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Structural and Serological Characterization of the Lipopolysaccharide from *Proteus penneri* 20 and Classification of the Cross-Reacting *Proteus penneri* Strains 10, 16, 18, 20, 32 and 45 in *Proteus* Serogroup O17

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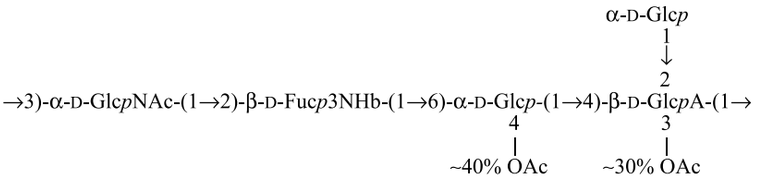
Abstract. O-specific polysaccharide (O-antigen) of the lipopolysaccharide of *Proteus penneri* 20 was studied using sugar analysis along with various one- and two-dimensional NMR spectroscopy techniques. The following structure of the polysaccharide was established:



It has the same carbohydrate backbone structure as that described earlier for *P. penneri* 16, in which the positions of the O-acetyl groups have not been determined. *P. penneri* 20 O-antiserum showed a strong cross-reactivity with the lipopolysaccharides of *P. penneri* 10, 16, 18, 32, 45 and *P. mirabilis* O17. These data enable classifying these strains together with *P. penneri* 20 in one *Proteus* serogroup, O17.

Key words: lipopolysaccharide; O-antigen; bacterial polysaccharide; O-acetyl group; serological classification; O-serogroup; *Proteus penneri.*

Introduction

Bacteria of the genus *Proteus* are a common cause of urinary tract infections, which may result in severe complications, such as acute or chronic pyelonephritis and formation of kidney and bladder stones. According to the serological classification based on the specificity of the outer-membrane lipopolysaccharide (LPS), strains of *P. mirabilis* and *P. vulgaris* are divided into 49 O-serogroups[7]. However, this classification does not

Abbreviations used: Fuc3NHb – 3,6-dideoxy-3-(3-hydroxybutyramido)galactose, HMQC – heteronuclear multiple-quantum coherence, LPS – lipopolysaccharide, OAc – O-acetyl group, OPS – O-specific polysaccharide.

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include the following strains: 1) two groups of *P. mirabilis* and *P. vulgaris* (20 strains in totally), which were described later[11]; 2) strains of two recently recognized medically important species *P. penneri* and *P. hauseri*, 3) the only representative of *P. myxofaciens* isolated from larvae of the gypsy moth[4]; and 4) three unnamed *Proteus* genomospecies 4, 5 and 6[6, 8–10]. Recently, based on the structural and serological investigation of the LPS, a number of additional O-serogroups have been established for strains of *P. penneri*[5, 12, 14, 19, 20]. In the present paper, we report on the structure of the O-specific polysaccharide (OPS) chain of the LPS of *P. penneri* 20 and serological cross-reactions of O-antiserum against this strain with the LPS of several other *P. penneri* strains as well as *P. mirabilis* O17. Based on these data, we propose classifying all these strains in the same *Proteus* serogroup, O17.

Materials and Methods

Bacterial strains and growth. Strains of *P. penneri* 10, 16, 18, 20, 32 and 45 were kindly provided by Prof. D. J. Brenner (Center for Disease Control, Atlanta, USA). *P. mirabilis* O17 was from the Collection of the Department of General Microbiology (Institute of Microbiology and Immunology, University of Łódź, Poland). The bacteria were grown on a nutrient broth (Warsaw Laboratory of Sera and Vaccines) supplemented with 1% glucose. Bacterial cells were separated by centrifugation, washed with distilled water, and lyophilized.

Isolation of the LPS and preparation of the alkali-treated LPS. The lipopolysaccharides were isolated by extraction of cells with hot aqueous phenol[18] and purified by treatment with trichloroacetic acid as described[22].

Alkali-treated LPS from *P. penneri* [10, 16, 32, 45] and *P. mirabilis* O17 were prepared by saponification of the LPS with 0.25 M NaOH (56°C, 2 h) followed by precipitation with ethanol. Alkali-treated LPS from *P. penneri* 18 and 20 were obtained by the action of aqueous 12% ammonia (12 h, 37°C) followed by removal of the reagent by concentration of the solution in vacuum and gel-permeation chromatography on a column of Sephadex G-50[15].

Preparation and O-deacetylation of the OPS. The LPS of *P. penneri* 20 was degraded with 2% acetic acid at 100°C until the precipitation of lipid A. High-molecular-mass OPS were isolated from the water-soluble portion by gel-permeation chromatography on Sephadex G-50 as described[15] using a Knauer differential refractometer (Germany) for monitoring.

O-deacetylation of the OPS was performed with aqueous 12% ammonia at 60°C for 2 h, and the modified polysaccharide was isolated by gel-permeation chromatography on Sephadex G-50.

Rabbit O-antiserum and serological assays. Polyclonal O-antiserum was obtained by immunization of rabbits with heat-inactivated bacteria of *P. penneri* 20 according to published procedure[21].

Agglutination and precipitation tests, SDS/PAGE, electrotransfer of LPS from gels to nitrocellulose sheets, immunostaining, passive immunohemolysis with increasing amounts (2–200 μg) of alkali-treated LPS, inhibition of passive immunohemolysis, absorption of O-antiserum and enzyme immunosorbent assay were carried out as described in detail previously[15].

NMR spectroscopy. NMR spectra were recorded on a Bruker DRX-500 spectrometer in solutions in D_2O at 50°C using acetone as the internal standard (δ_H 2.225 ppm, δ_C 31.45 ppm). Prior to measurements, the samples were deuterium-exchanged by freeze-drying from D_2O.

Results and Discussion

Structural study of the O-specific polysaccharide of Proteus penneri 20

The ^{13}C NMR spectrum of the OPS of *P. penneri* 20 (Fig. 1) was essentially identical to that of *P. penneri* 16[17] and, hence, the two polysaccharides have the same structure. This was additionally confirmed by comparison of the ^{1}H and ^{13}C NMR spectra of the O-deacetylated polysaccharides from both strains, which were also identical. The OPS of *P. penneri* 16 includes Glc, GlcA, GlcNAc, and a rarely occurring component, 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]-D-galactose (D-Fuc3NHb)[17]. It contains O-acetyl groups in minor quantities, but, although the structure of the repeating unit of the polysaccharide has been established (Fig. 2)[17], the sites of O-acetylation have not been determined.

In order to determine the location of the O-acetyl groups, the OPS of *P. penneri* 20 was studied in more details using NMR spectroscopy. Comparison of the one-dimensional ^{13}C NMR and two-dimensional ^{1}H, ^{13}C HMQC spectra of the initial OPS (Fig. 1) with the corresponding fully assigned spectra of the O-deacetylated polysaccharide[17] showed no significant changes in the chemical shifts of GlcNAc, Fuc3NHb, and the lateral glucose residue, whereas those of GlcA and the 6-substituted glucose were different. Compared with the O-deacetylated polysaccharide, in the initial OPS

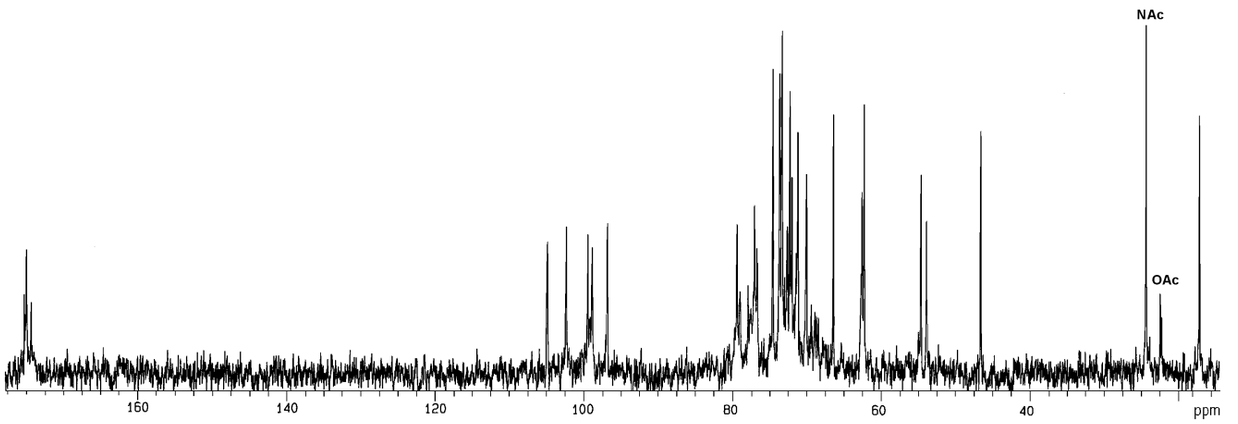

Fig. 1. ^{13}C NMR spectrum of the OPS of *P. penneri* 20. The region of CO resonances is not shown. NAc and OAc stand for the N-acetyl and O-acetyl groups, respectively

P. penneri 20

α-D-Glc*p*
1
↓
2
→3)-α-D-Glc*p*NAc-(1→2)-β-D-Fuc*p*3NHb-(1→6)-α-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→
4 3
| |
~40% OAc ~30% OAc

P. mirabilis O17

Etn*P*
|
6
→3)-α-D-Glc*p*NAc-(1→2)-β-D-Fuc*p*3NHb-(1→6)-α-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→

Fig. 2. Structures of the OPS of the cross-reactive LPS from *P. penneri* 20 and *P. mirabilis* O17[16]

the intensities of the signals of GlcA and the 6-substituted glucose residues were lower and additional signals for the corresponding O-acetylated residues appeared.

Position 3 is the only possible site of O-acetylation in GlcA, since positions 2 and 4 are glycosylated. Acetylation of this residue at O3 was confirmed by characteristic upfield displacements, due to β-effects of O-acetylation[13], of a part of the signals for C2 and C4 from δ 78.6 and 77.3 to δ 77.5 and 76.6, respectively. It was complicated to determine the effects of O-acetylation in the 6-substituted glucose residue owing to the coincidences of many signals for the O-acetylated residue with signals of other monosaccharides. However, the location of acetyl groups at O2 and O3 could be excluded, since neither β-effect on C1 and C4 nor an α-effect on C2 and C3 were observed. Hence, the 6-substituted Glc residue is O-acetylated at position 4. As judged by the relative intensities of the signals in the O-acetylated and non-acetylated residues, the degree of O-acetylation of GlcA and Glc is ~30 and ~40%, respectively. The full structure of the OPS of *P. penneri* 20 thus determined is shown in Fig. 2.

In *Proteus* OPS, the O-acetyl groups are rather common. In the most strains O-acetylation is non-stoichiometric and thus masks the regularity of the polymers. This feature is in accordance with the notion that O-acetylation is a post-polymerization modification of the OPS. The degree of O-acetylation varies from a negligible value to 95%, and it may be difficult to

determine the exact location of minor O-acetyl groups. For instance, in the OPS of *P. mirabilis* O23[3] and *P. vulgaris* O32[1], the location of the O-acetyl groups was determined only tentatively. The position of only one major O-acetyl group was determined in the polysaccharide of *P. penneri* 4[14], whereas the attachment sites of other, minor O-acetyl groups remain unknown. In the OPS of *P. penneri* 16, the positions of the O-acetyl groups, which are present in a minority of the repeating units, were not determined in previous studies[17], but were in this work.

Serological studies

When tested with various *Proteus* strains, in addition to the homologous bacteria, rabbit polyclonal *P. penneri* 20 O-antiserum agglutinated cells of *P. penneri* 10, 16, 18, 32, 45 and *P. mirabilis* O17. The specificity of the cross-reactivity was confirmed by a high reactivity of *P. penneri* 20 O-antiserum with the LPS from these strains in enzyme immunosorbent assay, passive immunohemolysis and its inhibition, and precipitation (Table 1).

Absorption of *P. penneri* 20 O-antiserum with alkali-treated LPS of each *P. penneri* strain (10, 16, 18, 32 and 45) completely removed both homologous and cross-reacting antibodies (Table 2). Absorption with *P. mirabilis* O17 antigen removed all antibodies to *P. mirabilis* O17 LPS and most antibodies to the LPS of *P. penneri* strains. These findings suggest the presence in *P. penneri* 20 O-antiserum of major antibodies, bound to an epitope(s) on the LPS of all strains studied, and minor antibodies to another epitope shared by all LPS except for that of *P. mirabilis* O17.

In Western blot (Fig. 3), *P. penneri* 20 O-antiserum recognized slowly migrating bands of all *P. penneri* strains, which correspond to high-molecular-mass LPS species consisting of the corelipid A moiety substituted with an OPS having a variable number of oligosaccharide repeating units. This finding is in accordance with the identity of the OPS structure, which was demonstrated by chemical studies of the OPS of *P. penneri* 16[17] and *P. penneri* 20 (this work). However, the banding pattern of the LPS of *P. penneri* 18 and 20 was markedly distinct from that of *P. penneri* 10, 16, 32 and 45 in the occurrence of both slowly and fast migrating bands of the OPS-containing LPS species. A lower average number of the repeating units in the OPS of *P. penneri* 18 and 20 compared with those of the other *P. penneri* strains was confirmed by gel-permeation chromatography on Sephadex G-50 of the alkali-degraded LPS (data not shown).

The presence in Western blot of band clusters for the LPS of *P. penneri* 10, 16, 32 and 45 indicates a model distribution of the O-antigen chain length, which is controlled by a genetically encoded protein regulator Wzz[2]. In contrast, the band pattern for the LPS of *P. penneri* 18 and 20 is characteristic for a random distribution of the O-antigen chain length, which

Table 1. Reactivity of the LPS of *Proteus* with *P. penneri* 20 O-antiserum

LPS from strain	EIA	PI	Precipitation	Inhibition of PI in the homologous test system
	reciprocal titre		minimal LPS dose	
			µg	ng
P. penneri 10	512 000	51 200	15.8	4
P. penneri 16	256 000	51 200	7.9	4
P. penneri 18	512 000	51 200	7.9	2
P. penneri 20	*512 000*	*51 200*	7.9	*4*
P. penneri 32	512 000	51 200	15.8	4
P. penneri 45	256 000	51 200	7.9	8
P. mirabilis O17	256 000	51 200	15.8	8

Data for the homologous LPS are italicsed. EIA – enzyme immunosorbent assay; PI – passive immunohemolysis.

Table 2. Passive immunohemolysis of the alkali-treated LPS of *Proteus* with absorbed *P. penneri* 20 O-antiserum

O-antiserum absorbed with strain	Reciprocal titre with alkali-treated LPS from						
	P.p. 10	*P.p.* 16	*P.p.* 18	*P.p.* 20	*P.p.* 32	*P.p.* 45	*P. p.* O17
Control	51 200	51 200	51 200	51 200	51 200	51 200	51 200
P. penneri 10, 16, 18, 20, 32 or 45	<100	<100	<100	<100	<100	<100	<100
P. mirabilis O17	3 200	3 200	6 400	6 400	3 200	6 400	<100

Sheep red blood cells were used as a control. *P.p.* and *P.m.* stand for *P. penneri* and *P. mirabilis*, respectively.

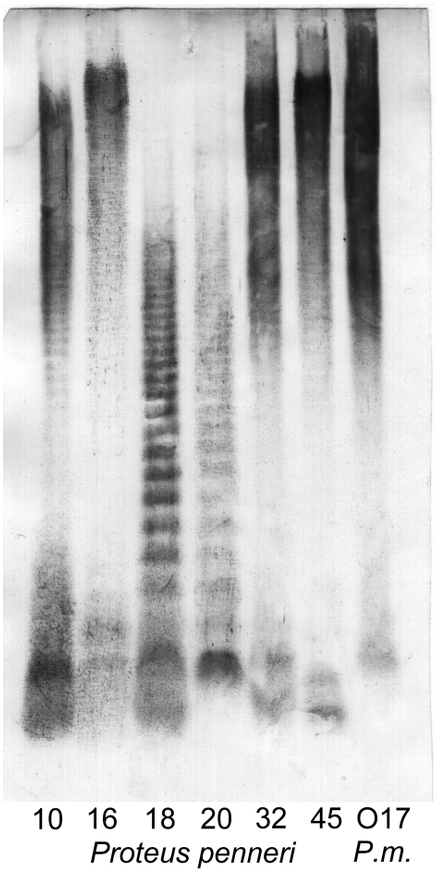

Fig. 3. Western blot of the LPS of *P. penneri* 10, 16, 18, 20, 32, 45 and *P. mirabilis* O17 with *P. penneri* 20 O-antiserum. *P.m.* stands for *P. mirabilis*

may result from a mutation affecting the *wzz* gene and, thus, the mechanism of the O-antigen modulation.

P. penneri 20 O-antiserum also recognized slowly migrating bands of *P. mirabilis* O17 LPS, thus showing that an epitope(s) shared by *P. penneri* 20 and *P. mirabilis* O17 is located on the OPS. The serological relatedness of these strains correlates with the similarity of the structures of their OPS, which have the same carbohydrate main chain and differ in the presence of a terminal glucose residue and O-acetyl groups in the former and ethanolamine phosphate in the latter strain (Fig. 2).

On the basis of these data, we propose to classify *P. penneri* 10, 16, 18, 20, 32 and 45 together with *P. mirabilis* O17 in one *Proteus* serogroup, O17, and to divide this serogroup into two subgroups, one for *P. mirabilis* O17 and the other for the strains of *P. penneri*. The common, major epitope (O17a) is associated with a partial structure of the OPS shared by all strains of serogroup O17, whereas a minor epitope present in the LPS of *P. penneri* could be linked to the lateral glucose residue or located on the LPS core region. The characterization of epitopes on the LPS of *P. mirabilis* O17 requires additional studies with *P. mirabilis* O-antiserum. *Proteus* O17 is the first serogroup described that combines strains of *P. mirabilis* and *P. penneri.*

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