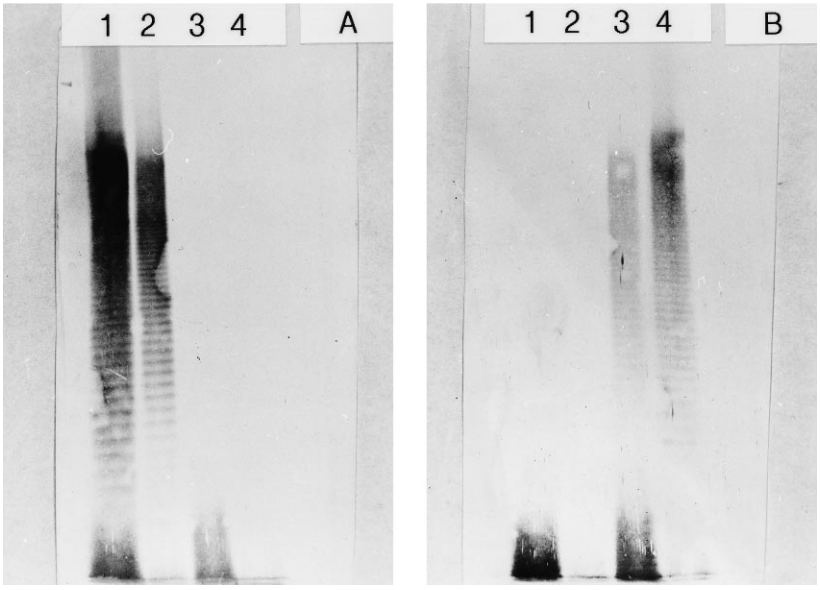

Fig. 2. Western immunoblot of *P. mirabilis* O24 and O29 LPSs (lanes 1 and 3, respectively) and whole cells (lanes 2 and 4, respectively) with rabbit anti-*P. mirabilis* O24 (A) and anti-*P. mirabilis* O29 (B) antibodies.

(80.4% and 67.9%, respectively). These data indicated a role of *P. mirabilis* O24 and O29 as pathological agents in humans.

P. mirabilis O24 LPS reacted with rabbit anti-*P. mirabilis* O24 serum at high titres of 1:102 400 in passive hemolysis (PH) and 1:128 000 in ELISA. Alkali-treated LPS demonstrated the same reactivity in ELISA as LPS, whereas *P. mirabilis* O24 OPS was less active (titre 1:16 000). A weaker reactivity of the OPS could be accounted for by the importance of the core-lipid A moiety of LPS in the exposure of the OPS chain optimal for antibodies binding or by the specificity of a significant fraction of antibodies to the LPS core (see below). A Smith-degraded LPS and a core oligosaccharide, obtained by mild acid degradation of the LPS, were inactive. The intact LPS and alkali-treated LPS at doses of 4.9 and 78 ng, respectively, inhibited the reaction in ELISA and PH in the homologous system of anti-*P. mirabilis* O24 serum/*P. mirabilis* O24 LPS. The OPS inhibited the reaction only in ELISA at as high dose as 625 ng, whereas the Smith-degraded LPS and the core oligosaccharide were inactive. Therefore, the lateral Gal residue, which is destroyed by Smith degradation, is crucial for manifesting of the O24 specificity.

LPSs from various *Proteus* strains representing more than 30 O-serogroups were tested in ELISA with rabbit anti-*P. mirabilis* O24 antiserum. A strong cross-reactivity was observed between anti-*P. mirabilis* O24 serum and *P. mirabilis* O29 LPS (titres 1:32 000 and 1:4000 with late and early sera, respectively). Vice versa, from rabbit anti-O sera against over 30 *Proteus* strains tested, only that to *P. mirabilis* O29 cross-reacted in ELISA with *P. mirabilis* O24 LPS to a high dilution of 1:16 000. The close serological relatedness of *P. mirabilis* O24 and O29 LPSs was confirmed by PH data (Table 2). Preincubation of anti-*P. mirabilis* O24 serum with the homologous LPS abolished the reactivity in PH with both *P. mirabilis* O24 and O29 LPSs. Preincubation of the same serum with the heterologous *P. mirabilis* O29 LPS reduced slightly the reactivity with *P. mirabilis* O24 LPS and abolished the reactivity with *P. mirabilis* O29 LPS. Absorption of anti-*P. mirabilis* O29 serum by the LPS of *P. mirabilis* O29 or O24 reduced significantly the reactivity with both antigens. Therefore the strong two-way cross-reactivity of *P. mirabilis* O24 and O29 is due to the presence of a common epitope(s) on the LPSs.

A cross-reactivity of *Proteus* strains belonging to different O-serogroups is not uncommon [5]. Most often cross-reactive antibodies recognise identical or similar oligosaccharide fragments on the OPS, from a disaccharide to a tetrasaccharide. The main chains of the OPSs of *P. mirabilis* O24 and O29 are structurally similar and, thus, might share a cross-reactive

Table 2
Data of passive hemolysis with absorbed anti-*P. mirabilis* O24 and anti-*P. mirabilis* O29 sera and alkali-treated LPSs

Serum against	Reciprocal titre with LPS of	
	P. mirabilis O24	*P. mirabilis O29*
P. mirabilis O24		
Non-absorbed	102 400	25 600
Absorbed with alkali-treated LPS of *P. mirabilis* O24	< 200	< 200
Absorbed with alkali-treated LPS of *P. mirabilis* O29	51 200	< 200
P. mirabilis O29		
Non-absorbed	204 800	409 600
Absorbed with alkali-treated LPS of *P. mirabilis* O29	800	800
Absorbed with alkali-treated LPS of *P. mirabilis* O24	3200	25 600

epitope(s). However, in Western immunoblotting, a ladder-like banding typical of LPSs with variable chain length OPSs was observed only with homologous anti-*P. mirabilis* O24 and anti-*P. mirabilis* O29 sera (Fig. 2A, B, respectively). The reaction of both anti-O sera with the heterologous LPSs of *P. mirabilis* O29 and O24 was limited to fast-migrating bands, which correspond to the core-lipid A moiety (Fig. 2, lanes 1 and 3). The corresponding heterologous whole cells did not react (Fig. 2, lanes 2 and 4) and, therefore, cross-reactive epitopes are not exposed on the cell surface.

These findings suggested that antibodies against the core-lipid A region of the LPS, rather than those against the OPS, are responsible for the cross-reactivity observed. A high level of anti-core-lipid A antibodies may be due to the exposure of OPS-lacking LPS species on the surface of *P. mirabilis* O24 and O29. A low degree of substitution of the core by the OPS has been demonstrated for *P. mirabilis* S1959 LPS (serogroup O3) [19]. A cross-reactivity has been reported for some other *Proteus* strains with structurally different OPSs and likewise accounted for by the presence of antibodies recognising the core region of LPS [20,21]. It can be suggested that the LPS core regions in the cross-reactive strains are similar or identical, but their exact structures remain to be determined.

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