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Carbohydrate Research 337 (2002) 1535–1540

CARBOHYDRATE RESEARCH

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Structure of the O-specific polysaccharide of *Proteus penneri* 103 containing ribitol and 2-aminoethanol phosphates

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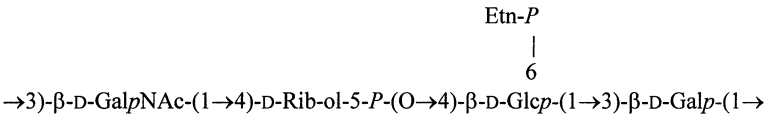
Received 1 February 2002; received in revised form 13 May 2002; accepted 6 August 2002

Abstract

The O-specific polysaccharide of the lipopolysaccharide of *Proteus penneri* strain 103 was studied using ^{1}H and ^{13}C NMR spectroscopy, including 2D COSY, TOCSY, NOESY, H-detected ^{1}H,^{13}C HMQC, ^{1}H, ^{31}P HMQC, and HMBC experiments. It was found that the polysaccharide is built up of oligosaccharide-ribitol phosphate repeating units and thus resembles ribitol teichoic acids of Gram-positive bacteria. The following structure of the polysaccharide was established:



where Etn and Rib-ol are ethanolamine and ribitol, respectively. This structure is unique among the known structures of *Proteus* O-antigens and, therefore, we propose classification of the strain studied into a new *Proteus* serogroup, O73. The molecular basis for cross-reactivity between O-antiserum against *P. penneri* 103 and O-antigens of *P. mirabilis* O33 and D52 is discussed.

Keywords: *Proteus penneri*; O-antigen; O-specific polysaccharide; Lipopolysaccharide; Ribitol phosphate, Ethanolamine phosphate; ^{1}H, ^{13}C, ^{31}P NMR

1. Introduction

Bacteria of the genus *Proteus* cause urinary tract infections, which can lead to severe complications, such as acute or chronic pyelonephritis and formation of bladder and kidney stones. The outer membrane lipopolysaccharide (LPS) is a somatic antigen and is considered as a potential virulence factor of *Proteus*.[1,2] The serological O-specificity of bacterial smooth forms is defined by the structure of the polysaccharide chain of LPS (O-antigen). Based on the somatic antigens, two species, *P. mirabilis* and *P. vulgaris*, have been classified into 49 O-serogroups.[3] However, this classification does not include two groups of representatives of *P. mirabilis* and *P. vulgaris* (totally 20 strains), which were described later,[3,4] nor two other recently recognised medically important species, *Proteus penneri* and *Proteus hauseri*, nor *Proteus* genomospecies 4, 5, and 6.[5–8] Recently, based on the immunochemistry of LPS, a number of additional O-serogroups for *P. penneri* strains have been created.[9–12] Here we report on the structure of the O-specific polysaccharide from LPS of *P. penneri* 103 and propose to classify this strain into a new *Proteus* serogroup O73.

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0008-6215/02/$ - see front matter
PII: S0008-6215(02)00265-3

2. Results and discussion

Structure of the O-specific polysaccharide.—LPS was isolated from dried bacterial cells of *P. penneri* 103 by the phenol–water extraction[13] and degraded with dilute acetic acid to give a high molecular mass polysaccharide. Sugar analysis of the polysaccharide revealed the presence of almost equal amounts of Glc and Gal as well as GalN, which were identified using sugar and amino acid analysers, respectively. GLC showed the presence of ribitol (Rib-ol). Determination of the absolute configurations by GLC of the acetylated (*S*)-2-butyl glycosides derived from sugars and (*S*)-2-butyl glycerate derived from ribitol showed that all components have the D configuration.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained signals for three anomeric carbons at δ 102.1, 104.0, and 105.0, one carbon linked to nitrogen at δ 52.1, one N-acetyl group at δ 23.3 (CH_3) and 175.7 (CO), one CH_2N-group of ethanolamine (Etn) at δ 41.0, six CH_2O groups at δ 61.7–66.3 (C-6 of three sugar residues, C-1 and C-5 of ribitol, and CH_2O-group of Etn), and 14 other carbons linked to oxygen at δ 68.7–83.3. Accordingly, the ^1H NMR spectrum contained signals for three anomeric protons at δ 4.51, 4.68, and 4.71, one Etn residue at δ 3.28 (CH_2N, t, 2H), and one N-acetyl group at δ 2.07 (CH_3, s, 3H). The ^{31}P NMR spectrum contained signals for two phosphate groups at δ 1.3 and 1.7. Therefore, the polysaccharide has a repeating unit containing one residue each of Glc, Gal, and GalNAc as well as Rib-ol and Etn. The evidence suggested that Rib-ol and Etn were linked to Glc via phosphate groups.

The ^1H and ^{13}C NMR spectra of the polysaccharide were assigned using 2D COSY, TOCSY, and $^1H,^{13}C$ HMQC experiments (Tables 1 and 2). Based on typical $J_{H,H}$ coupling constants values, the three sugar spin systems were assigned. The Glc*p* residue was identified by large $J_{3,4}$ and $J_{4,5}$ values of ~ 10 Hz, as compared with values ≤ 3 Hz for the Gal*p* and GalNAc*p* residues. GalNAc was distinguished from Gal by correlation of the proton at the nitrogen-bearing carbon (H-2) to the corresponding carbon (C-2) at δ 52.1, which was revealed by the $^1H,^{13}C$ HMQC experiment. The spin system of Rib-ol was identified based on $^1H,^{13}C$ correlations for five remaining oxygen-linked carbons in the $^1H,^{13}C$ HMQC spectrum.

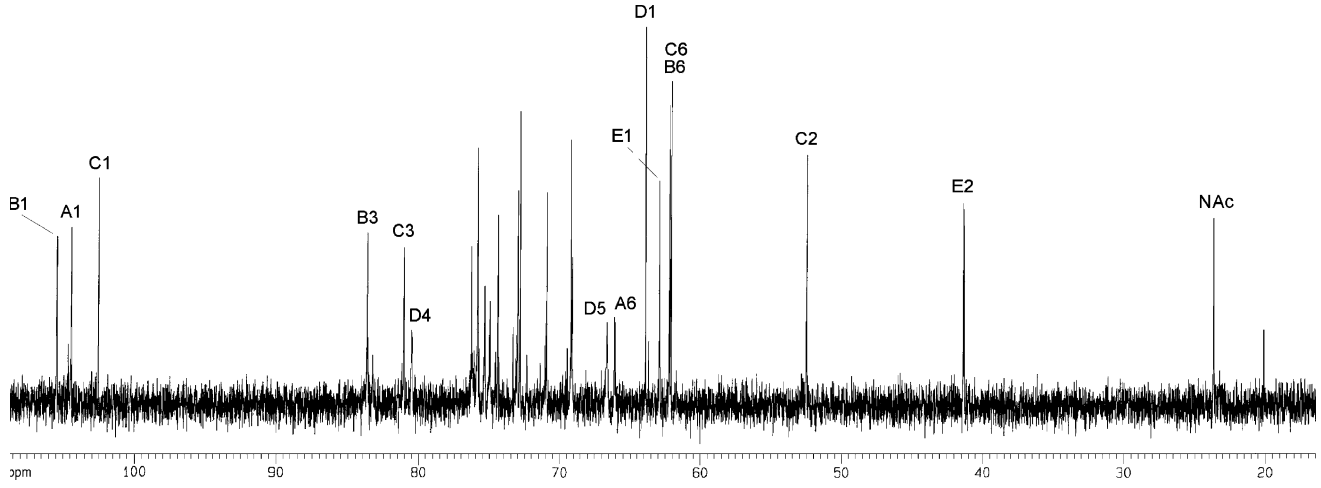

Fig. 1. ^{13}C NMR spectrum of the O-specific polysaccharide of *P. penneri* 103. Arabic numerals refers to carbons in sugar and ribitol residues denoted by letters as follows: A, Glc; B, Gal; C, GalNAc; D, Rib-ol; E, Etn.

Table 1
^1H NMR data (δ, p.p.m.) for the O-specific polysaccharide of *P. penneri* 103. Chemical shift for NAc group is δ 2.07

Residue	H-1a H-1b	H-2	H-3	H-4	H-5a H-5b	H-6a H-6b
Etn-*P*-(O→	4.11	3.28				
→3)-β-D-Gal*p*NAc-(1→	4.71	4.05	3.88	4.15	3.70	3.70–3.81
→4)-D-Rib-ol-5-*P*-(O→	3.63 3.81	3.82	3.82	4.13	3.99 4.18	
→4)-β-D-Glc*p*-(1→	4.68	3.43	3.70	3.94	3.71	4.24 3.99
→3)-β-D-Gal*p*-(1→	4.51	3.67	3.77	4.20	3.66	3.70–3.81

Table 2
^{13}C NMR data (δ, ppm; $J_{C,P}$ in Hz in parentheses) for the O-specific polysaccharide of *P. penneri* 103

Residue	C-1	C-2	C-3	C-4	C-5	C-6
Etn-*P*-(O→	62.5 (5.0)	41.0 (7.4)				
→3)-β-D-Gal*p*NAc-(1→	102.1	52.1	80.7	68.8	75.5	61.7[a]
→4)-D-Rib-ol-5-*P*-(O→	63.5 (~6)	72.5[b] (5.3)	72.6[b]	80.1	66.3	
→4)-β-D-Glc*p*-(1→	104.0	74.0	75.9	74.9 (6.5)	74.6 (6.1)	65.7 (~4)
→3)-β-D-Gal*p*-(1→	105.0	70.6	83.3	68.7	75.5	61.8[a]

Chemical shifts for NAc groups are δ 23.3 (CH_3) and δ 175.7 (CO).
[a,b] Assignment could be interchanged.

As judged by relatively large $J_{1,2}$ coupling constant values of 8–9 Hz determined from the ^1H NMR spectrum, all three sugar residues are β-linked. This conclusion was confirmed by a ROESY experiment, which revealed intraresidue correlations between H-1 and H-3, H-5 for all three monosaccharides, which is typical of β-pyranosides.

Low-field displacements (α-effects of glycosylation) of the signals for C-3 of Gal*p* (+9.5 ppm), C-3 of Gal*p*NAc (+8.3 ppm), and C-4 of Rib-ol (+6.6 ppm), as compared with their positions in the corresponding non-substituted compounds,[14] demonstrated the modes of substitution of the monosaccharides and ribitol in the repeating unit. Also positive but smaller effects of phosphorylation, which were observed for the signals for C-4 and C-6 of Glc*p* (+4.2 and +3.9 ppm, respectively) and C-5 of Rib-ol (+2.5 ppm), showed the phosphorylation sites.

The glycosylation pattern was confirmed and the sequence of the monosaccharides determined by ROESY and ^1H,^{13}C HMBC experiments. The ROESY spectrum showed the following interresidue correlations between the anomeric and linkage protons: Glc*p* H-1/Gal*p* H-3, Gal*p* H-1/Gal*p*NAc H-3, and Gal*p*NAc H-1/Rib-ol H-4 at δ 4.68/3.77, 4.51/3.88, and 4.71/4.13, respectively. Accordingly, the HMBC spectrum revealed the following correlations: Gal*p*NAc H-1/Rib-ol, C-4 Glc*p* H-1/Gal*p* C-3, and Gal H-1/Gal*p*NAc C-3 at δ 4.71/80.1, 4.68/83.3, and 4.51/80.7, respectively.

The ^1H,^{31}P HMQC experiment showed a correlation of the phosphorus signal at δ 1.7 with the signals for Etn at δ 4.11 and H-6a, H-6b of Glc*p* at δ 3.99 and 4.24. Hence, Etn-*P* is attached at position 6 of Glc*p*. The other phosphorus signal at δ 1.3 gave cross-peaks with the signals for H-5a, H-5b of Rib-ol at δ 3.99 and 4.18, as well as with the H4 signal of Glc*p* at δ 3.94. Therefore, ribitol and glucose residues are connected via a phosphate group. This conclusion was in agreement with splitting, due to coupling between carbon and phosphorus, of the signals for C-1 and C-2 of ethanolamine, C-4 and C-5 of ribitol, and C-4–C-6 of Glc*p* in the ^{13}C NMR spectrum (Table 2).

On the basis of these data, it was concluded that the O-specific polysaccharide of *P. penneri* 103 has the structure shown in Fig. 2, which is unique among the known structures of *Proteus* O-antigens. As the O-polysaccharide of *Proteus mirabilis* O16,[15] it resembles ribitol teichoic acids of Gram-positive bacteria, whereas the O-polysaccharide of *Proteus vulgaris* O12 is built up like a glycerol teichoic acid.[16]

Serological studies.—Lipopolysaccharides from 38 strains of *Proteus penneri* and 65 strains from 49 O-serogroups of *P. mirabilis* and *P. vulgaris* were examined in passive immunohemolysis with rabbit polyclonal O-antiserum against *P. penneri* 103. A positive reaction was observed for only three LPS, including the homologous LPS and those of *P. mirabilis* O33 and D52 (titres 1:25,600, 1:800–1600, and 1:800, respectively). In inhibition of passive immunohemolysis in the hemolytic system *P. penneri* 103 O-antiserum/*P. penneri* 103 alkali-treated LPS, the homologous LPS showed a strong inhibitory activity in a dose 4 ng, whereas two other LPS, which reacted with *P. penneri* 103 O-antiserum markedly weaker, were inactive in a dose 1000 ng. Similarly, in enzyme immunosorbent assay, *P. penneri* 103 O-antiserum showed the strongest reactivity with the homologous LPS, whereas LPS from *P. mirabilis* O33 and D52 reacted significantly weaker (Fig. 3).

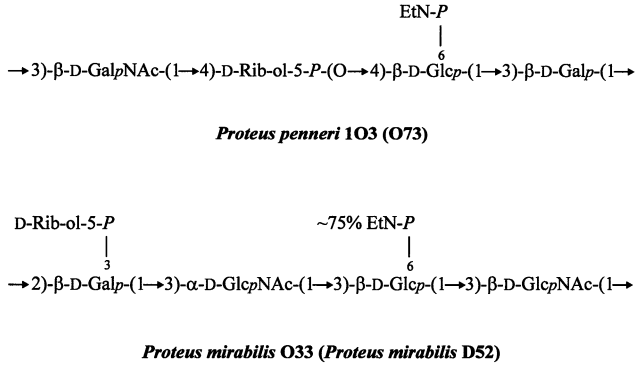

Fig. 2. Structures of the O-specific polysaccharides of *P. penneri* 103 (this work) and *P. mirabilis* O33 (*P. mirabilis* D52).[17]

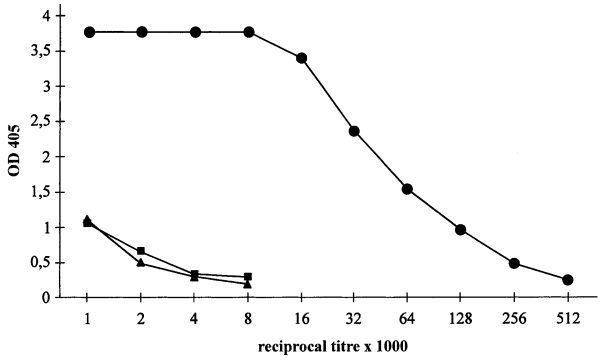

Fig. 3. Reactivity of *P. penneri* 103 O-antiserum with LPS–BSA complex in enzyme immunosorbent assay. ● LPS *P. penneri* 103; ■ LPS *P. mirabilis* O33; ▲ LPS *P. mirabilis* D52.

The reactivity of *P. penneri* 103 O-antiserum in passive immunohemolysis was completely abolished when the antiserum was absorbed with the homologous LPS (Table 3). Absorption with LPS of either *P. mirabilis* O33 or D52 abolished the binding of the LPS from both these strains but did not influence the reaction of the homologous LPS.

In Western blot (Fig. 4), *P. penneri* 103 O-antiserum bound strongly to both slow and fast migrating bands of the homologous LPS, which correspond to high- and low-molecular-mass LPS species consisting of the core-lipid A moiety with or without an O-chain polysaccharide attached. In contrast, only slow moving bands of *P. mirabilis* O33 and D52 bound slightly *P. penneri* 103 O-antiserum.

These data indicate relatedness between the O-antigen of *P. penneri* 103 on the one hand and those of *P. mirabilis* O33 and D52 on the other hand. Recently, it has been established that the O-antigens of *P. mirabilis* O33 and D52 strains are structurally and serologically identical, whereas the core parts of their LPS are different.[17] Comparison of the O-specific polysaccharide structures of *P. penneri* 103 with those of *P. mirabilis* O33 and D52 (Fig. 2) showed that they have a β-D-Glc*p*-6-*P*-Etn fragment in common, which can be responsible for the cross-reactivity. At the same time, *P.*

Fig. 4. Western blot of *Proteus* LPS with *P. penneri* 103 O-antiserum. P.m. and P.p. stand for *P. mirabilis* and *P. penneri*, respectively.

penneri 103 O-antiserum did not cross-react with *Proteus* LPS containing *P*-Etn linked to other monosaccharides, e.g. α-D-Glc*p*NAc-6-*P*-Etn in *P. mirabilis* O16,[15] β-D-Glc*p*NAc-6-*P*-Etn in *P. mirabilis* O27 and *P. penneri* 8[18] or β-D-Gal*p*NAc-6-*P*-Etn in *P. mirabilis* O41 (authors' unpublished data). This finding shows that the ethanolamine phosphate group alone is not sufficient for a serological relatedness but may create a cross-reactive epitope when the carrying monosaccharide is also the same. This conclusion is favoured by a strong cross-reactivity of *P. mirabilis* O14a,14b and O14a,14c, which both contain the same 2-[(*R*)-1-carboxyethylamino]ethyl phosphate group at position 6 of the same sugar (α-D-Gal*p*) but different composition and structures of the rest of repeating units.[19]

Table 3
Reactivity of absorbed *P. penneri* 103 O-antiserum with *Proteus* LPS in passive immunohemolysis. Sheep red blood cells were used as control.

Origin of alkali-treated LPS used for absorption	Reciprocal titre for alkali-treated LPS from:		
	P. penneri 103	*P. mirabilis* O33	*P. mirabilis* D52
Control	25,600	800–1600	800
P. penneri 103	<100	<100	<100
P. mirabilis O33	25,600	<100	<100
P. mirabilis D52	25,600	<100	<100

Taking into account that the cross-reactivity with *P. mirabilis* O33 and D52 is rather weak and that the O-specific polysaccharide has a unique structure, *P. penneri* 103 may be considered as a separate strain in respect to the O-antigenic specificity. Therefore, we propose to classify this strain into a new *Proteus* serogroup, O73.

3. Experimental

Bacterial strains.—*P. penneri* strain 103 (No CL 191/90 = 21B) was kindly provided by Dr. Barry Holmes (National Collection of Type Culture, Central Public Health Laboratory, London, UK). It was isolated from a mid stream urine of patient from Bristol (Avon, UK). Other *P. penneri* strains were from the collection of the Department of General Microbiology, University of Lódz, Poland. 37 strains of *P. mirabilis* and 28 strains of *P. vulgaris* were from the Czech National Collection of Type Cultures (CNCTC, Institute of Microbiology and Genetics, Prague, Czech Republic).

Dry bacterial cells of *P. penneri* 103 were obtained from an aerated culture as described previously.[20]

Isolation and degradation of lipopolysaccharide.—LPS was isolated from dried bacterial cells of *P. penneri* 103 by extraction with hot aqueous phenol[13] and purified by treatment with cold aqueous 50% CCl_3CO_2H followed by dialysis of the supernatant.[17] The yield of LPS was 10.9% of dried bacterial mass.

Acid degradation of LPS was performed with 0.1 M sodium acetate buffer (pH 4.5) at 100 °C for 1.5 h. The O-specific polysaccharide was isolated by gel-permeation chromatography on a column (3 × 65 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer (pH 4.5) in a yield 20% of the LPS weight.

Alkali-treated LPS was prepared by saponification of LPS with 0.25 M NaOH (56 °C, 2 h) followed by precipitation with ethanol.

Rabbit antiserum and serological assays.—Polyclonal *P. penneri* 103 O-antiserum was obtained by immunization of rabbits with heat-inactivated bacteria according to a published procedure.[21] SDS-PAGE, electrotransfer of LPS from gel to nitrocellulose sheets, immunostaining, and absorption experiments were carried out as described in detail elsewhere.[22] LPS was used as antigen in enzyme immunosorbent assay,[23] and passive immunohemolysis was performed with increasing amounts (2–200 μg) of alkali-treated LPS.[17]

Sugar and ribitol analysis.—The polysaccharide was hydrolysed with 3 M CF_3CO_2H (100 °C, 4 h), amino and neutral sugars were identified using a Biotronik LC-2000 amino acid and sugar analysers as described.[24] Ribitol was identified in the polysaccharide hydrolysate by GLC as the acetylated derivative using a Hewlett-Packard 5890 chromatograph equipped with an Ultra 2 capillary column. The absolute configuration of the monosaccharides was determined by GLC of acetylated (*S*)-2-butyl glycosides.[25,26] The absolute configuration of ribitol was determined by periodate oxidation followed by bromine oxidation, acid hydrolysis, and GLC of the derived acetylated (*S*)-2-butyl D-glycerate as described.[15]

NMR spectroscopy.—^1H, ^{13}C, and ^{31}P NMR spectra were recorded with a Bruker DRX-500 spectrometer in D_2O at 60 °C using internal acetone (δ_H 2.225, δ_c 31.45) or external aqueous 85% H_3PO_4 (δ_P 0) as reference. 2D NMR spectra were obtained using standard Bruker software, and the xwinnmr 2.1 program (Bruker) was used to acquire and process NMR data. Mixing times of 200 and 300 ms were used in TOCSY and ROESY experiments, respectively.

Acknowledgements

This work was supported by grants 6 PO4A 074 20 and 6 PO4A 045 21 of the Sciences Research Committee (KBN, Poland) and grant 02-04-48767 of the Russian Foundation for Basic Research.

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