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The O-chain structure from the LPS of the endophytic bacterium *Burkholderia cepacia* strain ASP B 2D

Serena Leone,[a] Antonio Molinaro,[a,*] Isak B. Gerber,[b] Ian A. Dubery,[b] Rosa Lanzetta[a] and Michelangelo Parrilli[a]

[a]*Dipartimento di Chimica Organica e Biochimica, Università di Napoli Federico II, via Cintia 4, 80126 Napoli, Italy*
[b]*Department of Biochemistry, University of Johannesburg, PO Box 524, Auckland Park 2006, South Africa*

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Abstract—The O-chain polysaccharide of the lipopolysaccharide from the endophytic bacterium *Burkholderia cepacia* strain was characterized. The structure was studied by means of chemical analysis and 2D NMR spectroscopy and shown to be the following:

$$\rightarrow 2)\text{-}\beta\text{-D-Rib}f\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow$$

Keywords: *Burkholderia cepacia*; Lipopolysaccharide; O-antigen; Ribose; Phytobacteria

1. Introduction

The *Burkholderia cepacia* complex (Bcc) consists of several species of closely related and extremely versatile Gram-negative bacteria found naturally in soil, water, and the rhizosphere of plants. The strains of Bcc have been used in the biological control of plant diseases and bioremediation, while some strains are plant pathogens or opportunistic pathogens of humans with cystic fibrosis.[1] The strains of *B. cepacia* can grow as phytopathogens or endophytes and soil species have an important role as biocontrol and plant growth promoting agents in the rhizosphere of several crop plants.[2] *B. cepacia* is able to colonize the surface of hyphae of *Fusarium oxysporum* sp. *cubense* and the fungal macrospores. In vitro colonization of the fungi by the endophyte results in mycelial deformation with terminal and intercalary swelling.[3]

Lipopolysaccharides play an essential role in the interaction of Gram-negative microorganisms with the surrounding environment, in particular, they play a major role in the molecular mechanisms of phytopathogenicity or symbiosis. LPSs are the main constituents of the outer leaflet of the outer cell membrane of Gram-negative bacteria, hence, they directly interact with the external environment.[4] They are made up of three chemically and biogenetically distinct regions,[5] namely, the glycolipid moiety of the Lipid A; the Core oligosaccharide region, and the O-specific chain (O-polysaccharide, O-chain).

LPS form *B. cepacia* elicits an enhanced defensitive capacity in treated tobacco plants.[6] This protection is accompanied by the synthesis of pathogenesis-related proteins with antimicrobial activity.[6] On a cellular level, LPS was found to lead to an influx of Ca^{2+} and extracellular alkalinization,[7] a burst in the generation of reactive oxygen species and NO,[7,8] and changes in the dynamics of protein phosphorylation.[9,10] The analysis of mRNA transcripts in LPS-treated cells revealed the up-regulation of genes involved in innate immunity.[8,11] The structural variation of the O-antigens of bacterial pathogen and symbiont LPS and its role in plant-microbe interactions was reviewed recently.[12] The composition and/or

* Corresponding author. Tel.: +39 081 674 123; fax: +39 081 674 393; e-mail: molinaro@unina.it

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size of the O-antigen might be reliable indicators of virulence potential and appears to be the part of a molecular communication between the bacterium and the host plant.[12]

This paper describes the structural elucidation of the O-polysaccharide fraction from the LPS of *B. cepacia* strain ASP B 2D.

2. Results and discussion

The cells of *B. cepacia* were extracted using the hot phenol–water procedure[13] and the LPS was detected in the water phase and further purified by digestion with nucleases, protease and by gel permeation chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoretic analysis of the purified LPS indicated a widespread O-antigen distribution in the characteristic ladder-like pattern formed by the repeating units (Fig. 1). The lipopolysaccharide was hydrolyzed using typical mild acidic conditions, and the O-polysaccharide fraction was collected, by centrifugation, as supernatant. Chemical analyses, carried out by GC–MS analysis of the acetylated *O*-methyl glycoside and *O*-octyl glycoside derivatives, yielded two different monosaccharides; ribose and glucose, both in D configuration. Methylation analysis showed the presence of the derivatives of 2-substituted ribofuranose and 6-substituted glucopyranose. The polysaccharide was eventually identified by NMR analysis, the ^{1}H NMR spectrum of (Fig. 2) appeared rather simple. In the anomeric region two signals were present and were related to two different spin systems, **A** and **B** at 5.10 and 5.04 ppm, respectively. The chemical shifts of each residue were assigned utilizing DQF-COSY, TOCSY, ROESY, HSQC and HMBC experiments (Table 1). The anomeric configurations were assigned on the basis of the chemical shifts, $^{1}J_{\text{H-1,H-2}}$ and $^{1}J_{\text{C-1,H-1}}$ values.

Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoretic analysis of purified LPS (in both lanes) from *Burkholderia cepacia* strain ASP B 2D showing the banding pattern of the repeating units of the O-antigen.

Spin system **A** was identified as the 2-substituted ribofuranose, on the basis of the correlations observed in the COSY and TOCSY spectra from the anomeric proton signal up to a diastereotopic methylene signal at position C-5. ^{13}C NMR data (Fig. 3) confirmed that ribose was present as 2-substituted furanose ring owing to the presence of the typical low field shifted signals (Table 1) and to a C-2 signal extra shifted due to substitution at that position.[14] The anomeric configuration of residue **A** was established by (i) the chemical shift of C-4 (around 84.0 ppm in case of β-configuration) and (ii) the low $^{3}J_{\text{H-1,H-2}}$ value derived from the DQF-COSY spectrum, indicative of β-configuration in aldofuranose rings (the $^{3}J_{\text{H-1,H-2}}$ value for β-configured rings is <2 Hz).[15,16] The second spin system, **B** residue, was identified as 6-substituted glucose, on the basis of the high $^{3}J_{\text{H,H}}$ vicinal coupling constant values (approximately 10 Hz),

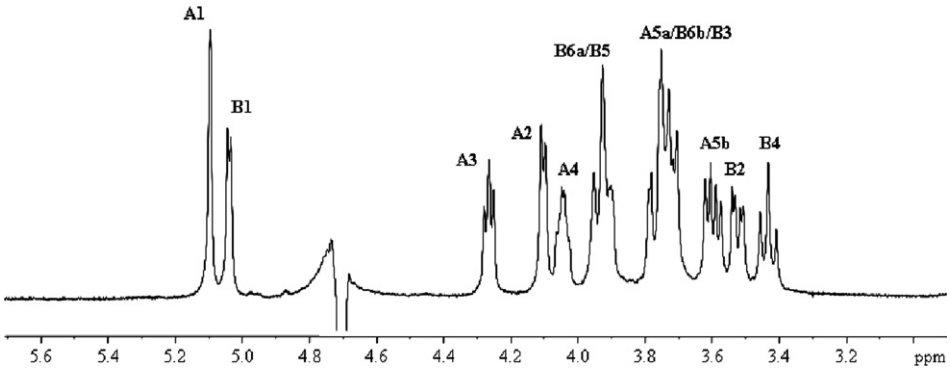

Figure 2. ^{1}H NMR spectrum of the purified O-polysaccharide from *B. cepacia* strain ASP B 2D. Letters refer to the assigned spin systems.

Table 1. ^{1}H and ^{13}C chemical shifts (ppm) of the O-chain from *B. cepacia* strain ASP B 2D

	1	2	3	4	5	6
2-β-Rib*f*	5.10	4.10	4.26	4.04	3.77/3.60	
A	105.7	79.8	70.8	83.9	62.8	
6-α-Glc	5.04	3.52	3.73	3.43	3.91	3.94/3.72
B	98.2	71.4	73.1	69.8	71.1	66.8

The values refer to internal standard acetone measured at 300 K (^{1}H 2.225, ^{13}C 31.45 ppm).

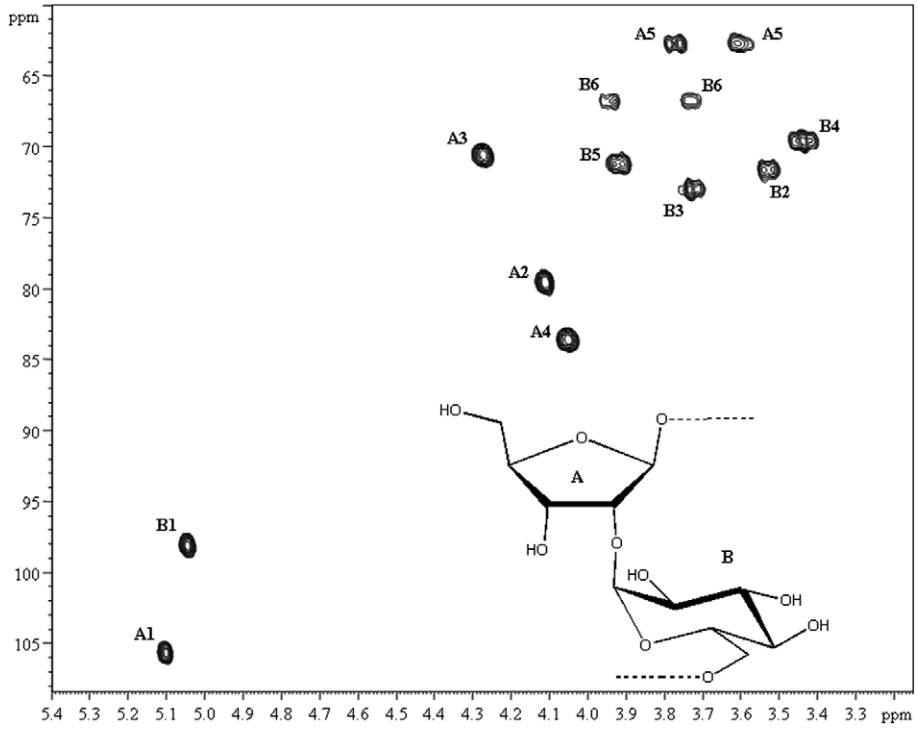

Figure 3. ^{1}H,^{13}C HSQC spectrum of the O-chain from *B. cepacia* strain ASP B 2D with all cross peaks assignment.

diagnostic of proton *trans*-di-axial orientation. On the basis of $^{3}J_{H\text{-}1,H\text{-}2}$, $^{1}J_{C\text{-}1,H\text{-}1}$ values (3.1 and 169 Hz, respectively) it was identified as α-glucose. From ^{13}C NMR chemical shifts it was possible to deduce that glucose was 6-substituted since the C-6 signal showed a downfield displacement because of glycosylation (Fig. 3).

In the ROESY spectrum (Fig. 4) it was possible to detect the dipolar correlations that allowed the establishment of the linkage of the two residues, and thus of the repeating unit of the O-polysaccharide. The anomeric proton of glucose **B** gave an NOE correlation with H-2 and H-1 proton signals of ribose **A**, whose anomeric signal showed an NOE correlation with the two H-6 signals of glucose. The HMBC experiment (not shown) only served as a confirmation of the O-polysaccharide repeating unit, since H-1/C-1 of ribose **A** showed inter-residual scalar connectivity with C-6/H-6 of glucose **B**, corroborating, in this way, the linkage between the two residues and, moreover, intra-residue connectivity with C-4/H-4, thus, also proving the presence of a fura-

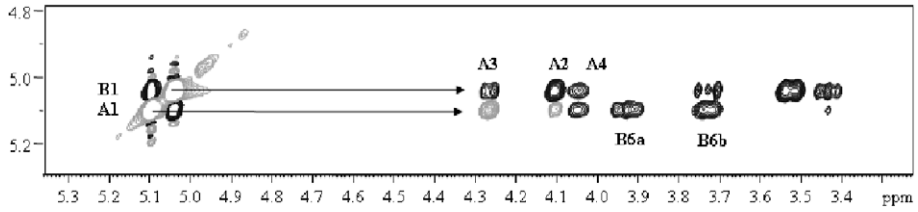

Figure 4. A section of the anomeric region of the 2D ROESY spectrum of the O-chain from *B. cepacia* strain ASP B 2D. Black levels indicate NOE and grey levels indicate TOCSY correlations. Letters refer to diagnostic inter-residual contacts.

nose ring. Likewise, the anomeric proton and the carbon signals of glucose **B** showed inter-residue scalar correlation with C-2/H-2 of ribose and intra-residue with their own C-5/H-5, thus confirming the repeating unit structure inferred on the basis of the NOE contacts.

In summary, the O-polysaccharide chain structure from *B. cepacia* was established by chemical analyses and 2D NMR spectroscopy and found to have the following repeating unit built up of 2-substituted-β-ribofuranose and 6-substituted-α-glucopyranose:

$$\rightarrow 2)\text{-}\beta\text{-D-Rib}f\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow$$

3. Experimental

3.1. Growth of bacteria, isolation of LPS and OPS

B. cepacia strain ASP B 2D, isolated from *Asparagus officinalis* were grown in Nutrient broth (Bio Lab) liquid medium at 25 °C on a continuous rotary shaker for 10 days.[6] The cells were collected by centrifugation, washed with saline and lyophilized. LPS were extracted from freeze dried bacterial cell walls using an adaptation of the phenol–water method, where the LPS fractionates into the water phase at 65 °C. The phase partitioning was repeated twice and the water phase, containing the LPS ad traces of RNA, was lyophilized. To further purify the LPS, Rnase (Roche), was added (50 μg enzyme to 0.5 g LPS in 20 mL distilled water) and the solution incubated at 37 °C for 2 h. An equal volume of 90% phenol was added to denature the enzyme and adhering proteins. The solution was vortexed and centrifuged at 12000*g* for 15 min. The upper water layer was removed, dialyzed against distilled water with three changes of water and lyophilized. The yield was 115 μg g^{-1} dried cell mass. The carbohydrate concentration was 0.87 mg mg^{-1} while LPS and Kdo concentration was 4.8 μg mg^{-1}. No protein was detected in the final preparation.

In order to obtain the O-polysaccharide chain, the LPS was hydrolyzed with aq 1% AcOH for 2 h at 100 °C and centrifuged (11,000 rpm, 4 °C, 1 h). The supernatant thus obtained (OPS fraction, 55 mg, 90% of LPS) was purified by gel permeation chromatography on a Sephacryl S100-HR column (90 cm × 1.5 cm) using 0.05 M ammonium bicarbonate as the eluent and monitored with a Waters differential refractometer.

3.2. NMR spectroscopy

1D and 2D ^{1}H NMR spectra were recorded on a solution of 3 mg in 0.6 mL of D_2O, at 25 °C. ^{1}H and ^{13}C NMR experiments were carried out using a Bruker DRX-400. The spectra were calibrated with internal acetone δ_H 2.225, δ_C 31.45. Rotating frame Overhauser enhancement spectroscopy (ROESY) was measured using data sets ($t1{\cdot}t2$) of 4096·1024 points, and 16 scans were acquired. A mixing time of 200 ms was used. Double quantum-filtered phase-sensitive COSY experiments were performed with 0.258 s acquisition time, using data sets of 4096·1024 points, and 64 scans were acquired. Total correlation spectroscopy experiments (TOCSY) were performed with a spinlock time of 120 ms, using data sets ($t1{\cdot}t2$) of 4096·512 points, and 16 scans were acquired. In all homonuclear experiments the data matrix was zero-filled in the F1 dimension to give a matrix of 4096·2048 points and was resolution enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. Heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments were measured in the ^{1}H-detected mode with proton decoupling in the ^{13}C domain, using data sets of 2048·512 points, and 100 scans were acquired for each $t1$ value. Experiments were carried out in the phase-sensitive mode according to the method of States et al.[17] A 60 ms delay was used for the evolution of long-range connectivity in the HMBC experiment. In all heteronuclear experiments the data matrix was extended to 2048·1024 points using forward linear prediction extrapolation.

3.3. Gas chromatography

GC was performed on a Hewlett–Packard 5890 instrument, SPB-5 capillary column (0.25 mm × 30 m, Supelco), for compositional and methylation analyses the temperature program was 150 °C for 5 min, then 5 °C min^{-1} to 300 °C, for absolute configuration analysis was 150 °C for 8 min, then 2 °C min^{-1} to 200 °C for 0 min, then 6 °C min^{-1} to 260 °C for 5 min.

3.4. Compositional and methylation analysis

The monosaccharides were identified as acetylated *O*-methyl glycosides derivatives, briefly, samples were methanolyzed with 2 M HCl/MeOH at 85 °C 20 h, dried under reduced pressure and then acetylated with acetic anhydride in pyridine at 80 °C for 30 min. After work-up, the sample was analyzed by the GLC–MS. The absolute configuration of ribose and glucose was determined by GLC of acetylated glycosides of (+)-2-octanol according to the published method.[18] Methylation was carried out with methyl iodide in dimethyl sulfoxide in the presence of sodium hydroxide.[19] Hydrolysis of the methylated O-polysaccharide was carried out with 2 M TFA (120 °C, 1 h). The partially methylated monosaccharides were reduced with $NaBD_4$ and converted to their alditol acetates with acetic anhydride in pyridine at 80 °C for 30 min and analyzed by GLC–MS.

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