Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Research 343 (2008) 392–396
Carbohydrate RESEARCH

Note

The structure of the O-specific polysaccharide of the lipopolysaccharide from *Pantoea agglomerans* strain FL1

Alessio Cimmino,[a,c] Guido Marchi,[b] Giuseppe Surico,[b] Anna Hanuszkiewicz,[c] Antonio Evidente[a] and Otto Holst[c,*]

[a]*Dipartimento di Scienze del Suolo, della Pianta, dell' Ambiente e delle Produzioni Animali, Universitá Degli Studi di Napoli Federico II, I-80055 Portici (NA), Italy*
[b]*Dipartimento di Biotecnologie Agrarie-Patologia Vegetale, Università di Firenze, I-50144 Firenze, Italy*
[c]*Division of Structural Biochemistry, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, D-23845 Borstel, Germany*

Received 13 August 2007; received in revised form 11 October 2007; accepted 24 October 2007
Available online 1 November 2007

Abstract—A neutral O-specific polysaccharide consisting of D-rhamnose was obtained by mild acid hydrolysis of the lipopolysaccharide of the plant pathogenic bacterium *Pantoea agglomerans* strain FL1, a common epiphyte of many plant species, and associated with *Pseudomonas savastanoi* pv. *savastanoi* in young and apparently intact olive knots. By means of compositional and methylation analyses, and NMR spectroscopy, the chemical repeating unit of the polymer was identified as a linear tetrasaccharide of the structure:

→2)-α-D-Rha*p*-(1→2)-β-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→

Keywords: Olive knot; *Pantoea agglomerans*; Lipopolysaccharide; O-specific polysaccharide; NMR spectroscopy

The phytopathogenic bacterium *Pseudomonas savastanoi* pv. *savastanoi* (*Ps. savastanoi*) is a common resident in the olive phyllosphere being able to switch from an epiphytic phase sensu Laben[1] to an invasive phase every time wounds of various nature allow the penetration of the bacterium into the host tissues.[2,3] The macroscopic result, in both, natural and artificial environment, of an interaction of the bacterium with a susceptible host is the development of knots. Results of a survey carried out in olive orchards of central Italy in 2002–2003 showed that *Pantoea agglomerans* (*P. agglomerans*) was associated with *Ps. savastanoi* in 70% of olive knots examined.[4] Pathogenicity tests carried out by coinoculation of the two species on the stem of 1-year-old olive plants in ratios of 1:1, 1:100 and 100:1 showed that the population growth of *P. agglomerans* was supported by the presence of an actively growing population of *Ps. savastanoi*. At the same time, the presence of a prevailing population of *P. agglomerans* in the inoculation site may have a detrimental effect on the population growth of *Ps. savastanoi*. Moreover, in certain situations the association of *P. agglomerans* with *Ps. savastanoi* quantitatively increases the host cells' proliferation process.[5]

It is well established that LPSs play an important role in host-pathogen interactions in animals, however, this holds also true for such interactions between pathogenic bacteria and plants,[6–9] including the elicitation of host defense mechanisms. Recently, the effects of LPS from a pathogenic strain of *Xanthomonas campestris* pv. *campestris* and its fragments on *Arabidopsis thaliana* and *Nicotiana tabacum* have been reported,[10,11] proving LPS-induced plant defense responses. Also, specific

* Corresponding author. Tel.: +49 4537 188472; fax: +49 4537 188745; e-mail: oholst@fz-borstel.de

0008-6215/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2007.10.023

structural features of LPSs may have chemotaxonomic value among strains and species of the same genus.

In this paper the isolation and the structural determination of the O-specific polysaccharide (OPS) of the LPS from *P. agglomerans* cells are reported.

After cultivation and harvest, the bacterial cell mass was lyophilized (5.70 g) and then extracted utilizing the hot phenol/water method.[12] The resulting aqueous phase was dialyzed then freeze-dried (900 mg, 16% of bacterial dry mass), dissolved in water, and the LPS was precipitated by ultracentrifugation and lyophilized (360 mg, 6.3% of bacterial dry mass).

Mild acid hydrolysis of the LPS and centrifugation gave a lipid sediment (lipid A, 32.9 mg, 2.1% of the bacterial dry mass) and a water-soluble carbohydrate fraction (60.1 mg, 3.8% of the bacterial dry mass). This carbohydrate fraction was separated by gel-permeation chromatography (GPC) on Sephadex G-50, from which two main fractions were obtained, the first of which contained the OPS which was lyophilized (31.6 mg, 2% of the bacterial dry mass) and used in further investigations, the second fraction contained core fraction which is not investigated in this work.

Sugar analysis of the OPS identified D-rhamnose as the major constituent and minor quantities of glucose and galactose, in an approximate molar ratio of Rha:Glc:Gal of 30:3:1. Methylation analyses gave mainly 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-rhamnitol and 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-rhamnitol in an approximate molar ratio of 3:1, which revealed the presence of 2- and 3-substituted rhamnopyranose residues.

The ^{1}H NMR spectrum (Fig. 1) of the OPS showed four anomeric signals at δ 5.20 (**A**), δ 4.84 (**B**), δ 5.05 (**C**), and δ 5.12 (**D**), and four overlapping methyl signals characteristics for 6-deoxy-sugars in the region δ 1.28–1.31. The sugar residues were labeled **A**–**D** according to the sequence determined for the repeating unit.

The ^{13}C NMR spectrum contained 22 signals, however, two of them possessed double intensity (δ 70.52 and δ 17.80), thus, a total of 24 carbon atoms were present, confirming a repeating unit comprising four hexoses. A ^{1}H,^{13}C HMQC experiment identified four anomeric carbon signals at δ 101.41 (**A**), δ 97.85 (**B**), δ 103.10 (**C**), and δ 102.04 (**D**).

The anomeric configuration of all rhamnose residues was assigned from the coupling constants $^{1}J_{\mathrm{H\text{-}1,C\text{-}1}}$, which were identified in another ^{1}H,^{13}C HMQC experiments without decoupling. The $^{1}J_{\mathrm{H\text{-}1,C\text{-}1}}$ values of 176 Hz, 175 Hz, and 174 Hz for residues **A**, **C** and **D**, and 159 Hz for residue **B**, revealed their α and β (residue B) configurations, respectively.[13]

The complete structural characterization of the O-chain was achieved by 1D and 2D ^{1}H and ^{13}C NMR spectroscopy. ^{1}H,^{1}H COSY, TOCSY, ROESY, and NOESY, as well as ^{1}H,^{13}C HMQC and HMBC spectra allowed the complete assignment of all ^{1}H and ^{13}C chemical shifts (Table 1). Low-field shifted signals of carbon atoms C-2 (**A** δ 79.72, **B** δ 79.09, and **D** δ 78.95) and C-3 (**C** δ 78.18) compared with those of unsubstituted rhamnose proved the glycosylation at O-2 of residues **A**, **B**, and **D**, and at O-3 of **C**.[14] These data were consistent with that of methylation analysis. The NOESY, ROESY, and HMBC experiments revealed the sequence of the sugar residues in the repeating unit. Strong inter-residual NOE contacts were observed in NOESY spectrum between protons **A**-1/**B**-2, **B**-1/**C**-3, **C**-1/**D**-2, and **D**-1/**A**-2 (Fig. 2). Also, intra-residual NOE contacts were found between protons **B**-1/**B**-2, **B**-1/**B**-3, and **B**-1/**B**-5, resulting from the *syn*-diaxial orientation of those β-rhamnopyranosyl ring protons. Thus, the sequence →**A**→**B**→**C**→**D** was established which was confirmed by the HMBC experiment (not shown) identifying the inter-residual proton-carbon correlations, that is, between H-1 of **A** and C-2 of **B**, H-1 of **B** and C-3 of **C**, H-1 of **C** and C-2 of **D**, H-1 of **D** and C-2 of **A**, as well as H-2 of **A** and C-1 of **D**, H-2 of **B** and C-1 of **A**, H-3 of **C** and C-1 of **B**, H-2 of **D** and C-1 of **C**. Thus, the structure of the OPS from LPS of *P. agglomerans* was established as

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

→2)-α-D-Rha*p*-(1→2)-β-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→

In conclusion, the chemical repeating unit structure of the OPS of the LPS from *P. agglomerans* was established, representing a novel repeating unit which is different from that described for another strain of the same species that grows symbiotically in wheat.[15] Rhamnose is a common sugar in OPS of plant-pathogenic bacteria, a number of rhamnan backbones have been identified in OPS of LPS from plant-pathogenic *Xanthomonas* and *Pseudomonas* species.[16–19] As described above, several studies have demonstrated a strict cooperation of *P. agglomerans* and *Ps. savastanoi* during the induction of olive knot formation, affecting the proliferation process.[5] Whether the LPS of *P. agglomerans* alone or those of both bacteria play(s) an important role in this process, is currently being investigated.

1. Experimental

1.1. Cultivation of bacteria

Strain *P. agglomerans* FL1 was isolated from young and apparently intact olive knots collected from orchards located in the province of Grosseto in Tuscany.[5] The

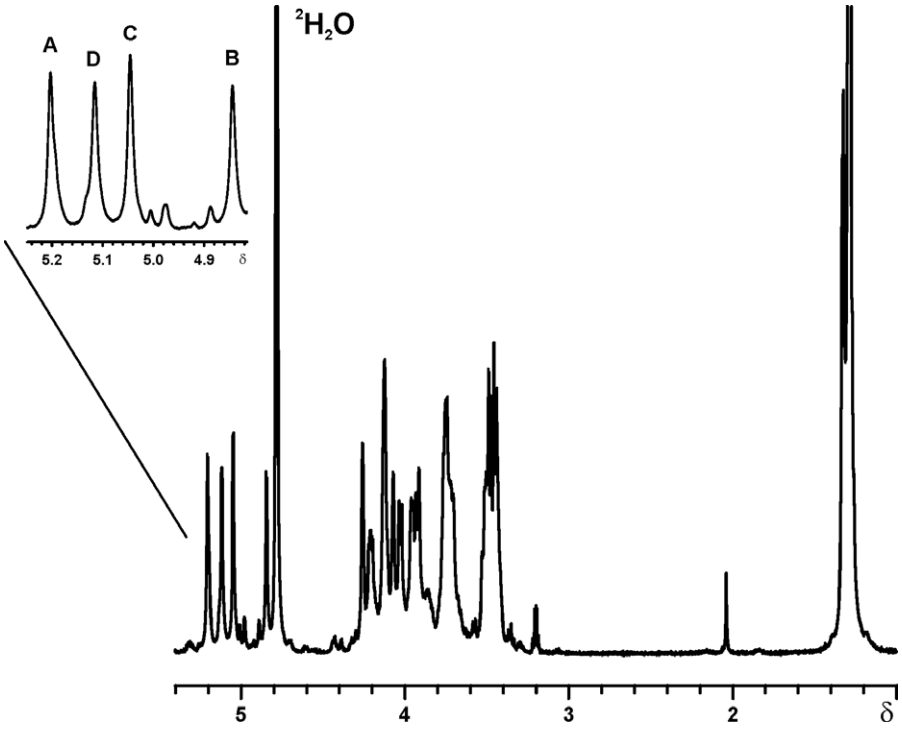

Figure 1. The ^{1}H NMR spectrum of the OPS of the LPS from *Pantoea agglomerans* strain FL1. The spectrum was recorded at 600 MHz and 27 °C. The sugar residues were labeled **A** to **D** according to the sequence in the repeating unit.

Table 1. ^{1}H and ^{13}C NMR data of the OPS isolated from *Pantoea agglomerans* strain FL1

Residue	Chemical shift of proton and carbons (δ)					
	1	2	3	4	5	6
→2)-α-D-Rha*p*-	5.20	4.13	4.01	3.45	4.22	1.28
A	101.41	<u>79.72</u>	70.94	73.42	70.09	17.80
→2)-β-D-Rha*p*-	4.84	4.07	3.71	3.51	3.44	1.31
B	97.85	<u>79.09</u>	74.48	71.59	73.47	17.98
→3)-α-D-Rha*p*-	5.05	4.25	3.95	3.52	3.74	1.28
C	103.10	68.41	<u>78.18</u>	71.57	70.52	17.80
→2)-α-D-Rha*p*-	5.12	4.11	3.91	3.46	3.74	1.29
D	102.04	<u>78.95</u>	71.10	73.29	70.52	17.91

Spectra were recorded at 27 °C in ^2H_2O relative to internal acetone (δ_H 2.225; δ_C 31.45). Underlined chemical shifts indicate substituted positions.

strain was stored in 15% aqueous glycerol at −70 °C and regrown on nutrient sucrose agar plates at 27 °C for 48 h. Erlenmeyer flasks (1 L volume) containing 200 mL of King's B[20] were inoculated with 0.5 mL of 10^8 log-phase bacterial cells of *P. agglomerans* FL1 and incubated under shaking at 27 °C for 3 days. The bacterial cells were harvested by centrifugation at 10,000*g* for 10 min and washed three times with 0.85% (w/v) NaCl. Harvested cells were frozen at −40 °C and lyophilized.

1.2. Isolation and analyses of LPS

The lyophilized bacteria were extracted with hot phenol/water,[12] the water phase of which was dialyzed and lyophilized. The sample was suspended in 10 mL of ultrapure Mili-Q water and centrifuged (4 °C, 100,000*g*, 6 h). The LPS was recovered from the pellet, which was washed with ultrapure Mili-Q water, ultracentrifuged again, and freeze-dried. Both obtained supernatants were combined and lyophilized. The lipopolysaccharide (100 mg) was hydrolyzed with acetate buffer (pH 4.4) at 100 °C for 2 h, the lipid precipitate (lipid A) was removed by ultracentrifugation (100,000*g*, 4 °C, 4 h) and lyophilized. The supernatant was also lyophilized to yield the polysaccharide which was fractionated by gel-permeation chromatography on a column (70 cm × 2.6 cm) of Sephadex G-50 using 0.1 M pyridinium acetate buffer as eluent and monitoring with a differential refractometer (Knauer). Two main fractions were obtained, the first of which (O-specific

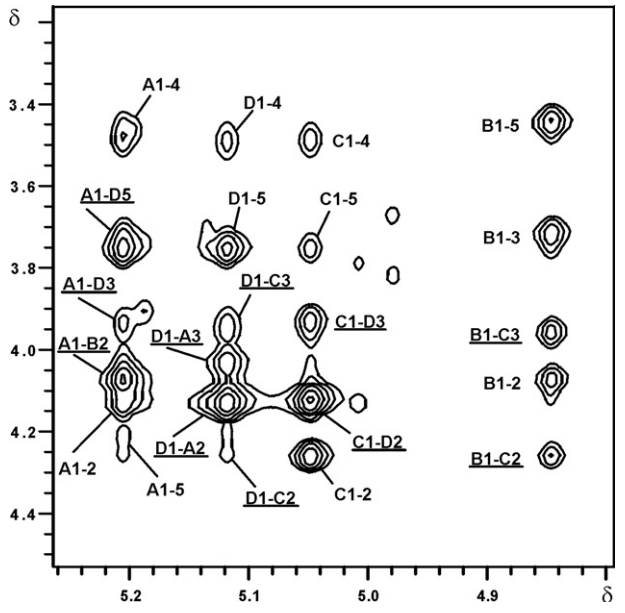

Figure 2. Section of the NOESY spectrum of the OPS of the LPS from *Pantoea agglomerans* strain FL1. The spectrum was recorded at 600 MHz and 27 °C. The *letters* refer to the carbohydrate residues as defined in Table 1, and the *arabic numerals* refer to the protons in the respective residues. The inter-residual NOE contacts are underlined.

chain) was lyophilized and used in further investigations. For compositional analysis, O-specific polysaccharide was hydrolyzed with 0.1 M HCl (100 °C, 48 h). Rhamnose was identified as alditol acetate as the main sugar by GLC using a Hewlett-Packard 5880 instrument equipped with a capillary column (30 m × 0.25 mm, 0.25 μm film thickness) of SPB-5 and applying a temperature gradient of 150 °C (3 min) to 320 °C at 3 °C/min. The absolute configuration of Rha was determined by GLC of its acetylated (*S*)-2-butyl glycoside applying the same chromatographic conditions as above.[21,22]

1.3. Methylation analysis

Methylation of the polysaccharide was carried out according to Ciucanu and Kerek.[23] The methylated sample was extracted from DMSO three times with chloroform, organic fractions were pooled, passed through 25 mL Mili-Q water, and evaporated. Permethylated sample was hydrolyzed (2 M CF_3CO_2H, 120 °C, 2 h), reduced with $NaBH_4$, acetylated, and products were analyzed by GLC–MS using a Hewlett-Packard 5989A instrument equipped with a HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness) and applying a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C/min.

1.4. NMR spectroscopy

NMR spectra were obtained on solutions of ^2H_2O with a Bruker DRX Avance 600 MHz spectrometer (operating frequencies 600.31 MHz for ^1H NMR and 150.96 MHz for ^{13}C NMR) at 27 °C. Chemical shifts were reported relative to internal acetone (δ_H 2.225; δ_C 31.45). One-dimensional ^1H and ^{13}C NMR, and 2D $^1H,^1H$ COSY, TOCSY, NOESY, ROESY, as well as the $^1H,^{13}C$-heteronuclear HMQC and HMBC experiments were recorded applying standard Bruker software. The spectral width for ^1H, ^{13}C correlations was 6009 Hz in F2 dimension and 31,694 Hz in F1 dimension. A mixing time of 250 ms was used for NOESY and ROESY experiments. The spin-lock field strength corresponded to a 90° pulse with 35 μs and the mixing time of 100 ms was used for TOCSY.

Acknowledgments

We thank H. Moll for help with GLC–MS and H. Käßner for recording NMR spectra.

References

1. Laben, C. *Plant Dis.* **1981**, *65*, 633–637.
2. Ercolani, G. L. *Phytopathol. Mediterr.* **1971**, *10*, 130–132.
3. Lavermicocca, P.; Surico, G.; Varvaro, L.; Babelegoto, N. M. *Phytopathol. Mediterr.* **1987**, *26*, 65–72.
4. Marchi, G.; Viti, C.; Giovanetti, L.; Surico, G. *Eur. J. Plant Pathol.* **2005**, *112*, 101–112.
5. Marchi, G.; Sisto, A.; Cimmino, A.; Andolfi, A.; Cipriani, M. G.; Evidente, A.; Surico, G. *Plant Pathol.* **2006**, *55*, 614–624.
6. Medzhitov, R.; Janeway, C. A., Jr. *Curr. Opin. Immunol.* **1998**, *10*, 12–15.
7. Medzhitov, R.; Janeway, C. A., Jr. *Cell* **1997**, *91*, 295–298.
8. Down, J. M.; Newman, M. A.; von Roepenack, E. *Ann. Rev. Phytopathol.* **2000**, *38*, 241–261.
9. Newman, M.-A.; Dow, J. M.; Molinaro, A.; Parrilli, M. *J. Endotoxin Res.* **2007**, *13*, 69–84.
10. Silipo, A.; Molinaro, A.; Sturiale, L.; Dow, J. M.; Herbs, J.; Lanzetta, R.; Newman, M. A.; Parrilli, M. *J. Biol. Chem.* **2005**, *280*, 33660–33668.
11. Kaczyński, Z.; Braun, S.; Lindner, B.; Niehaus, K.; Holst, O. *J. Endotoxin Res.* **2007**, *13*, 101–108.
12. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.
13. Bock, K.; Lundt, I.; Pedersen, C. *Tetrahedron Lett.* **1973**, *13*, 1037–1040.
14. Jansson, P. E.; Kenne, L.; Widmalm, G. *Carbohydr. Res.* **1989**, *188*, 169–191.
15. Kohchi, C.; Inagawa, H.; Nishizawa, T.; Yamaguchi, T.; Nagai, S.; Soma, G. *J. Biosci. Bioeng.* **2006**, *102*(6), 485–496.
16. Knirel, Y. A.; Kochetkov, N. K. *Biochemistry (Moscow)* **1994**, *59*, 1325–1383.
17. Jansson, P. E. In *Endotoxin in Health and Disease*; Brade, H., Opal, S. M., Vogel, S. N., Morrison, D. C., Eds.; Marcel Dekker: New York, 1999; pp 155–178.
18. Corsaro, M. M.; De Castro, C.; Molinaro, A.; Parrilli, M. *Recent Res. Dev. Phytochem.* **2001**, *5*, 119–138.

19. Evidente, A.; Motta, A. In *Studies in Natural Products Chemistry*; Atta-ur-Rahman, Ed.; Elsevier: Amsterdam, 2002; Vol. 26, pp 581–629.
20. King, E. O.; Ward, M. K.; Raney, D. E. *J. Lab. Clin. Med.* **1954**, *62*, 301–307.
21. Leontein, K.; Lindberg, B.; Lönngren, J. *Carbohydr. Res.* **1978**, *62*, 359–362.
22. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1979**, *77*, 1–7.
23. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.