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Note

The structure of a putative exopolysaccharide of *Burkholderia gladioli* pv. *agaricicola*

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Abstract—A putative capsular polysaccharide containing D-rhamnose was isolated from the phytopathogenic bacterium *Burkholderia gladioli* pv. *agaricicola* by phenol/water extraction followed by ultracentrifugation of the separated water phase and gel-permeation chromatography of the thus obtained supernatant. By means of chemical analyses and NMR spectroscopy, the repeating unit of the polymer was shown to be a linear tetrasaccharide with the structure.

→4)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→3)-β-D-Rha*p*-(1→

Keywords: Mushroom disease; *Agaricus bitorquis*; *Burkholderia gladioli* pv. *agaricicola*; Exopolysaccharide; NMR spectroscopy

Sporophores of the edible mushroom *Agaricus bitorquis* are subject to infection by *Burkholderia gladioli* pv. *agaricicola*, which causes soft rot disease. The disease is manifested by a rapid development of deep oozing lesions on the pileal surface, which renders the mushroom unmarketable. Several devastating cases of wet or soft rot of mushrooms have been observed in the UK.[1,2] Due to the practical and economical importance of the disease, studies were initiated into the structures and toxicity of the lipopolysaccharide (LPS) and the exopolysaccharide (EPS) in order to clarify their role in the plant–pathogen interaction and the disease. Other studies on the isolation and chemical and biological characterization of toxic metabolites are also in progress. Preliminary results suggest that these metabolites belong to the class of lipodepsipeptides.

In this paper, the isolation and the structural determination of a putative EPS from *B. gladioli* pv. *agaricicola* are reported.

After cultivation and harvest, the bacterial cell mass was lyophilized (4.57 g) and then extracted utilizing the hot phenol/water method.[3] The lipopolysaccharide was precipitated from the obtained water phase by ultracentrifugation, the supernatant of which was lyophilized (184.8 mg, 4% of the bacterial dry mass). This material was then purified by gel-permeation chromatography on Sephadex G-50, from which one fraction eluting with the void volume was obtained and lyophilized (145 mg, 3.2% of the bacterial dry mass).

Sugar analyses of the polysaccharide fraction dentified D-rhamnose as the major constituent and minor quantities of Gal and Man originating from the

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O-specific polysaccharide of the LPS (manuscript in preparation). The approximate molar ratio of Rha:Gal:Man was 40:3:3. The major products of methylation analysis were 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-rhamnitol and 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-rhamnitol in the molar ratio of ~3:1. This data indicated the presence of a putative EPS being composed of 3- and 4-substituted rhamnopyranose residues.

The ^{1}H NMR spectrum (Fig. 1) of the EPS showed four anomeric signals at δ 5.064 (**A**), δ 5.049 (**B**), δ 5.028 (**C**), and δ 4.754 (**D**), and four overlapping methyl signals characteristic for 6-deoxy-sugars in the region δ 1.350–1.304. The sugar residues were labeled **A**–**D** in order to decrease chemical shifts of the anomeric protons. Additional minor resonances originated from the O-specific polysaccharide of the LPS, which was not further characterized in this investigation (anomeric signals at δ 5.17 and δ 4.58).

The ^{13}C NMR spectrum contained 20 signals, however, since four of them possessed double intensity (δ 103.1, δ 71.1, δ 70.4, and δ 17.7), a total of 24 carbon atoms were present, thus confirming a repeating unit comprising four hexoses. A ^{1}H,^{13}C-heteronuclear multiple-quantum coherence (HMQC) experiment identified four anomeric carbon signals at δ 103.11 (**A**), δ 103.14 (**B**), δ 103.21 (**C**), and δ 101.24 (**D**).

The anomeric configurations of all rhamnose residues were assigned from the coupling constants $^{1}J_{\text{H-1,C-1}}$, which were identified in another ^{1}H,^{13}C HMQC experiment without decoupling. The $^{1}J_{\text{H-1,C-1}}$ values 172 Hz for residues **A**, **B**, and **C**, and 161 Hz for residue **D** revealed their α and β configuration, respectively.[4]

The complete structural characterization of the EPS was achieved by 1D and 2D ^{1}H and ^{13}C NMR spectroscopy. ^{1}H,^{1}H COSY, double-quantum-filtered COSY (DQFCOSY), and TOCSY, as well as ^{1}H,^{13}C HMQC spectra allowed the complete assignment of all ^{1}H and ^{13}C chemical shifts (Table 1). Low-field shifted signals of carbon atoms C-3 (**B** δ 79.90, **C** δ 79.40, and **D** δ 81.73) and C-4 (**A** δ 83.56) compared with those of unsubstituted rhamnose proved glycosylation at O-3 of residues **B**, **C**, and **D**, and at O-4 of **A**.[5] These data confirmed those of methylation analysis.

The 2D rotating frame nuclear Overhauser spectroscopy (ROESY) and ^{1}H,^{13}C-heteronuclear multi bond correlation (HMBC) experiments revealed the sequence of the sugar residues in the repeating unit. Strong *inter*residual NOE contacts were observed between protons **A-1/B-3**, **B-1/C-3**, **C-1/D-3**, and **D-1/A-4** (Fig. 2). Weak NOE contacts were also found between **A-1/B-4**, **B-1/C-4**, **C-1/D-4**, and **D-1/A-3**. Thus, the sequence →**A**→**B**→**C**→**D**→ was established, which was confirmed by the HMBC experiment identifying the *inter*residual proton–carbon correlations, H-1 of **A** and C-3 of **B**, H-1 of **B** and C-3 of **C**, H-1 of **C** and C-3 of **D**, and H-1 of **D** and C-4 of **A**, as well as H-4 of **A** and C-1 of **D**, H-3 of **B** and C-1 of **A**, H-3 of **C** and C-1 of **B**, H-3 of **D** and C-1 of **C**.

In summary, the data identified the structure of a putative EPS from *B. gladioli* pv. *agaricicola* as

A **B** **C** **D**

→4)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→3)-β-D-Rha*p*-(1→

The role of EPS produced by plant pathogenic bacteria has not been completely clarified, however, EPS are considered either being able to avoid or delay the activation of plant defence or to act as signal substances in plant–pathogen cross-talk. In the last decades, chemical compositions and structures of several EPS have been characterized. Many plant-related pseudomonads produce alginate and levan.[6] Other EPS possess a very complex structure, being branched heteropolymers. Such compositional and structural differences of the EPS purified from different plant pathogens suggest that these molecules possess specific and different roles in plant–pathogen interactions.[7]

1. Experimental

1.1. Growth of bacteria

Type strain ICMP11096 of *B. gladioli* pv. *agaricicola* was grown at 25 °C under shaking (180 rpm). 500 ml Erlenmeyer flasks were filled with 150 mL of liquid King's B medium and inoculated with 1.5 mL of a bacterial suspension containing 10^{8} cfu/mL.[8] After 48 h, cultures were centrifuged (20,000*g* for 15 min) and the resulting supernatants were tested for antimicrobial activity against *Bacillus megaterium* according to a procedure established earlier,[9] then lyophilized and stored at −20 °C prior to further processing.

1.2. Isolation of the polysaccharide

The lyophilized bacteria (4.57 g) were extracted with hot phenol/water,[3] the water phase of which was lyophilized (335 mg, 7.3% of the bacterial dry mass), then suspended in 10 mL of ultrapure Mili-Q water and centrifuged (4 °C, 100,000*g*, 6 h). The pellet was washed with ultrapure Mili-Q water and centrifuged again. Both obtained supernatants were combined and lyophilized (184.8. mg, 4% of the bacterial dry mass). This material was purified

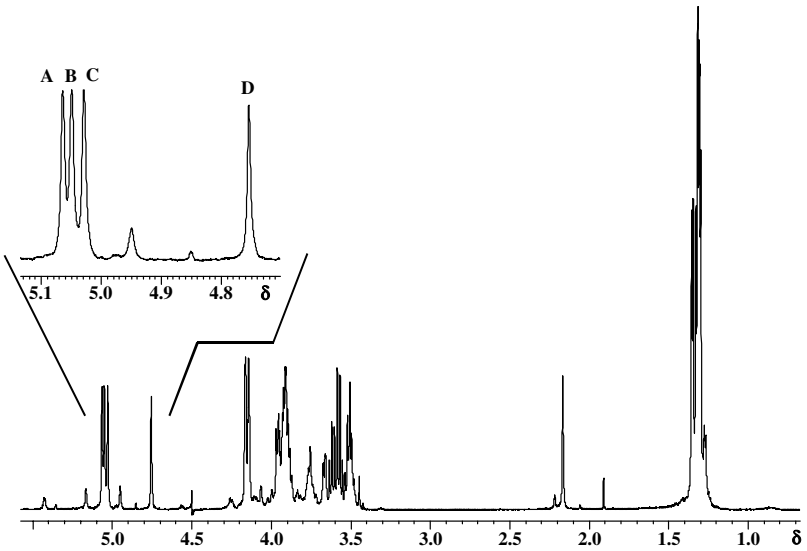

Figure 1. The ^{1}H NMR spectrum of the capsular polysaccharide isolated from *Burkholderia gladioli* pv. *agaricicola*. The spectrum was recorded at 600 MHz and 50 °C. The sugar residues were labeled **A**–**D** according to the decreasing chemical shifts of the anomeric protons.

Table 1. ^{1}H and ^{13}C NMR data of the capsular polysaccharide from *Burkholderia gladioli* pv. *agaricicola*

Residue	Chemical shift of proton and carbon [δ]					
	1	2	3	4	5	6
→4)-α-D-Rha*p*-	5.064	4.147	3.960	3.618	3.958	1.323
A	103.11	70.62	70.09	<u>83.56</u>	68.65	17.51
→3)-α-D-Rha*p*-	5.049	4.164	3.917	3.564	3.902	1.315
B	103.14	71.08	<u>79.90</u>	72.33	70.38	17.70
→3)-α-D-Rha*p*-	5.028	4.164	3.924	3.583	3.902	1.304
C	103.21	71.08	<u>79.40</u>	72.39	70.38	17.70
→3)-β-D-Rha*p*-	4.754	4.138	3.667	3.508	3.500	1.350
D	101.24	71.69	<u>81.73</u>	72.09	73.23	17.83

Spectra were recorded at 50 °C in $^{2}H_2O$ relative to internal acetone (δ_H 2.225; δ_C 31.45). Underlined values indicate positions of substitution.

by gel-permeation chromatography on a column (70 × 2.6 cm) of Sephadex G-50 (Pharmacia) using 0.1 M pyridinium acetate buffer (pH 4.2) as eluent and monitoring with a Knauer differential refractometer. One fraction was obtained by eluting with the obtained void volume, lyophilized (145 mg, 3.2% of the bacterial dry mass) and used in further investigations.

1.3. Compositional analyses

The capsular polysaccharide was hydrolyzed with 0.1 M HCl (48 h, 100 °C). Rhamnose was identified as alditol acetate by GLC using a Hewlett–Packard 5880 instrument equipped with a SPB-5 capillary column (30 m × 0.25 mm × 0.25 μm) and applying a temperature gradient of 150 (3 min)–260 °C at 3 °C/min. The absolute configuration of Rha was determined by GLC of its acetylated (*S*)-2-butanol glycoside utilizing the same chromatographic conditions as above.[10,11]

1.4. Methylation analysis

Methylation was carried out according to Ciucanu and Kerek.[12] The methylated sample was extracted from DMSO by chloroform, then hydrolyzed, reduced with $NaBH_4$ and acetylated, and the products were analyzed by GLC–MS using a Hewlett–Packard 5989 instrument equipped with a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) and applying a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C/min.

1.5. NMR spectroscopy

NMR spectra were obtained for solutions in $^{2}H_2O$ with a Bruker DRX Avance 600 MHz spectrometer (operating frequencies 600.31 MHz for ^{1}H NMR and 150.96 MHz for ^{13}C NMR) at 50 °C. Chemical shifts were reported relative to internal acetone (δH 2.225; δC 31.45). One-dimensional ^{1}H and ^{13}C NMR, and

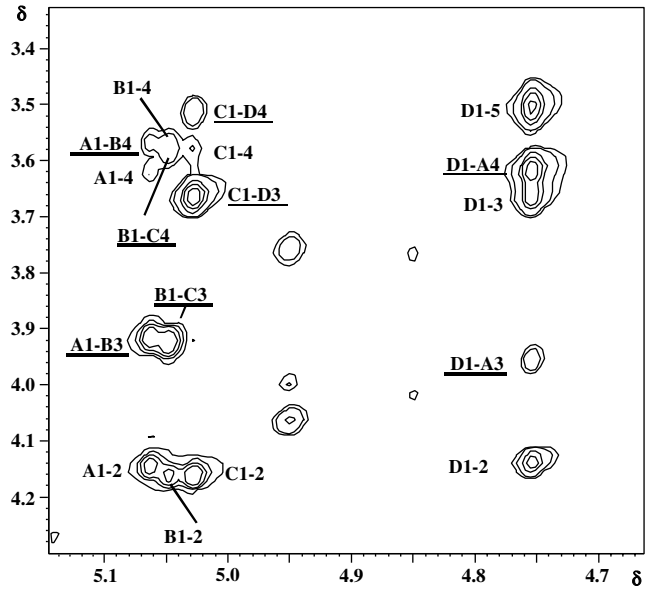

Figure 2. Sections of the ROESY spectrum of *Burkholderia gladioli* pv. *agaricicola* capsular polysaccharide. The spectrum was recorded at 600 MHz and 50 °C. The *letters* refer to the carbohydrate residues as shown in Figure 1, and the *arabic numerals* refer to the protons in the respective residues. The *inter*residual NOE contacts are underlined.

COSY, TOCSY, ROESY, DQFCOSY, as well as the $^{1}H,^{13}C$-heteronuclear HMQC and HMBC experiments were recorded applying standard Bruker software. A mixing time of 100 ms for TOCSY was used, and the spinlock field strength was 9400 Hz. A spinlock field strength of 4100 Hz for 150 ms was applied in ROESY measurements. The spectral widths were 5388 Hz in both dimensions for $^{1}H,^{1}H$ correlations, and 5388 Hz in F2 and 16,600 Hz in F1 for $^{1}H,^{13}C$ correlations. A total of 512 experiments each consisting of 16 free induction decays (FID) for homocorrelations and 96 FID for heterocorrelations with 2048 data points were acquired.

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