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Note

Structure of the O-specific polysaccharide from the legume endosymbiotic bacterium *Ochrobactrum cytisi* strain ESC1^{T}

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ABSTRACT

The O-specific polysaccharide was obtained from the lipopolysaccharide of the legume-endosymbiotic bacterium *Ochrobactrum cytisi* strain ESC1^{T} and studied by chemical analyses and 1D and 2D NMR spectroscopy. The polysaccharide was found to have a disaccharide repeating unit containing α-D-fucose and β-N-acetyl-D-galactosamine residues connected via (1→3)-glycosidic bonds, resulting in the following structure:

→3)-α-D-Fuc*p*-(1→3)-β-D-Gal*p*NAc-(1→

The D-Gal*p*NAc residue was nonstoichiometrically substituted with a 4-O-methyl group (~10%) or with a 4,6-O-(1-carboxy)-ethylidene residue (pyruvyl group) (~10%).

Gram-negative bacteria from the genus *Ochrobactrum* are classified in the family *Brucellaceae*, which also includes important animal and human pathogens, e.g. *Brucella abortus*. The genus *Ochrobactrum* currently comprises approximately twenty species, including human pathogens (*Ochrobactrum anthropi*),[1] soil rhizospheric bacteria (*Ochrobactrum tritici* or *Ochrobactrum grignonense*)[2] as well as legume endosymbionts, such as *Ochrobactrum lupini*[3] and *Ochrobactrum cytisi*[4] nodulating *Lupinus albus* and *Citisus scoparius*, respectively. Bacterial glycoconjugates released into the environment or exposed on the cell surface play a crucial role in establishing effective symbiosis. Lipopolysaccharide (LPS), an integral component of the cell envelope of most Gram-negative bacteria, is of particular importance in symbiotic interactions between bacteria and plants.[5,6] The correct and complete architecture of the outer membrane is essential in the stage of root hair colonization and formation of the infection thread and effective nodules in which nitrogen fixation takes place.[7] Moreover, LPS protects microsymbiont cells against the defense mechanisms of the host plant.[8–10]

In this work, we report on the chemical structure of the O-specific polysaccharide (OPS) obtained from the LPS of *O. cytisi* strain ESC1^{T}.

LPS preparations were obtained from the bacterial cell mass using the classical hot-phenol/water method and were found in the phenol phase (LPS-PP) and in the interphase (LPS-IP). The SDS-PAGE analysis of *O. cytisi* LPS (Fig. 1) showed heterogeneity of the material typical of rhizobial and mesorhizobial LPSs.[11,12] Three intensely stained zones representing different forms of LPS were observed. The fast-, and slow-migrating fractions were present in the regions corresponding to the R- and S-form of *Salmonella* LPS, respectively. In addition, a few very intense bands corresponding to the species with a molecular mass of approximately 6 kDa were detected. These intermediate fractions represented incomplete LPS with only a few repeating units attached.

Mild acid hydrolysis of the LPS-IP of *O. cytisi* resulted in cleavage of the polysaccharide from the lipid A part. High-molecular-weight material was obtained by fractionation of the hydrolysate by GPC on a Sephadex G-50 fine column. (Amino)sugars liberated from the OPS by hydrolysis were further converted to (amino)alditol acetates and analyzed by GC–MS. Fucose (Fuc) and galactosamine (GalN) were identified as the main sugar components. A peak with mass spectrum characteristic of 2-amino-2-deoxy-hexositol acetate methylated at position C-4 was also observed on the chromatogram. The area of this peak was estimated at about 10% of GalN-ol peak. Analysis of the ethylated OPS (because of the presence of O-methylated sugar) showed that Fuc and GalN were substituted at position C-3. Terminal fucose, 3-substituted-4-*O*Me-hexosamine and 3,4,6-trisubstituted hexosamine, were also noticed.

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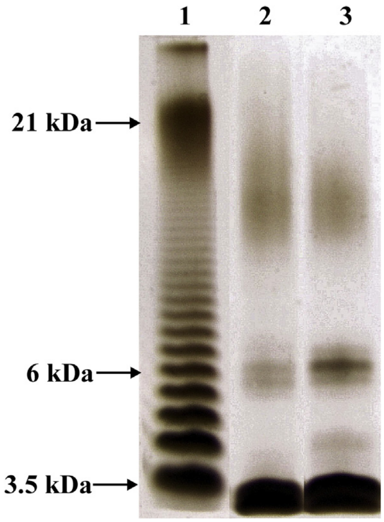

Fig. 1. Silver-stained SDS-PAGE of the LPS from *Ochrobactrum cytisi* ESC1^T (lane 2, LPS-PP, 2 µg; lane 3, LPS-IP, 2 µg) and *Salmonella enterica* sv. Typhimurium (Sigma) (lane 1, 2 µg). M_w values were calculated based on the known structure of the *Salmonella* LPS.

The GC–MS analysis of trimethylsililated *R*-(−)-2-butyl glycosides showed that Fuc and GalN had the D-configuration.

The structure of *O. cytisi* OPS was studied in detail by 2D NMR spectroscopy, including ^1H, ^1H COSY, TOCSY, NOESY, ^1H, ^{13}C HMQC, and ^1H, ^{13}C HMBC experiments. As a result, all proton and carbon chemical shifts were assigned (Table 1). The ^1H, ^{13}C NMR spectrum (HMQC, Fig. 2) of the O-polysaccharide contained signals for anomeric carbons (δ_C 97.1–103.9), nitrogen-bearing carbons (δ_C 52.2–53.5), sugar ring carbon atoms (δ_C 62.1–80.5), *C*-methyl groups (C-6) of 6-deoxyhexose residues (δ_C 16.6), and also signals for O-methyl (δ_C 59.8) and N-acetyl groups at δ_C 23.6 ($-\mathbf{C}H_3$) and 176.2 ($=\mathbf{C}=O$ from acetamido group). Additionally, the presence of signals characteristic of the pyruvyl substituent at δ_C 26.4 ($-\mathbf{C}H_3$), 102.5 ($=\mathbf{C}=O$) and 177.0 ($-\mathbf{C}OO$) was also observed. The ^{13}C NMR data showed that all the sugar residues were in the pyranose form.[13]

While tracing the connectivities between signals in the ^1H, ^1H COSY, TOCSY, and NOESY spectra, two main spin systems were assigned. The first one for D-Gal*p*NAc and the other belonged to D-Fuc*p* residues. They were marked as **A** and **B**, respectively. The chemical shift for the H-1, H-2, and $J_{C1,H1}$ coupling constant for D-Gal*p*NAc 162.1 Hz pointed that this sugar had β configuration,[14] whereas the chemical shifts for H-1, H-5, and C-5, as well as the relatively high value (170.0 Hz) of the $J_{C1,H1}$ coupling constant for D-Fuc*p* indicated its α-configuration.[14] The presence of the N-acetamido sugar was confirmed by correlations of **A**H-2 at δ_H 4.08 with the corresponding carbon-bearing nitrogen at δ_C 52.3, as revealed by the HMQC experiment, as well as with carbonyl at δ_C 176.2 observed in the HMBC spectrum. The downfield shift of signals for C-3 of both sugar residues (in comparison with the chemical shift values of the non-substituted D-Gal*p*NAc and D-Fuc*p*) indicated their linkage position.[13,15]

The sequence of the polysaccharide components was established by analysis of NOESY and HMBC spectra. Strong interresidue NOE signals were observed between proton **A**H-1 (δ_H 4.69) and proton **B**H-3 (δ_H 3.86) as well as between proton **B**H-1 (δ_H 5.03) and **A**H-3 (δ_H 3.79). In addition, strong interactions via glycosidic bonds were detected in the HMBC experiment between proton **B**H-1 (δ_H 5.03) and carbon **A**C-3 (δ_C 77.2) and between proton **A**H-1 (δ_H 4.69) and carbon **B**C-3 (δ_C 80.5). Thus, the data above show that the O-specific polysaccharide from *O. cytisi* ESC1^T LPS has a disaccharide repeating unit containing one residue of α-D-fucose and one of β-D-galactosamine linked via a (1→3)-glycosidic bond.

Furthermore, two additional spin systems for D-Gal*p*NAc were also noticed. The first one (marked as **a**′) was attributed to 4-O-methylated D-Gal*p*NAc and the second one (**a**″) to the D-Gal*p*NAc decorated with a pyruvyl residue. The occurrence of the O-methyl substituent at position C-4 of the D-Gal*p*NAc residue was confirmed by the presence of a correlation between O-methyl protons and H-4 of D-Gal*p*NAc (spin system **a**′) in the NOESY spectrum (δ_H 3.41/3.70). Moreover, H-4 and H-6 protons of D-Gal*p*NAc (**a**″) showed correlations with carbon from the chiral center (carbon C-2) of the pyruvyl residue (HMBC spectrum). This indicated that the pyruvyl substituted positions C-4 and C-6 of some amino sugar residues. The chemical shift of the pyruvate methyl group (δ_C 26.4) indicated that the chiral center of the pyruvate (carbon C-2) had the *R*-configuration. The pyruvate residue with the *S*-configuration has axial methyl signals at ca. 17 ppm.[16,17]

There were several overlapping anomeric signals corresponding to D-Gal*p*NAc at the ^1H NMR spectrum. In the DQF-COSY spectrum, the region between (F2) 4.65–4.75 ppm and (F1) 3.95–4.15 ppm was occupied by at least five cross-peaks resulting from correlation between H-1 and H-2 of D-Gal*p*NAc (data not shown). Among them, the **A**H-1/**A**H-2 cross-peak was the most abundant. Such amount of signals could indicate that D-Gal*p*NAc residues were substituted with pyruvyl and methyl groups in a random manner. Moreover, all anomeric protons of D-Gal*p*NAc showed NOE correlations with H-3 of α-D-Fuc*p* (including spin system **b**), as well as all anomeric protons of α-D-Fuc*p* (**B** and **b**) were connected by glycosidic bonds

Table 1
^1H and ^{13}C chemical shifts [δ, ppm] and coupling constants [*J*, Hz] of the components of the O-specific polysaccharide from *Ochrobactrum cytisi* ESC1^T LPS

Residue	$J_{C1,H1}$ $J_{H1/H2}$	H1 C1	H2 C2	H3 C3	H4 C4	H5 C5	H6; H6, H6′ C6	O-Methyl[-O-CH_3]	2-N-Acetyl[-CH_3]	2-N-Acetyl[-C=O]
A	162.1	4.69	4.08	3.79	4.14	3.66	3.79; 3.79		2.04	—
→3)-β-D-Gal*p*NAc-(1→	8.8	103.9	52.3	77.2	65.4	75.8	62.1		23.6	176.2
B	170.0	5.03	3.86	3.86	4.04	3.97	1.23			
→3)-α-D-Fuc*p*-(1→	3.8	97.1	67.9	80.5	72.7	68.0	16.6			
b	170.5	4.90	3.88	4.08	4.04	3.95	1.23			
→3)-α-D-Fuc*p*-(1→	3.7	101.7	68.1	80.3	72.6	68.8	16.6			
a′	165.7	4.72	4.02	4.08	3.70	3.88	3.81; 3.81	3.41	2.04	—
→3)-β-D-Gal*p*NAc4OMe-(1→	8.4	103.8	53.5	80.3	72.3	n.d.	62.1	59.8	23.6	176.2
a” 4┐										
→3)-β-D-Gal*p*NAc-(1→	164.4	4.73	4.09	3.87	4.35	3.59	3.95; 4.04		2.04	—
6┘	8.4	103.8	52.2	77.0	72.0	67.1	66.3		23.6	176.2
Pyr		—	—	1.50						
R-pyruvyl		177.0	102.5	26.4						

n.d.—Not determined.