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Note

Structural analysis of the O-polysaccharide from the lipopolysaccharide of *Azospirillum brasilense* S17

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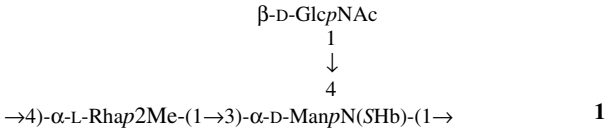
Abstract—A mixture of two structurally distinct neutral O-polysaccharides was obtained by mild acid degradation of the lipopolysaccharide isolated by the phenol/water extraction from the asymbiotic diazotrophic rhizobacterium *Azospirillum brasilense* S17. The following structures of the O-polysaccharides were established by composition and methylation analyses, Smith degradation, and ^{1}H and ^{13}C NMR spectroscopy, including a 2D NOESY experiment:



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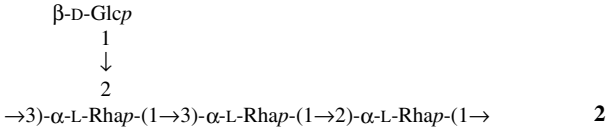

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where L-Rha2Me stands for 2-*O*-methyl-L-rhamnose and *S*Hb for the (*S*)-3-hydroxybutanoyl group. The occurrence of two distinct polysaccharides is reported for the first time in *Azospirillum* spp.

Keywords: Lipopolysaccharide; Bacterial polysaccharide structure; *Azospirillum brasilense*; 2-*O*-Methyl-L-rhamnose

Azospirillum species colonize the rhizosphere and have the potential to promote plant growth. On the part of the bacteria, the establishment of plant–microbial associations may be realized by the action of pectolytic enzymes,[1] the production of phytohormones, which causes morphological and physiological changes in inoculated plants,[2] and the action of surface components eliciting plant response to inoculation.[3]

The lipopolysaccharide (LPS) is the major antigen of the bacterial outer membrane of the *Azospirillum* cell envelope. Together with other surface polysaccharides of *Azospirillum*, the LPS is thought to be essential for the plant–bacterium interaction but the structural basis of this interaction remains to be elucidated. Previously,[4–6] we had reported structures of the O-polysaccharides from the LPS of strains belonging to three

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Azospirillum species. In this paper, we describe structures of two O-polysaccharides isolated from the LPS of *A. brasilense* S17.

Strain S17 was isolated from the roots of pearl millet. When cultivated in a liquid synthetic malate medium to the end of the exponential phase of growth, it produces at least two capsular polysaccharide-containing complexes. These polysaccharides and the LPS are involved in the interaction of the bacteria with wheat roots and with other rhizobacteria (*Paenibacillus polymyxa* 1460 lectins and *Rhizobium leguminosarum* 252 agglutinins).[7,8] The capsular polysaccharides are composed of mannose, galacturonic acid, galactose, rhamnose, glucosamine, and galactosamine.[7] No data on the LPS structure of *A. brasilense* S17 have been hitherto reported.

Bacterial cells were extracted with aq 45% phenol, and the LPS was recovered from the aqueous phase. Degradation of the LPS under mild acid conditions afforded a lipid sediment and a water-soluble carbohydrate portion, which was fractionated by GPC on Sephadex G-50 to give a high-molecular-mass polysaccharide preparation (PS). Sugar analysis by GLC of the alditol acetates obtained after full acid hydrolysis of the PS revealed rhamnose, glucose, GlcNAc, ManNAc, and a component with a short retention time later identified as 2-*O*-methylrhamnose (Rha2Me) in the ratios of 3.7:2.2:1.1:1:1, respectively, as well as smaller amounts of fucose, xylose, mannose, galactose, a heptose, and 2-deoxy-2-(3-hydroxybutanoylamino)mannose (ManNHb). The last sugar was identified by electron impact mass spectrometry of the corresponding alditol acetate. The mass spectrum of the ManNHb derivative contained diagnostic ion peaks at *m*/*z* 230 and 446 for the C-1–C-2 and C-2–C-6 fragments, respectively, as well as peaks for the corresponding secondary fragments (Fig. 1).

Methylation analysis of the PS by GLC–MS of the partially methylated alditol acetates resulted in the identification of 2,3,4,6-tetra-*O*-methylglucose, 2,3-di-*O*-methylrhamnose, 2,4-di-*O*-methylrhamnose, 3,4-di-*O*-methylrhamnose, 4-*O*-methylrhamnose, 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methyl)acetamidoglucose (from GlcNAc), and 2-deoxy-2-(3-methoxybutanoylamino)-6-*O*-methylmannose (from ManNHb). When CD_3I was used for methylation instead of CH_3I, the corresponding fully trideuteromethylated derivatives were identified, except for 2-*O*-methyl-3-*O*-trideuteromethylrhamnose rather than 2,3-di-*O*-trideuteromethylrhamnose. Therefore, the PS contains Rha2Me. The PS is branched with terminal glucose and GlcNAc residues in the side chains and rhamnose and ManNHb at the branching points.

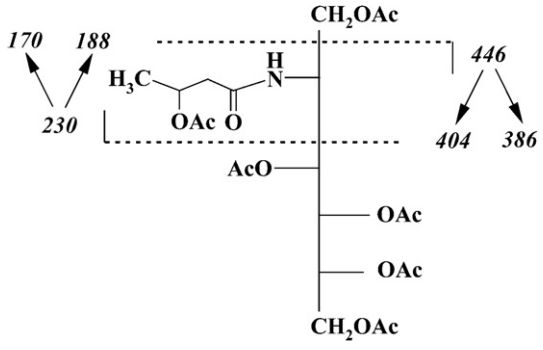

Figure 1. Fragmentation of the ManNHb-derived alditol acetate in electron impact MS.

Determination of the absolute configurations of the monosaccharides by GLC of the acetylated glycosides with (*S*)-2-octanol indicated the presence of D-Glc, D-GlcN, D-ManN, and L-Rha. The absolute configuration of Rha2Me was inferred from the ^{13}C NMR data using the known regularities in the glycosylation effects[9] (see below). The *S* configuration of the Hb group was established by GLC of the O-trifluoroacetylated (*S*)-2-octyl ester of 3-hydroxybutyric acid released by acid hydrolysis of the PS.

The ^1H and ^{13}C NMR spectra of the PS showed signals of different intensities, thus indicating a structural heterogeneity. The ^{13}C NMR spectrum (Fig. 2) contained *inter alia* signals for seven anomeric carbons at δ 94.9–105.5, a number of CH_3–C groups (C-6) of Rha at δ 17.9–18.2, three $HOCH_2$–C groups (C-6 of Glc, GlcN, and ManN) at δ 61.3–63.4, two nitrogen-bearing carbons (C-2 of GlcN and ManN) at δ 51.0 and 57.1, and 26 other sugar ring carbons in the region δ 68.7–82.1. In addition, there were signals for one *N*-acetyl and one *N*-(3-hydroxybutanoyl) groups (CH_3 at δ 23.4–23.5, CO at δ 175.4–176.0, CH_2 and CHOH of Hb at 46.4 and 66.2, respectively). These data confirmed the composition of the PS determined by sugar and methylation analyses. The absence of signals from the region of δ 83–88 characteristic of furanosides[10] showed that all monosaccharide residues are in the pyranosidic form.

A low-field region of the ^1H NMR spectrum of the PS (Fig. 3) contained eight signals at δ 4.48–5.34, including those for seven anomeric protons and H-2 of ManNHb at δ 4.68. A high-field region of the spectrum included signals for one *N*-acetyl group at δ 2.06, CH_3–C groups of Rha (H-6) and Hb at δ 1.22–1.32, one CH_2 group of Hb at δ 2.37 and 2.47, and one *O*-methyl group at δ 3.45.

The ^1H and ^{13}C NMR spectra of the PS were assigned using 2D COSY, TOCSY, NOESY, and ^1H, ^{13}C HSQC experiments (Tables 1 and 2) and spin systems for one residue each of glucose, GlcN, ManN, Hb, and four rhamnose residues (Rha^I–Rha^{III} and Rha2Me) were revealed. The assignment was based on correlations between H-1 and H-2 to H-6 for Glc and GlcN, H-2 to H-4 for Rha^I–Rha^{III}, and H-2 for ManN and Rha2Me, between H-2 and H-3 to H-5 for ManN, and between H-6 and H-5 to H-2 for Rha^I–Rha^{III} and

Rha2Me in the TOCSY spectrum. The assignment within each spin system was performed using COSY, and relative configurations of the monosaccharides were identified based on $^{3}J_{H,H}$ coupling constants. The H-6

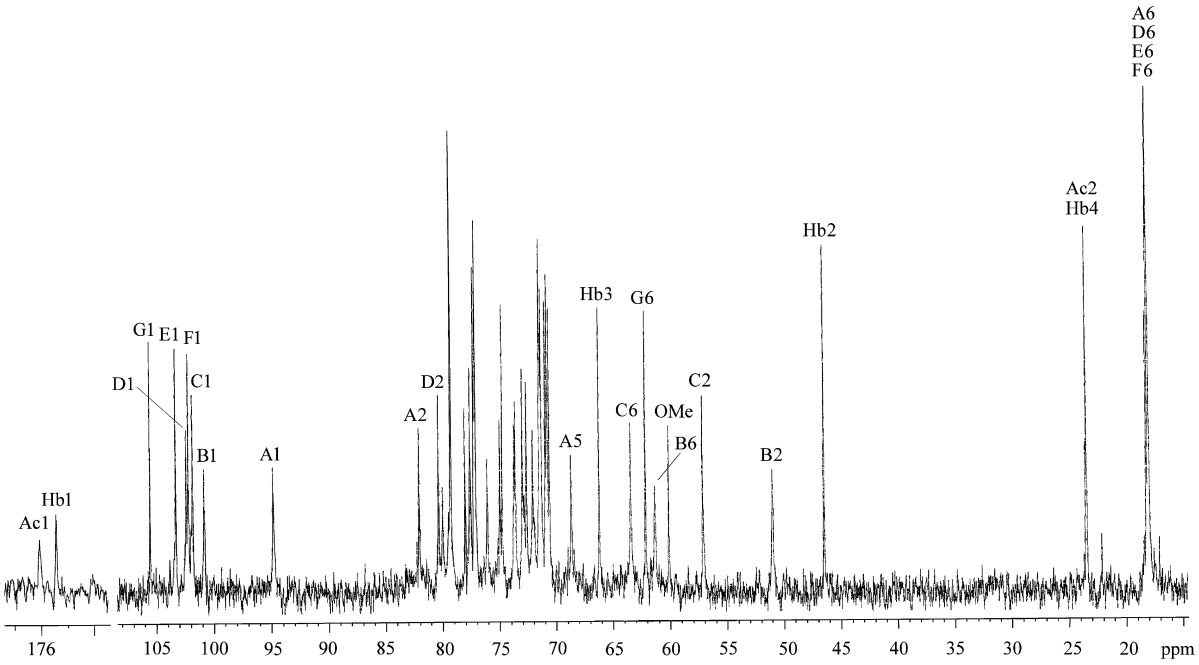

Figure 2. ^{13}C NMR spectrum of the polysaccharide from *A. brasilense* S17. For abbreviations see Chart 1.

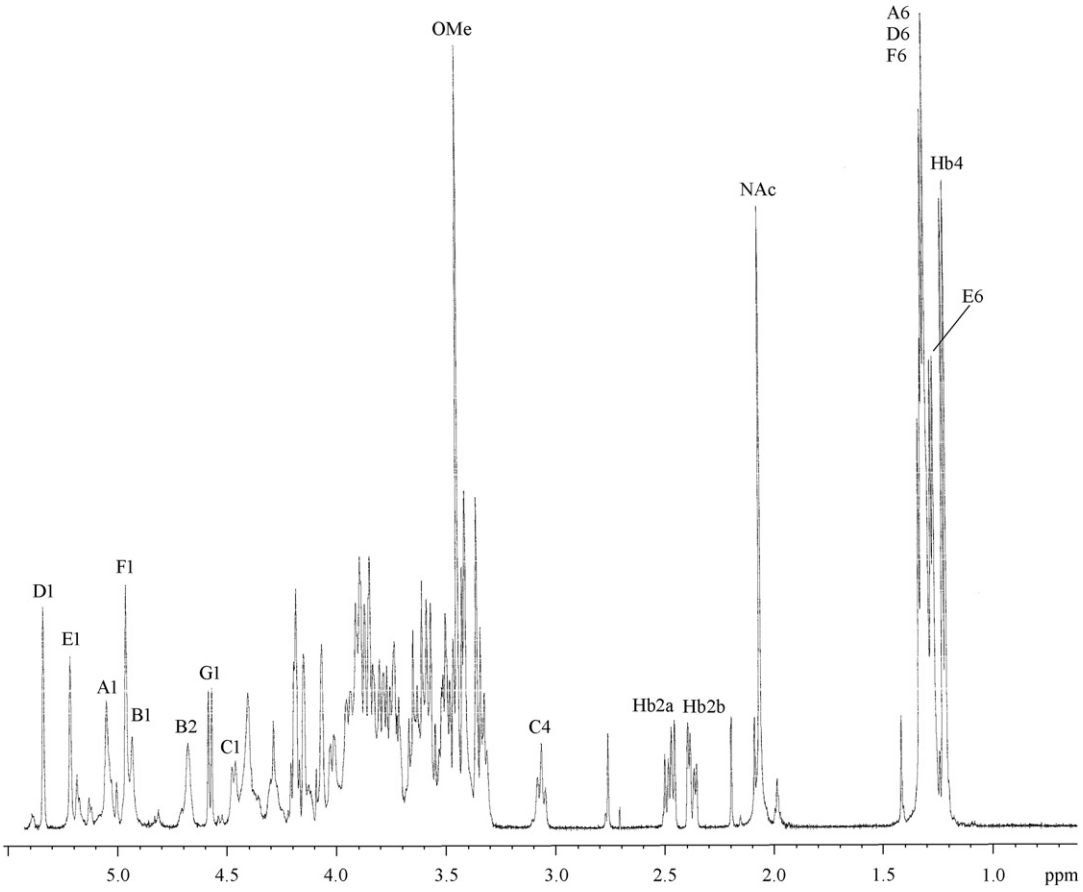

Figure 3. ^{1}H NMR spectrum of the polysaccharide from *A. brasilense* S17. For abbreviations see Chart 1.

Table 1. 500-MHz ^{1}H NMR data (δ, ppm)

Residue	Sugar							NAc	N(*S*Hb)				OMe
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	H-2	H-2a	H-2b	H-3	H-4	
Polysaccharide **1**													
→4)-α-L-Rha*p*2Me-(1→(**A**)	5.04	3.36	3.95	3.58	4.28	1.30							3.45
→3,4)-β-D-Man*p*N(*S*Hb)-(1→(**B**)	4.93	4.68	3.91	3.80	3.33	3.93	3.77		2.47	2.37	4.19	1.21	
β-D-Glc*p*NAc (**C**)	4.48	3.64	3.51	3.06	3.40	3.60	3.90	2.06					
Polysaccharide **2**													
→2,3)-α-L-Rhap^{I}-(1→(**D**)	5.34	4.19	4.02	3.65	3.86	1.31							
→3)-α-L-Rhap^{II}-(1 → (**E**)	4.95	4.15	3.84	3.58	3.74	1.27							
→2)-α-L-Rhap^{III}-(1→(**F**)	5.21	4.07	3.90	3.49	3.77	1.32							
β-D-Glc-(1→(**G**)	4.58	3.34	3.49	3.42	3.41	3.72	3.85						
Smith-degraded polysaccharide **3**													
→4)-α-L-Rha*p*2Me-(1→	5.15	3.37	3.91	3.61	3.99	1.31							3.47
→3,4)-β-D-Man*p*N(*S*Hb)-(1→	4.97	4.70	3.92	3.83	3.47	3.85	3.99		2.45	2.37	4.19	1.22	
Rest of destroyed GlcNAc	4.87	4.75	3.79, 3.64	3.71, 3.71	3.78	3.65	3.71	2.06					
Monosaccharide L-*Rha2Me*													
α-L-Rha*p*2Me	5.26	3.56	3.82	3.35	3.83	1.27							3.46
β-L-Rha*p*2Me	4.85	3.65	3.63	3.29	3.35	1.27							3.46

Table 2. 125-MHz ^{13}C NMR data (δ, ppm)

Monosaccharide residue	Sugar						NAc		N(*S*Hb)				OMe
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-1	C-2	C-3	C-4	
Polysaccharide **1**													
→4)-α-L-Rha*p*2Me-(1→(**A**)	94.9	82.1	71.5	80.0	68.7	17.9							60.1
→3,4)-β-D-Man*p*N(*S*Hb)-(1→(**B**)	100.8	51.0	76.1	72.1	77.1	61.3			175.4	46.4	66.2	23.4	
β-D-Glc*p*NAc (**C**)	101.8	57.1	75.0	72.6	77.6	63.4	176.0	23.5					
Polysaccharide **2**													
→2,3)-α-L-Rhap^{I}-(1→(**D**)	102.4	80.4	78.0	73.6	70.6	17.9							
→3)-α-L-Rhap^{II}-(1→(**E**)	103.3	71.3	79.3	73.0	70.8	18.0							
→2)-α-L-Rhap^{III}-(1→(**F**)	102.2	79.3	71.5	73.7	70.8	18.2							
β-D-Glc-(1→(**G**)	105.5	74.8	77.1	71.0	77.3	62.1							
Smith-degraded polysaccharide **3**													
→4)-α-L-Rha*p*2Me-(1→	94.5	82.1	71.5	80.9	68.9	18.0							62.0
→3,4)-β-D-Man*p*N(*S*Hb)-(1→	100.8	50.1	75.6	71.9	77.1	61.5			175.4	46.4	66.4	23.0	
Rest of destroyed GlcNAc	104.5	57.1	61.2	62.6	83.4	63.2	176.0	23.5					
Monosaccharide L-*Rha2Me*													
α-L-Rha*p*2Me	92.2	82.2	71.1	74.0	69.5	18.3							60.8
β-L-Rha*p*2Me	96.0	82.9	74.5	73.6	73.7	18.3							60.8

signals for ManN were not found directly as no H-5,H-6 correlation in the COSY or TOCSY spectrum and no H-6, C-6 correlation in the ^{1}H, ^{13}C HSQC spectrum were observed; however, their position could be inferred from a NOESY experiment (see below).

Relatively large $^3J_{\text{H-1,H-2}}$ coupling constant values (7–8 Hz) showed that Glc and GlcNAc are β-linked. This was confirmed and the β configuration of ManNHb established by relatively low-field positions of the C-5 signals at δ 77.1–77.6 in the ^{13}C NMR spectrum. Similarly, the α configuration of Rha^{I}–Rha^{III} and Rha2Me was inferred by relatively high-field positions of the C-5 signals at δ 68.7–70.8 (compare published data[11] δ 70.0 and 73.2 for C-5 of α- and β-rhamnopyranose, respectively).

Relatively low-field positions of the signals for C-2 and C-3 of Rha^{I}, C-3 of Rha^{II}, C-2 of Rha^{III}, C-2 and C-4 of Rha2Me, C-3 and C-4 of ManNHb at δ 80.4, 78.0, 79.3, 79.3, 80.0, 76.1, and 72.1, respectively, as compared with their positions in the spectra of the corresponding non-substituted monosaccharides,[11] were in agreement with the substitution pattern determined by the methylation analysis. In accordance with their terminal positions, the C-2 to C-6 chemical shifts of Glc and GlcNAc were close to those in the corresponding non-substituted sugars.[11]

The NOESY experiment revealed the occurrence of repeating units of two types, and the monosaccharide sequence in each of them was demonstrated by interresidue correlations between the anomeric protons and

protons at the linkage carbons. Taking into account the ^{13}C NMR chemical shift data (see above), these were assigned as follows: Rha2Me H-1, ManNHb H-3, ManNHb H-1, Rha2Me H-4, and GlcNAc H-1, ManNHb H-4 at δ 5.04/3.91, 4.93/3.58, and 4.48/3.80, respectively, in the repeating unit **1**; Rha^{I} H-1, Rha^{II} H-3, Rha^{II} H-1, Rha^{III} H-2, Rha^{III} H-1, Rha^{I} H-3, and Glc H-1, Rha^{I} H-2 at δ 5.34/3.84, 4.95/4.07, 5.21/4.02, and 4.58/4.19, respectively, in the repeating unit **2**. There were additional interresidue NOE correlations, including Rha^{III} H-1, Rha^{II} H-5 and Rha2Me H-1, ManNHb H-2 at δ 5.21/3.74 and 5.04/4.68, which were typical of the linkage types in the α-Rha^{II}-(1→2)-Rha^{III} and α-Rha2Me-(1→3)-ManNHb disaccharide fragments, respectively. Cross-peaks at δ 4.48/3.77 and 4.48/3.90 were assigned to GlcNAc H-1, ManNHb H-6a, 6b correlations expected for the β-GlcNAc-(1→4)-ManNHb disaccharide. A correlation between OMe and H-2 of Rha2Me at δ 3.45/3.36 again demonstrated Rha2Me.

In the α-Rha2Me-(1→3)-D-ManNHb disaccharide fragment, the glycosylation effect on C-1 of Rha2Me is relatively small (+2.7 ppm as compared with free α-Rha*p*2Me, see Table 2) and β-effect on C-2 of ManNHb relatively large by the absolute value (−3.3 ppm as compared with published data for β-ManNAc[11]). These values are characteristic for different absolute configurations of the constituent monosaccharides,[9] and, hence, Rha2Me is L.

On the basis of these data, it was concluded that the PS from the LPS of *A. brasilense* S17 consists of two polysaccharides having structures **1** and **2** shown in Chart 1.

To further confirm the structures, the PS was subjected to Smith degradation. The products of mild acid hydrolysis (aq 2% AcOH, 100 °C, 2 h) of the degraded polysaccharide (DPS) were extracted by GPC on TSK HW-40, and an isolated polymer was studied by NMR spectroscopy. Its ^{1}H and ^{13}C NMR spectra showed three signals in the region of anomeric atoms at δ_H 4.87–5.15 and δ_C 94.5–104.5. This and the presence of a number of other signals additional to those expected for Rha2Me and ManNHb, including the signals for three additional $HOCH_2$–C groups at δ_C 61.2–62.6, showed that the destroyed GlcNAc residue was not cleaved by mild acid hydrolysis, most likely, owing to sterical hindrance at the branching point. Full assignment of the ^{1}H and ^{13}C NMR spectra of the DPS (Tables 1 and 2) followed by linkage and sequence analyses using NOESY, as described above for the PS, demonstrated structure **3** of the DPS.

Full acid hydrolysis of the DPS with 2 M CF_3CO_2H (120 °C, 2 h), followed by the treatment of the products with a cation-exchange resin to remove ManN, resulted in Rha2Me in the free form, which was characterized by ^{1}H and ^{13}C NMR chemical shifts (Tables 1 and 2) assigned using COSY, TOCSY, and ^{1}H, ^{13}C HSQC

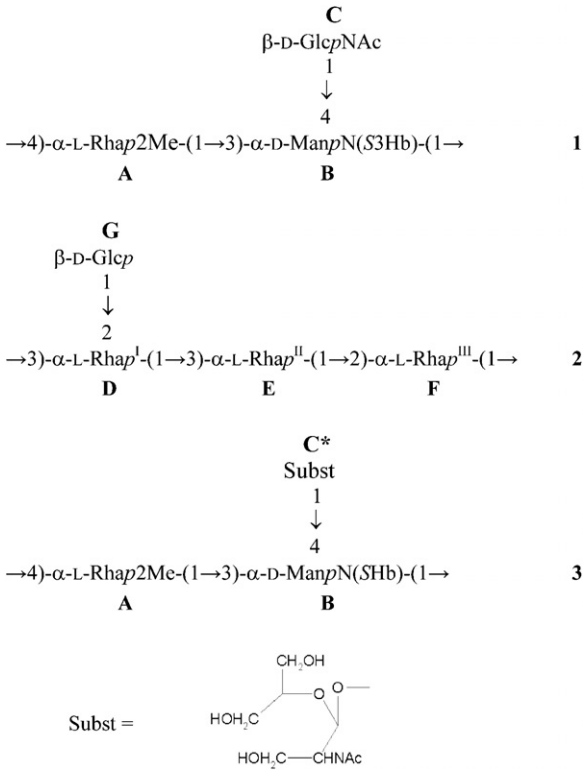

Chart 1. Structures of the polysaccharides from *A. brasilense* S17.

experiments. These data confirmed structure **1** of the initial polysaccharide, including the identity of Rha2Me. As expected, upon Smith degradation the other polysaccharide present in the PS having structure **2** gave low-molecular-mass products, which were not studied further.

The LPS of *A. brasilense* S17 provides the first example in *Azospirillum* spp. of a simultaneous expression of two structurally distinct polysaccharides. It is unclear if they are two independent chains of the LPS or represent long blocks within the single chain. One of the polysaccharides from *A. brasilense* S17 having an L-rhamnan main chain is structurally similar to the O-polysaccharide of *Azospirillum lipoferum* SpBr17 studied earlier.[12] Both L- and D-rhamnose are widespread among microorganisms that establish various types of relationships with plants, which may attest to their important role in plant–microbial interactions.[13] The second polysaccharide is distinguished by the presence of O-methylated L-rhamnose and an N-linked (*S*)-3-hydroxybutanoyl group. Various O-methylated monosaccharides have been found in bacterial polysaccharides, including 2-*O*-methylfucose, 3-*O*-methylfucose, 3-*O*-methylrhamnose, and 2-*O*-methylgalactose, an *Azospirillum* LPS.[14] No 3-hydroxybutanoyl group has been hitherto reported as a component of *Azospirillum* polysaccharides. Unusual for azospirilla polysaccharides is also the occurrence of GlcNAc, a specific hapten of wheat germ agglutinin. One can speculate that the *A. brasilense*

S17 polysaccharide with the GlcNAc residue occupying a lateral position in the polymer chain can function as a specific carbohydrate receptor for plant lectins.

1. Experimental

1.1. Bacterial strain, growth, isolation, and degradation of the lipopolysaccharide

A. brasilense strain S17 isolated from pearl millet was kindly provided by Dr. Lahiri (India). The culture was continuously grown in a 10-L ANKUM-2M fermentor at 30 °C in a liquid malate medium[7] to the late exponential phase. The cells were separated by centrifugation and dried with acetone. The dried cells (15 g) were extracted with phenol–water,[15] and the isolated LPS was purified by repeated ultracentrifugation (105,000*g*, 2 × 4 h). The yield of the LPS was 1.8% of the dry cell weight.

An LPS sample (95 mg) was hydrolyzed with aq 1% HOAc at 100 °C for 4 h, a lipid precipitate (12 mg) was removed by centrifugation (13,000*g*, 20 min), and the carbohydrate portion fractionated by GPC on a column (56 × 2.6 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer pH 4.5 monitored by a Knauer differential refractometer. The yield of the high-molecular-mass PS was 23.7% of the LPS weight.

1.2. Chemical analyses and methylation

Hydrolysis was performed with 2 M CF_3CO_2H (120 °C, 2 h). The monosaccharides were analyzed by GLC as the alditol acetates[16] on an Ultra 2 capillary column using a Hewlett–Packard 5880 instrument and a temperature gradient of 180 °C (1 min) to 290 °C at 10 °C min^{-1}. The absolute configurations of the monosaccharides were determined by GLC of the acetylated glycosides with (*S*)-2-octanol as described.[17] The absolute configuration of 3-hydroxybutyric acid was determined by the published method[18] modified as described.[19]

Methylation of the PS was carried out with CH_3I or CD_3I in dimethyl sulfoxide in the presence of sodium methylsulfinylmethanide.[20] Hydrolysis of the methylated polysaccharide was performed with 2 M CF_3CO_2H (100 °C, 2 h), and the partially methylated monosaccharides were reduced with $NaBH_4$, acetylated, and analyzed by GLC–MS on a Hewlett–Packard HP 5989A instrument equipped with an HP-5ms column, using a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C min^{-1}.

1.3. Smith degradation

A PS sample (20 mg) was oxidized with 0.1 M $NaIO_4$ in the dark at 20 °C for 48 h. After the addition of an excess of ethylene glycol, reduction with $NaBH_4$, and desalting on a column (80 × 1.6 cm) of TSK HW-40 (S) in water, the product was hydrolyzed with aq 2% AcOH at 100 °C for 2 h, reduced with $NaBH_4$, and desalted with a KU-2 cation-exchange resin (H^+-form). Smith-degraded polysaccharide (DPS) (8.4 mg) was isolated by GPC on TSK HW-40 (S).

A DPS sample (8 mg) was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h). The hydrolysate was freed from ManN (2.7 mg) by treatment with a KU-2 cation-exchange resin (H^+-form). The remaining Rha2Me (3.8 mg) was studied by NMR spectroscopy.

1.4. NMR spectroscopy

Samples were deuterium-exchanged by freeze-drying from D_2O. ^1H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer for solutions in D_2O at 27 °C. Chemical shifts are reported with internal sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00). A mixing time of 200 and 150 ms was used in the TOCSY and NOESY experiments, respectively.

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