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The structure of the polysaccharide produced by *Proteus vulgaris* (ATCC 49990)

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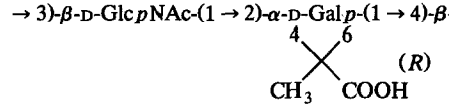
ABSTRACT

An exocellular polysaccharide produced by a clinical isolate of *Proteus vulgaris* (ATCC 49990) was shown by composition, methylation, periodate oxidation, and nuclear magnetic resonance analyses to be composed of repeating trisaccharide units containing D-galactose, 2-acetamido-2-deoxy-D-glucose and pyruvic acid (2:1:1), and having the structure:



The homologus smooth lipopolysaccharide of the *P. vulgaris* strain was determined to have an O-polysaccharide component having the same structure as the above extracellular polysaccharide.

INTRODUCTION

The genus *Proteus* contains two major species, *P. mirabilis* and *P. vulgaris* defined by Wenner and Rettger[1]. The organisms are widely distributed in nature and under certain conditions grow in the animal body and cause septic infections. In particular they are an important cause of urinary tract infections. Our interest in *Proteus* infections is directed towards discovering the possible role of *Proteus* lipopolysaccharide (LPS) and polysaccharide (PS) in the pathogenesis of urinary tract infections and in urinary stone formation. As part of this study the fine chemical structural analysis of the LPS and CPS produced by clinical isolates of virulent strains of *P. mirabilis, P. vulgaris*, and related *P. rettgeri*, and *Morganella morgani* has been undertaken.

The classification scheme of Kaufmann and Perch defined 49 different *P. mirabilis* and *P. vulgaris* serotypes based on the specific structures of the O-poly-

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saccharide components of respective somatic LPS[2,3]. Penner and Hennessy[4] developed separate O-grouping schemes for the serotyping of clinical isolates of *P. vulgaris* and *P. mirabilis*. In contrast to the extensive studies which have been made of the structures of the LPS produced by different genera of the *Enterobacteriaceae*, only the analysis of the LPS O-polysaccharides *P. vulgaris* O19[5] and 5/43 belonging to OX19 group[6] have been reported.

Our preliminary analysis data on several clinical strains of *P. mirabilis* and *P. vulgaris* have shown them to produce acidic polysaccharides whose structures have been indicated to be identical with their homologous LPS O-polysaccharides, giving rise to the question of their biological origin, serological significance and role in the pathogenesis of urinary infections. In this paper we describe the characterization of the acidic PS produced by *P. vulgaris* (NRCC 4418, ATCC 49990).

RESULTS AND DISCUSSION

Cells of a clinical isolate of *Proteus vulgaris* (NRCC 4418, ATCC 49990) were extracted by a modified[8] hot aqueous phenol method to give an aqueous phase LPS (ca. 8% yield) recovered and purified by ultracentrifugation (105 000*g*, 12 h, 4°C) as previously described[7]. No LPS was obtained from the phenol phase of the extraction.

A polysaccharide (PS) precipitated by ethanol from the ultracentrifugate remaining after the removal of the precipitated gel of LPS, was obtained as the void volume fraction by Sephadex G-50 gel filtration chromatography (ca. 4% yield).

The PS had $[\alpha]_D$ +45° (*c* 9.6, H_2O). Anal. Found: C, 41.34; H, 5.03; N, 2.41; and ash, 3.4%. The PS on hydrolysis and quantitative analysis by GLC[9] was shown to be composed of D-galactose (D-Gal) and 2-amino-2-deoxy-D-glucose (D-GlcN) (2:1), the configurations being determined by capillary GLC of their trimethylsilylated (2*R*)-butyl glycoside derivatives[10]. Quantitative analysis of the hydrolysis products by HPLC showed that pyruvic acid was present corresponding to approximately one part per trisaccharide unit. The pyruvic acid constituent was released by autohydrolysis of the PS (pH 2.0, 16 h, 80°C), indicating that it was present as a ketal substituent.

The ^1H NMR spectrum of the PS confirmed the identity of the pyruvic acid substituent since it showed characteristic methyl proton signals at δ 1.57 (s, 3 H) of ketal-linked pyruvate. A methyl signal at δ 2.02 (s, 3 H), due to an acetamido substituent, showed that the D-GlcN residues were present as *N*-acetyl derivatives. The spectrum also showed *inter alia*, three H-1 signals at δ 5.18 (1 H, $J_{1,2}$ 3.0 Hz), 4.64 (1 H, $J_{1,2}$ 8.4 Hz), and 4.47 (1 H, $J_{1,2}$ 7.5 Hz), indicating that the PS was composed of a repeating trisaccharide unit containing one α-D- and two β-D-hexopyranosyl residues.

Consistent with the above conclusions, the ^{13}C NMR spectrum of the PS (Fig. 1A) showed resonances at δ 174.1, 99.5 and 25.7 attributed to the carbonyl, tertiary carbon, and methyl resonances of the ketal-substituted pyruvate residue. The

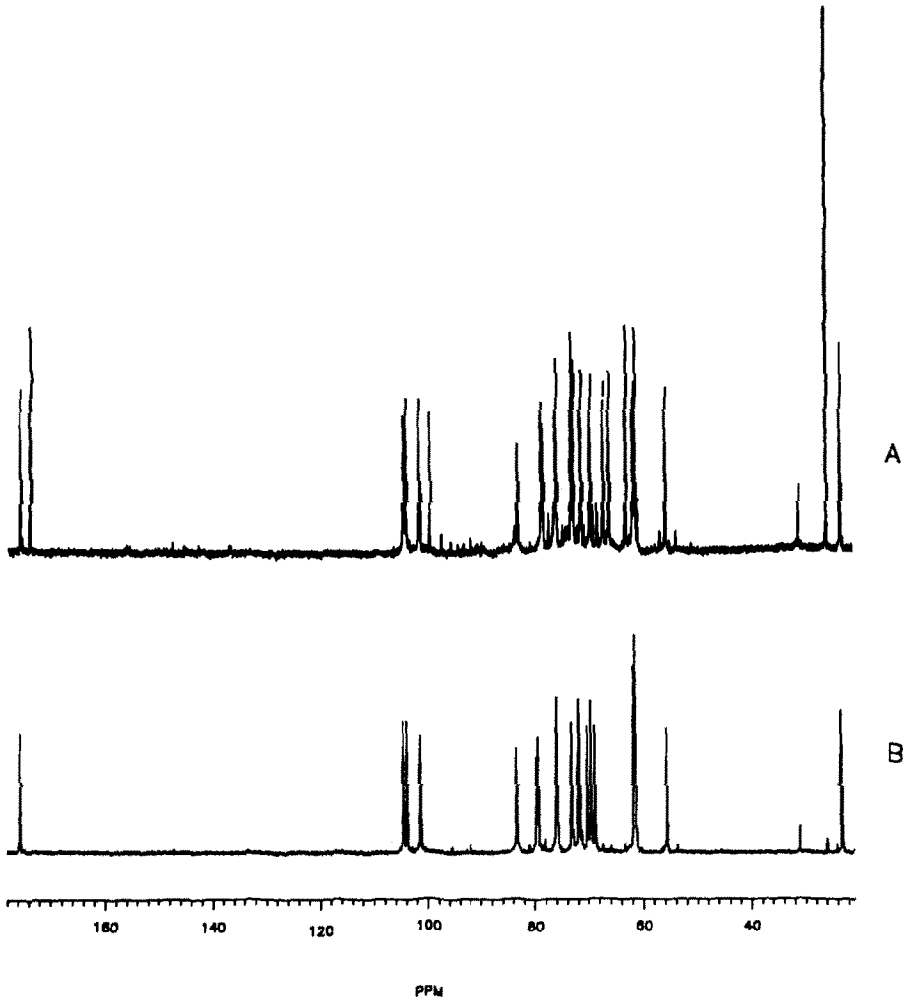


Fig. 1. ^{13}C NMR spectrum (125 MHz) of (A) Native PS and (B) Depyruvylated PS of *P. vulgaris* (ATCC 49990).

chemical shift of the latter resonance is characteristic of an axially orientated methyl group of a 4,6-*O*-substituted pyruvic ketal on a D-Gal*p* residue[11,12], thus allowing the substituent to be assigned the *R* configuration. These signals were absent from the spectrum of the depyruvylated PS (Fig. 1B). The spectrum of the PS also showed *inter alia* three C-1 signals at δ 104.4 ($J_{C,H}$ 164 Hz), 103.5 ($J_{C,H}$ 163 Hz), and 101.5 ($J_{C,H}$ 175 Hz), together with signals at δ 175.8 (CH_3CONH) and 23.2 (CH_3CONH) from the D-GlcNAc residue.

The depyruvylated PS obtained on autohydrolysis had $[\alpha]_D$ +58° (*c* 6.0, H_2O) and gave ^1H and ^{13}C NMR spectra (Fig. 1B) in which the chemical shifts and coupling constants of the anomeric resonances (Tables I and II) were similar to those observed in the spectra of the native PS.

TABLE I

^{1}H NMR data for the H-1 protons of the polysaccharide produced by *P. vulgaris* (ATCC 49990) and its degradation products [a]

Carbohydrate	Glycosyl residue		
	→ 2)-α-D-Gal*p*-(1 →	→ 3)-β-D-Glc*p*NAc-(1 →	→ 4)-β-D-Gal*p*-(1 →
Native O-PS	5.18 (3.0)	4.64 (8.4)	4.47 (7.5)
Depyruvylated O-PS	5.19 (3.5)	4.66 (8.4)	4.47 (7.5)
Oligosaccharide *B*	5.29 (3.1)	4.69 (8.3)	
Oligosaccharide *A*1		4.67 (8.3)	

[a] Chemical shifts are recorded in ppm at 27°C using acetone as an internal standard (δ 2.225) and coupling constants ($J_{1,2}$) measured in Hertz are given in parentheses.

Methylation analysis of the depyruvylated PS (Table III) gave 3,4,6-tri-*O*-methyl-D-Gal, 2,3,6-tri-*O*-methyl-D-Gal, and 4,6-di-*O*-methyl-D-GlcNMe showing that the polysaccharide was an unbranched linear polymer of a repeating trisaccharide unit containing 1,2- and 1,4-linked D-Gal*p* residues and a 1,3-linked D-Glc*p*N residue. Similar methylation analysis of the native PS (Table III) indicated that the 1,4-linked D-Gal and 1,3-linked D-GlcNAc residues were unsubstituted, but the detection of 3-*O*-methyl-D-Gal, which must originate from the 1,2-linked D-Gal residues, indicates that these residues are substituted as 1-carboxyethylidene acetals at the C-4 and C-6 positions.

Determination of the glycose linkage sequence and anomeric configurations were made through the characterization of the periodate oxidation products of the native PS and its depyruvylated derivative (Scheme 1).

Periodate oxidation[13] of the depyruvylated PS (Scheme 1), followed by reduction ($NaBH_4$) and mild hydrolysis, gave on Bio-Gel P-2 chromatography, an oligosaccharide *A* (K_{av} 0.15) having $[\alpha]_D$ −25° (*c* 2.0, H_2O), which on reduction ($NaBH_4$) gave oligosaccharide *A*1 composed of D-GlcNAc and glycerol (1:1). Methylation analysis of *A1* gave 3,4,6-tri-*O*-methyl-D-GlcNMe and 1,3-di-*O*-methylglycerol. The ^{1}H NMR spectrum of *A*1 showed an H-1 signal at δ 4.67 ($J_{1,2}$ 8.3 Hz), and its ^{13}C spectrum showed a C-1 resonance at δ 101.9 ($J_{C,H}$ 163 Hz)

TABLE II

^{13}C NMR data for the C-1 carbon atoms of the polysaccharide produced by *P. vulgaris* (ATCC 49990) and is degradation products [a]

Carbohydrate	Glycosyl residue		
	→ 2)-α-D-Gal*p*-(1 →	→ 3)-β-D-Glc*p*NAc-(1 →	→ 4)-β-D-Gal*p*-(1 →
Native O-PS	101.5 (175)	103.5 (163)	104.4 (164)
Depyruvylated O-PS	101.5 (175)	104.0 (163)	104.8 (163)
Oligosaccharide *B*	100.8 (173)	101.9 (163)	
Oligosaccharide *A*1		101.9 (163)	

[a] Chemical shifts are measured in ppm at 27°C using acetone as an internal standard (δ 31.07) and coupling constants ($J_{C,H}$) measured in Hertz are given in parentheses.

TABLE III

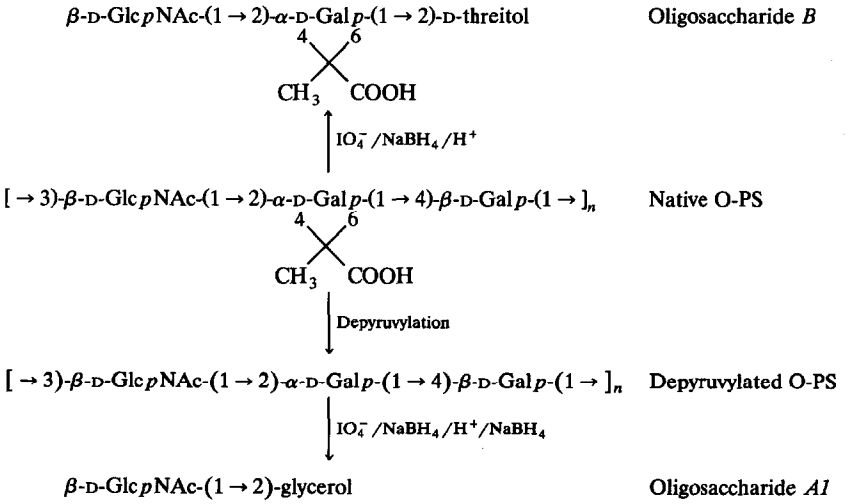
Methylation data for the analysis of the polysaccharide produced by *P. vulgaris* (ATCC 49990) and its degradation products [a]

Methylated glycitol	T_{GM}	Native O-chain	Depyruvylated O-chain	Oligo *B*
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol	1.35	1.00	1.00	
1,2,5-Tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-galactitol	1.40		1.02	
1,2,4,5,6-Tetra-*O*-acetyl-3-*O*-methyl-D-galactitol	2.47	0.97		1.00
1,5-Di-*O*-acetyl-2-deoxy-2-(*N*-methylacetamido)-3,4,6-tri-*O*-methyl-D-glucitol	2.50			0.79
1,3,5-Tri-*O*-acetyl-2-deoxy-2-(*N*-methylacetamido)-4,6-di-*O*-methyl-D-glucitol	2.76	0.90	0.88	

[a] GLC capillary column DB-17 (0.32 mm × 30 m) using a program of start 200°C (2 min) at 1°C/min to 220°C. Retention times are quoted relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (T_{GM} = 1.00).

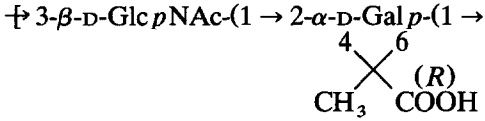
characteristic of a β-D linkage[14]. Since *A*1 can be assigned the structure β-D-Glc*p*NAc-(1,2)-glycerol the sequence of the PS can be established as →3)-β-D-Glc*p*NAc-(1→2)-D-Gal*p*-(1→4)-D-Gal*p*-(1→. The anomeric configurations of the D-Gal*p* residues remained to be determined.

Periodate oxidation of the native PS resulted in oxidation of the 1,4-linked D-Gal residues. Reduction ($NaBH_4$) and mild hydrolysis of the oxidation product afforded, on Bio-Gel P-2 chromatography, an oligosaccharide (*B*) (K_{av} 0.64) which had $[\alpha]_D$ +58° (*c* 0.8, H_2O) and was composed of D-GlcNAc, D-Gal, threitol, and pyruvic acid (1:1:1:1) Methylation analysis of *B* (Table III) showed it to have a nonreducing terminal D-Glc*p*NAc residue linked 1,2 to the 4,6-*O*-pyruvic-substituted D-Gal*p* residue that was linked to a threitol end group originating from



Scheme 1. Degradation studies on the polysaccharide produced by *P. vulgaris* (ATCC 49990).

the original 1,4-linked D-Gal*p* residue. Since the ^{1}H and ^{13}C NMR spectra of *B* (Tables I and II) showed that it contained an α-D and a β-D linkage, *B* can be characterized as having the structure β-D-Glc*p*NAc-(1 → 2)-α-D-Gal*p*-(1 → 2)-threitol in which the 1,2-linked D-Gal*p* residue was substituted at the 4,6-positions by a ketal-linked pyruvic acid substituent having the *R* configuration. Consideration of the structures of *A* and *B* leads to the conclusion that the repeating trisaccharide unit of the PS has the structure:



The PS is acidic due to the ketal substituent of pyruvic acid and this acid nature appears to be a characteristic of PS produced by pathogenic strains of *Proteus* species[7] where initial analyses have indicated that their acidic natures can originate from hexuronic acid, phosphate, pyruvate, or other possible acidic substituents.

Partial hydrolysis of the *P. vulgaris* LPS with hot dilute acetic acid afforded an insoluble lipid A (40%), and Sephadex G-50 gel filtration chromatography of the concentrated water-soluble products gave a polysaccharide (K_{av} 0.03, 21%), a core oligosaccharide (K_{av} 0.55, 22%), and a fraction (K_{av} 0.94, 15%) containing 3-deoxy-D-*manno*-octulosonic acid. The O-polysaccharide had $[\alpha]_D$ +44° (*c* 0.21, H_2O) and the same glycose compostion as the PS. On structural analysis, the O-chain was found to have the same structure as the homologous PS. The core oligosaccharide had $[\alpha]_D$ +85° (*c* 0.15, H_2O) and was composed of D-Glc, D-Gal, D-*glycero*-D-*manno*-heptose, and L-*glycero*-D-*manno*-heptose (4:2:1:1).

The investigated strain of *P. vulgaris* showed a positive reaction with reference *P. vulgaris* O19 antisera. The only common structural feature between the currently reported structure and the previously determined *P. vulgaris* O19 LPS polysaccharide structure[5], is a 3-*O*-glycosidically substituted β-D-Glc*p*NAc residues contained in both linear glycans. Further studies on the polysaccharide antigens of *Proteus* species are required to reveal the specific immunobiological importance of glycose residues and substituents in pathogenesis and serological classifications.

EXPERIMENTAL

Cells of *P. vulgaris* (ATCC 49990; NRCC 4418) were grown in brain–heart infusion (Difco) at 37°C in a Microferm fermenter (New Brunswick, 28 L) and were extracted by a modified enzyme–aqueous phenol method[8]. Subsequent isolation procedures involving ultracentrifugation (105 000*g*, 12 h, 4°C) to precipitate LPS were the same as those previously recorded[7]. Fission of LPS (1%) in 2% acetic acid (2.5 h, 100°C) released insoluble lipid A, which was removed by centrifugation. The lyophilized water-soluble product was fractionated by Sephadex

G-50 chromatography, and the eluate was monitored by refractive index measurement.

Polysaccharide (PS) that remained in the ultracentrifugate following the collection of LPS was precipiated by the stirred addition of EtOH (6 vol). The aqueous solution of the crude product was dialysed, lyophilized, and subjected to Sephadex G-50 chromatography to yield an essentially pure PS (3% yield from cells) eluting at the void volume of the system.

Glycan hydrolyses, aldose characterizations, periodate oxidations, methylation analyses, and ^{1}H and ^{13}C NMR spectroscoscopy were made under previously described conditions[14]. GLC–MS analyses were made using a Hewlett–Packard 5985 GLC–MS system using an ionization potential of 70 eV.

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