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Structure of the major exopolysaccharide produced by *Azotobacter beijerinckii* B-1615

Leonid M. Likhosherstov, Sofia N. Senchenkova*, Alexander S. Shashkov, Varvara A. Derevitskaya,
N. D. Zelinsky Institute of Organic Chemistry, Academy of Sciences of the U.S.S.R., Moscow (U.S.S.R.)
Irina V. Danilova, and Irina V. Botvinko
Faculty of Biology, Moscow State University, Moscow (U.S.S.R.)

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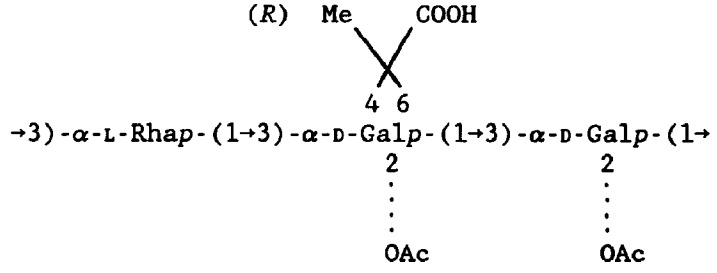
ABSTRACT

A. beijerinckii strain B-1615 produced two acidic exopolysaccharides in the ratio ~9:1. The minor polysaccharide contained mannuronic and guluronic acids in the ratio 2.3:1 and is a bacterial alginate. The major polysaccharide consisted of D-galactose, L-rhamnose, and pyruvic acid in the ratios 2:1:1 and was acetylated. On the basis of methylation analysis, and ^{1}H- and ^{13}C-n.m.r. spectroscopy of the polysaccharide before and after removal of the pyruvic acid residues and *O*-deacetylation, it was concluded that the major polysaccharide had the structure



with up to 1.5 OAc groups per repeating unit.

INTRODUCTION

Bacterial exopolysaccharides play an important role in the processes of recognition and adhesion, and some of them find applications in industry and medicine[1]. The nitrogen-fixing bacteria *Azotobacter* produce alginate-like polysaccharides, but the other exopolysaccharides have been little studied[2,3]. *Azotobacter beijerinckii* strain B-1615 produces alginic acid and another acidic exopolysaccharide, the structure of which is reported.

* To whom correspondence should be addressed.

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RESULTS AND DISCUSSION

The acidic polysaccharides, isolated from the culture fluid by precipitation with Cetavlon[4], were sonicated in order to decrease their viscosity[5], then fractionated by ion-exchange chromatography on DEAE-Trisacryl M to give PS-I and PS-II in the ratio ~9:1.

Hydrolysis of PS-II with acid gave only mannuronic and guluronic acids in the ratio 2.3:1, identified by ion-exchange chromatography in a sodium phosphate buffer[6]. Therefore, PS-II was an alginic acid and was not studied further.

Sugar analysis of PS-I and determination[7] of the absolute configurations of the monosaccharides revealed D-galactose and L-rhamnose in the ratio ~2:1. The ^{13}C-n.m.r. spectrum of PS-I (Fig. 1) contained signals for an acetal-linked pyruvic acid residue (Me at 26.2 p.p.m.) and OAc groups (CH_3CO at 21.4 p.p.m.). PS-I did not have a regular structure, most probably due to non-stoichiometric *O*-acetylation.

The ^{13}C-n.m.r. data indicated that the degree of *O*-acetylation varied from ~0.4 (PS-IA) to ~1.5 (PS-IB) per rhamnose residue (Me at 18.0 p.p.m.) in two samples that were isolated from different batches of culture fluid. The presence of OAc groups was confirmed by the ^{1}H-n.m.r. spectrum of PS-IB (Fig. 2), which contained signals (2 s) at 2.06 and 2.13 p.p.m. each with an integrated intensity of 0.7–0.8 as compared to the signal for Me-5 of rhamnose (1.25 p.p.m., d, $J_{5,6}$ 6 Hz). The pyruvic acid methyl group gave signals (2 s) at 1.43 and 1.40 p.p.m. in the ratio 3:1 that probably corresponded to

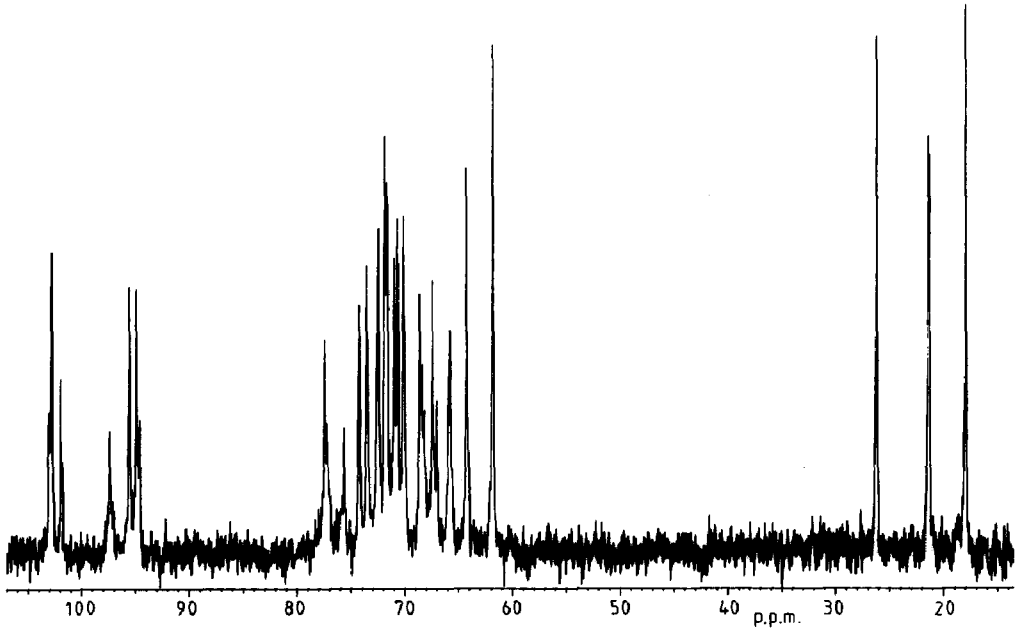


Fig. 1. ^{13}C-N.m.r. spectrum of PS-IB.

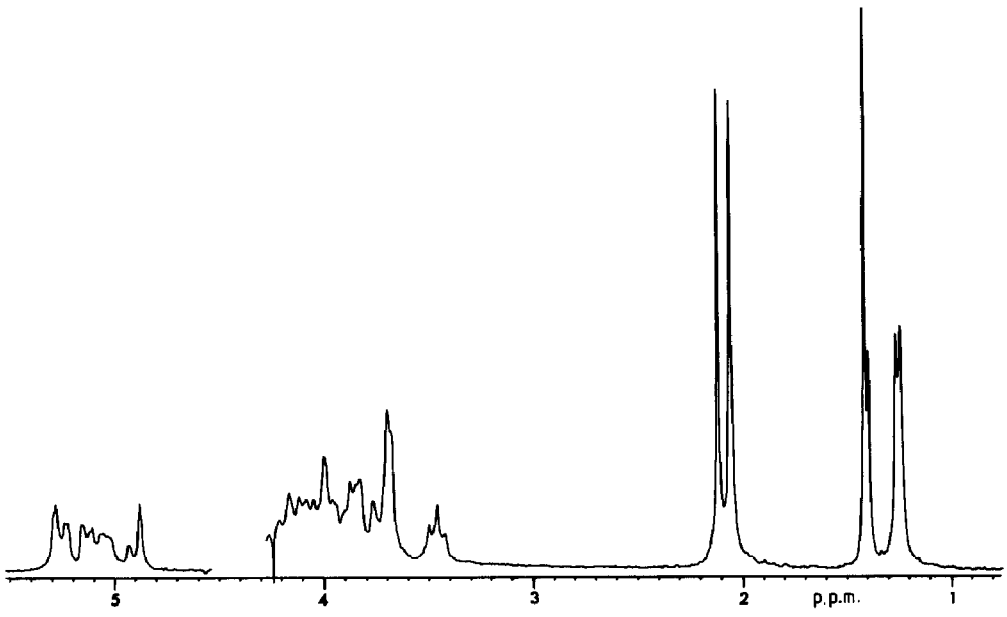


Fig. 2. ^{1}H-N.m.r. spectrum of PS-IB.

TABLE I

^{13}C-N.m.r. data[a,b] (δ in p.p.m.)

Sugar unit	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
PS-III						
→3)-α-L-Rha*p*-(1→ (A)	102.9	68.1	77.0	71.8	70.4	18.0
	(103.5)	(68.3)	(76.8)	(71.8)	(70.0)	(18.0)
→3)-α-D-Gal*p*-(1→ (B)	96.8	68.8	78.4	70.4	72.2	62.3
	(96.3)	(68.9)	(78.5)	(70.6)	(72.2)	(62.4)
→3)-α-D-Gal*p*-(1→ (C)	97.0	68.1	75.9	67.0	71.9	62.3
	(96.8)	(68.1)	(75.3)	(66.9)	(72.2)	(62.4)
PS-IA						
→3)-α-L-Rha*p*-(1→ (A)	102.8	67.9	76.7	71.5	70.0	18.0
→3)-α-D-Gal*p*-(1→ (B)	96.9	68.5	75.8	72.7	64.0	65.9
4 6						
X						
→3)-α-D-Gal*p*-(1→ (C)	96.9	67.8	75.9	67.0	71.9	61.9

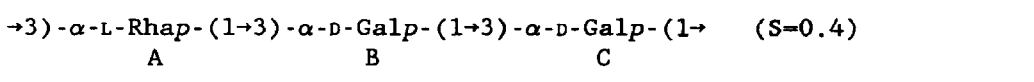
[a] Additional signals: OAc at 21.4 (Me) and 175.3 (CO) p.p.m.; pyruvic acid at 26.2 (C-3), 101.8 (C-2), and 176.1 (C-1) p.p.m. [b] Calculated[9] data are given in parentheses.

the presence and absence of an OAc group at the sugar residue which carried the pyruvic acid moiety. The difference in the content of OAc groups in the two samples of PS-I may be accounted for by differences in the preparation of nutrient medium, *e.g.*, in the mode of sterilisation of components.

O-Deacetylation of PS-IA with aqueous ammonia and removal of pyruvic acid by mild acid hydrolysis afforded a neutral polysaccharide (PS-III). The ^{13}C-n.m.r. data (Table I) were typical for a regular polymer and contained signals for three anomeric carbons (96.8, 97.0, and 102.9 p.p.m.), Me-5 of the Rha residue (18.0 p.p.m.), two hydroxymethyl groups of the Gal residues (a signal at 62.3 p.p.m. of double intensity), and twelve other sugar carbons in the region 67.0–68.4 p.p.m. The absence of signals in the region 82–88 p.p.m. proved that all the sugar residues were pyranosidic.

Therefore, PS-III had a trisaccharide repeating unit that included one Rha*p* and two Gal*p* residues. Methylation analysis[8] of PS-III gave 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylrhamnitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol in the ratio 1:2. Hence, PS-III was linear and all the constituent sugars were 3-substituted.

The computer-assisted analysis[9] of PS-III revealed two structures **1** and **2** which were characterised by the smallest sums of squared deviations of chemical shifts in the calculated and experimental spectra (S = 0.4 or 0.6 per sugar residue, respectively). These structures differed in the position of substitution (2 or 3) of one of the Gal residues (unit B). For all other possible structures, the value S exceeded 2.2 and, hence, these structures were inconsistent with the experimental spectrum of PS-III.



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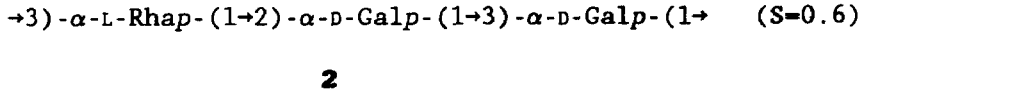

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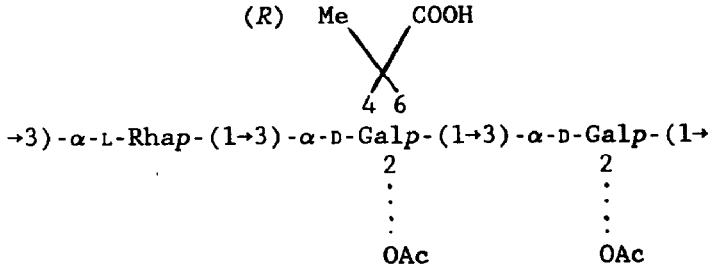
Structure **2** was not in accord with the results of methylation analysis (see above) and, therefore, PS-III had structure **1**. The computer-assisted analysis also allowed the ^{13}C-n.m.r. data (Table I) of PS-III to be assigned tentatively.

The location of the pyruvic acid moiety was established bycomparison of the ^{13}C-n.m.r. data for PS-III and PS-IA (Table I). The signals at 62.3, 70.4, 72.2, and 78.4 p.p.m. for PS-III were shifted to 64.0, 65.9, 72.7, and 75.8 p.p.m. for PS-IA, whereas other signals were shifted insignificantly. The downfield displacement of the signal for C-6 at 62.3 p.p.m. reflected an α-effect, and, thus, the pyruvic acid moiety was 4,6-linked to one of the Gal residues. The signals at 78.4 p.p.m., which was shifted upfield due to a β-effect, evidently belonged to C-3 of unit B (C-3 of unit C in the spectrum of PS-III resonated at 75.9 p.p.m., Table I), and, hence, the pyruvic acid moiety was located in unit B. The chemical shift (26.2 p.p.m.) of the signal for the pyruvic acid methyl group in the spectrum of PS-I proved the *R* configuration[10,11].

Comparison of the ^{13}C-n.m.r. spectra of PS-IA and PS-IB showed that *O*-acetylation shifted the signals for C-1 of units B and C from 96.9 to 94.9–95.6 p.p.m. (the β-effects of *O*-acetylation[12]). The presence of the AcO-2 in the Gal residues was confirmed by the corresponding shifts of the signals for C-2,3 of units B and C from 67.8–68.5 and 75.8–75.9 p.p.m. to 70.0–71.2 and 73.4–74.2 p.p.m., respectively (α- and β-effects of *O*-acetylation[12]). The appearance of the signals for the non-acetylated residues of galactose in the spectra of PS-IA and PS-IB was due to a non-stoichiometric content of OAc groups (see above).

Thus, it was concluded that PS-I had the structure **3** with up to 70–80% acetylation of each Gal residue.



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EXPERIMENTAL

General. — The n.m.r. spectra were recorded with Bruker WM-250 (^1H) and AM-300 (^{13}C) spectrometers for solutions in D_2O at 70° (internal acetone; ^1H, 2.24 p.p.m.; ^{13}C, 31.45 p.p.m.). The optical rotation was measured with a JASCO DIP 360 polarimeter for a solution in water at 25°. Ultrasonication was done with a USDN-2T instrument at 44 kHz and 0°.

G.l.c. was performed with a Hewlett–Packard 5890 instrument equipped with a flame-ionisation detector and a glass capillary column (0.2 mm × 25 m) coated with OV-1. Gel-permeation chromatography was performed on a column (70 × 2.2 cm) of Sephadex G-15 in water, and fractions were monitored by a 2138 Uvicord S at 206 nm. Ion-exchange chromatography was carried out on a column (10 × 3 cm) of DEAE-Trisacryl M (Whatman) with stepwise gradients of 0.01M→0.1M sodium phosphate (pH 6.3) and then 0→0.5M sodium chloride. The eluate was monitored by the orcinol–sulfuric acid reaction, using a Technicon sugar analyser. Analysis of neutral sugars and uronic acids was performed with a Biotronik LC-2000 analyser, using a column (15 × 3.7 cm) of Dionex Ax8-II in 0.5M sodium borate buffer (pH 8.0) at 65° or 0.04M sodium phosphate buffer (pH 2.4) at 70°, respectively; detection was effected with copper 2,2′-bicinchoninate[13].

Production of the exopolysaccharides. — *A. beijerinckii* strain B-1615, obtained from the All-Union Collection of Microorganisms of the Academy of Sciences of the

U.S.S.R., was grown for 5 days at 28–30° in Eshby's liquid nutrient medium containing D-glucose (20 g), K_2HPO_4 (0.2 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), NaCl (0.2 g), and $CaCO_3$ (5 g) in 5 L of water. Bacterial cells were separated by centrifugation for 30 min at 101,000*g*, and proteins were removed from the supernatant solution by the method of Sevag[14]. The solution was then concentrated, and the acidic polysaccharides were precipitated with Cetavlon[4], collected by centrifugation at 10,000*g*, and dissolved in M saline. The residual Cetavlon was removed by treatment with chloroform, and the aqueous solution was dialysed against water and freeze-dried.

Nothing was precipitated by the addition of 3 vol. of ethanol from an aqueous solution of the isolated material kept overnight at 5°, which demonstrated the absence of neutral polysaccharides. An aqueous solution (20 mg/mL) of the acidic polysaccharides was sonicated for 1 h, then concentrated, and subjected to ion-exchange chromatography to give PS-I (~90%) and PS-II (~10%), which were eluted with 0.2 and 0.5M sodium chloride in 0.1M sodium phosphate buffer, respectively, and deionised by gel-permeation chromatography on Sephadex G-15.

Monosaccharide composition. — PS-I and PS-II (1 mg of each) were hydrolysed in sealed tubes with 2M trifluoroacetic acid for 1 h at 121° and the hydrolysates were concentrated *in vacuo*.

Removal of the pyruvic acid and O-deacetylation. — A solution of PS-I in aqueous 2% acetic acid was heated for 2 h at 100°. The polymeric material was isolated by gel-permeation chromatography on Sephadex G-15, freeze-dried, and dissolved in distilled water (2 mg/mL). The solution was treated overnight at room temperature with an equal volume of conc. ammonia, and the solvent was evaporated *in vacuo* at 40°. PS-III was isolated by gel-permeation chromatography on Sephadex G-15 and had $[\alpha]_D$ +152°.

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