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Carbohydrate Research 338 (2003) 2025–2027
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Note

Structure of the O-polysaccharide of *Erwinia carotovora* ssp. *carotovora* GSPB 436☆

Sof'ya N. Senchenkova,[a] Yuriy A. Knirel,[a,*] Alexander S. Shashkov,[a] Mamdoh Ahmed,[b] Athanasios Mavridis,[b] Klaus Rudolph[b]

[a] *N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospeckt 47, 119991 Moscow, Russian Federation*
[b] *Institut für Pflanzenpathologie und Pflanzenschutz, Georg-August-Universität, D-37077 Göttingen, Germany*

Received 20 May 2003; accepted 17 June 2003

Abstract

The O-polysaccharide of a phytopathogenic bacterium, *Erwinia carotovora* ssp. *carotovora* GSPB 436, was studied by sugar and methylation analysis, along with ^{1}H and ^{13}C NMR spectroscopy. The following structure of the branched tetrasaccharide repeating unit of the O-polysaccharide was established:

α-D-Glc*p*-(1→3)┐
→3)-β-L-Rha*p*-(1→4)-α-L-Rha*p*-(1→3)-α-D-Fuc*p*-(1→

The O-polysaccharide contains a minor proportion of 4-*O*-methylrhamnose, which is suggested to terminate the polymer main chain.

Keywords: Phytopathogenic bacteria; *Erwinia carotovora*; O-polysaccharide structure; Lipopolysaccharide

The lipopolysaccharide of Gram-negative phytopathogenic bacteria is implicated in plant pathogenesis, such as soft rot in vegetables caused by *Erwinia carotovora.* The core structure was established in an R-type lipopolysaccharide of *E. carotovora* FERM P-7576,[1] whereas no data on the O-polysaccharide chain structure are available. We report herein on the structure of the O-polysaccharide of *E. carotovora* ssp. *carotovora* GSPB 436, which was isolated from the lipopolysaccharide by mild acid degradation.

Sugar analysis of the polysaccharide revealed rhamnose, fucose and glucose in the ratios 1.85:1:1.04, as well as a small amount of 4-*O*-methylrhamnose. Determination of the absolute configurations of the monosaccharides by GLC of the acetylated (+)-2-octyl glycosides showed that rhamnose has the L configuration and fucose and glucose have the D configuration.

Methylation analysis of the polysaccharide revealed 2,4-di-*O*-methylrhamnose, 2-*O*-methylrhamnose, 2,4-di-*O*-methylfucose and 2,3,4,6-tetra-*O*-methylglucose in the ratios 1:1.08:1.05:0.98, respectively, together with a minor proportion of 2,3,4-tri-*O*-methylrhamnose. Therefore, the polysaccharide is branched with a terminal glucopyranose residue and a 3,4-disubstituted rhamnose residue at the branching point. Another rhamnose residue and a fucose residue are 3-substituted. Methylation using CD_3I showed that 4-*O*-methylrhamnose is not glycosylated.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained signals for four anomeric carbons at δ 95.7–102.6, three CH_3–C groups (C-6 of Rha and Fuc) at δ 16.1–18.3, one $HOCH_2$–C group (C-6 of Glc) at δ 61.9 and sugar ring carbons linked to oxygen in the region of δ 67.6–79.5. In addition, there were present a minor signal at δ 60.9, which could be assigned to an *O*-methyl group, and a number of minor sugar signals, which, most likely, belonged to 4-*O*-methylrhamnose. The ^{1}H

☆ Data presented at the XIth European Carbohydrate Symposium, September 2–7, 2001 Lisbon, Portugal.

* Corresponding author. Tel.: +7-095-9383613; fax: +7-095-1355328.
E-mail address: knirel@ioc.ac.ru (Y.A. Knirel).

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doi:10.1016/S0008-6215(03)00326-4

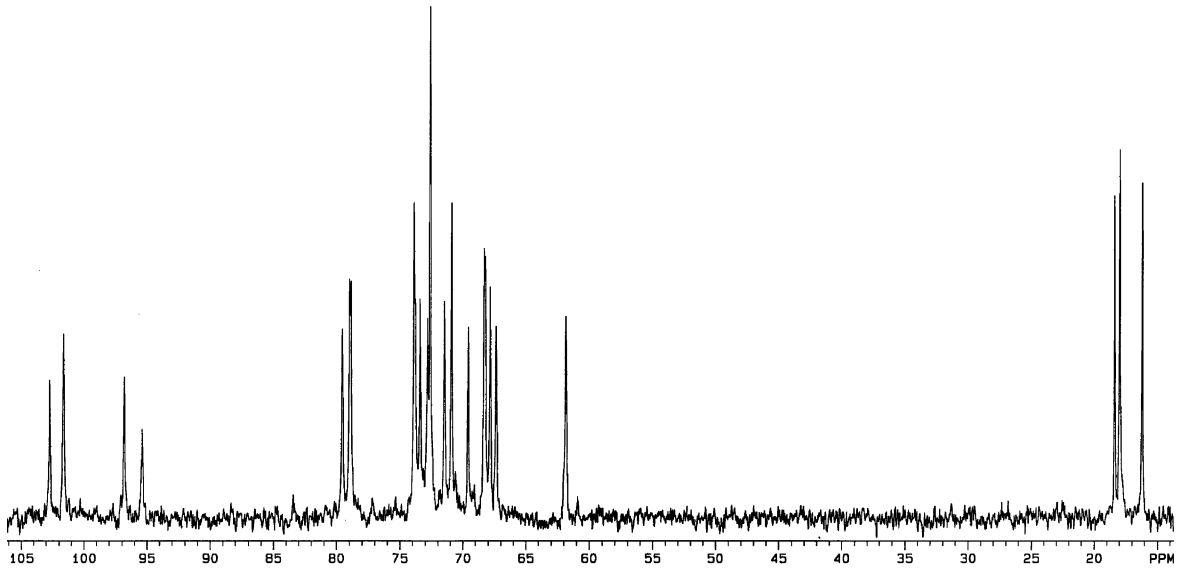

Fig. 1. ^{13}C NMR spectrum of the O-polysaccharide from *E. carotovora* subsp. GSPB 436.

NMR spectrum of the polysaccharide contained signals for four anomeric protons at δ 4.73–5.13, three CH_3–C groups (H-6 of Rha and Fuc) at δ 1.18–1.37 and other sugar protons in the region of δ 3.42–4.32, and a minor signal for an *O*-methyl group at δ 3.55 (data of the $^1H,^1H$ COSY spectrum).

The ^1H and ^{13}C NMR spectra of the polysaccharide were assigned using 2D COSY, ROESY and H-detected $^1H,^{13}C$ HMQC experiments (Table 1). In the COSY spectrum, connectivities could be traced between all protons of the four sugar spin systems, except for H-4 and H-5 of Fuc, which, instead, showed a correlation in the ROESY spectrum. The spin systems were assigned to particular sugars by typical $J_{2,3}$, $J_{3,4}$ and $J_{4,5}$ coupling constant values, which demonstrated also that all sugars are in the pyranose form. Relatively small $J_{1,2}$ coupling constant values of $\sim$3 Hz indicated that Glc and Fuc are α-linked. As judged by the ^{13}C NMR chemical shifts, one of the Rha residues is α-linked and the other β-linked (e.g., compare δ 69.6 and 73.4 for C-5 of α-Rha*p* and β-Rha*p* in the polysaccharide with δ 69.5 and 73.2 in the corresponding free monosaccharides,[2] respectively). The configurations of the glycosidic linkages were confirmed by intraresidue cross-peaks for H-1, H-2 of the α-linked monosaccharides and for H-1,H-3 and H-1,H-5 of β-Rha*p* in the ROESY spectrum.

The ^{13}C NMR chemical shifts for C-2–C-6 of Glc in the polysaccharide were close to the chemical shifts of α-glucopyranose,[2] and demonstrated the terminal position of the Glc residue. Downfield displacements of the signals for C-3 of α-Fuc and β-Rha, C-3 and C-4 of α-Rha in the ^{13}C NMR spectrum of the polysaccharide, as compared with their positions in the spectra of the corresponding non-substituted monosaccharides,[2,3] showed that α-Fuc and β-Rha are 3-substituted and α-Rha is at the branching point and is 3,4-disubstituted. The ROESY spectrum of the polysaccharide showed correlations between the following anomeric protons and protons at the linkage carbons: β-Rha H-1,α-Rha H-4, α-Rha H-1,α-Fuc H-3, α-Fuc H-1,β-Rha H-3, and Glc H-1,α-Rha H-3. These data defined the monosaccharides sequence in the repeating unit.

Table 1
500-MHz ^1H and 125-MHz ^{13}C NMR data of the O-polysaccharide from *E. carotovora* subsp. *carotovora* GSPB 436 (δ, ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6 (H-6a,6b)
→3)-β-L-Rha*p*-(1→	4.73	4.21	3.63	3.48	3.42	1.37
→3,4)-α-L-Rha*p*-(1→	5.08	4.30	4.12	3.78	3.95	1.32
→3)-α-D-Fuc*p*-(1→	5.07	3.94	4.00	3.85	4.32	1.18
α-D-Glc*p*-(1→	5.13	3.57	3.99	3.43	4.15	3.74, 3.81
	C-1	C-2	C-3	C-4	C-5	C-6
→3)-β-L-Rha*p*-(1→	101.5	68.3	79.1	71.6	73.4	18.3
→3,4)-α-L-Rha*p*-(1→	102.6	67.6	74.2	79.5	69.6	18.0
→3)-α-D-Fuc*p*-(1→	96.8	68.3	78.8	72.8	67.9	16.1
α-D-Glc*p*-(1→	95.7	72.6	74.0	71.0	72.7	61.9

Therefore, the O-polysaccharide of *E. carotovora* ssp. *carotovora* GSPB 436 has the structure shown below, which is unique among bacterial polysaccharide structures. Remarkably, *Pseudomonas fluorescens* IMV 472 produces an O-polysaccharide with the same main chain but a different side chain.[4]

α-D-Glc*p*-(1→3)┐
→3)-β-L-Rha*p*-(1→4)-α-L-Rha*p*-(1→3)-α-D-Fuc*p*-(1→

The ROESY spectrum of the O-polysaccharide showed correlation of the signal for the OMe group with three minor sugar signals at δ 3.49 (strong), 3.61 and 3.44 (both weak). These signals are close to those of H-4, H-3 and H-5 of β-Rha in the major series (δ 3.48, 3.63 and 3.42, respectively) and could be assigned thus to the minor 4-*O*-methyl-β-rhamnopyranose residue, which may terminate the main chain of the polysaccharide. Minor O-methylated monosaccharides occur typically in bacterial homo- or heteropolysaccharides with a homopolymer main chain and in a few cases are confirmed to terminate the main chain,[5–7] whereas methylation of the terminal sugar in a heteropolymer main chain, as in the O-polysaccharide of *E. carotovora* ssp. *carotovora* GSPB 436, seems to be less common.

1. Experimental

1.1. Isolation of lipopolysaccharide and polysaccharide

E. carotovora ssp. *carotovora* GSPB 436 was cultivated as described earlier.[8] Bacterial cells were suspended in deionised water at 70 °C, mixed (1:1) with warm aq 90% phenol (70 °C), and stirred for 30 min at 70 °C.[9] The mixture was stored on ice for 12 h and centrifuged for 20 min at 17,000*g*. The aq phase was dialyzed against deionised water for 7 days and lyophilised.

The polysaccharide was prepared by degradation of the lipopolysaccharides with aq 2% HOAc for 1.5 h at 100 °C, followed by GPC on a column (70 × 2.6 cm) of Sephadex G-50 using 0.05 M pyridinium acetate buffer pH 4.5 as eluent and monitoring with a Knauer differential refractometer.

1.2. Sugar and methylation analysis

The polysaccharide (0.5 mg) was hydrolyzed with 2 M CF_3CO_2H (100 °C, 2 h), and the monosaccharides were identified by GLC as the alditol acetates[10] using a Hewlett–Packard 5880 instrument on a DB-5 column with a temperature gradient of 160 (1 min) to 250 °C at 3 °C min^{-1} or GLC–MS on a Carlo Erba Fractovap 4200 chromatograph equipped with an Ultra-1 column and a Finnigan MAT ITD-700 mass spectrometer, using a temperature gradient of 150 (1 min) to 280 °C at 5 °C min^{-1}. The absolute configurations of the monosaccharides were determined by GLC of the acetylated glycosides with (+)-2-octanol[11] under the same chromatographic conditions as above.

Methylation was carried out with CH_3I or CD_3I in DMSO in the presence of methylsulphinylmethanide.[12] Hydrolysis was performed with 2 M CF_3CO_2H (100 °C, 2 h), and the partially methylated monosaccharides were reduced with $NaBH_4$, acetylated, and analysed by GLC–MS as above.

1.3. NMR spectroscopy

A sample of the polysaccharide was deuterium-exchanged by freeze-drying three times from D_2O and then examined in a solution of 99.96% D_2O. NMR spectra were recorded using a Bruker DRX-500 spectrometer at 50 °C. A mixing time of 200 ms was used in a 2D ROESY experiment. Chemical shifts are reported with internal sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) and external acetone (δ_C 31.45).

Acknowledgements

This work was supported by the RFBR (grant 02-04-48271).

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