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Note

Structure of the O-polysaccharide of *Pseudomonas mandelii* CYar1 containing 3,6-dideoxy-4-*C*-[(*S*)-1-hydroxyethyl]-D-*xylo*-hexose (yersiniose A)

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ABSTRACT

The O-polysaccharide isolated by mild acid hydrolysis of the lipopolysaccharide of *Pseudomonas mandelii* CYar1 was studied by sugar analysis and 1D and 2D ^{1}H and ^{13}C NMR spectroscopies. The following structure of the O-polysaccharide was established:

α-Yer*p*
1
↓
4
→3)-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→3)-β-D-Glc*p*NAc-(1→

where Yer indicates 3,6-dideoxy-4-*C*-[(*S*)-1-hydroxyethyl]-D-*xylo*-hexose (yersiniose A).

Lipopolysaccharide is the major component of the outer membrane of Gram-negative bacteria and has been known as a pathogen-associated molecular pattern recognized by receptors of the immune system. The O-specific polysaccharide chain of the lipopolysaccharide (O-antigen) is expressed on the cell surface and defines the serospecificity of bacteria. The O-antigen is the most variable cell constituent, and its structural diversity is believed to be important for adaptation of bacteria for specific niches.

Pseudomonas mandelii is a fluorescent, Gram-negative, rod-shaped psychrotrophic bacterium, which has been placed into the *Pseudomonas fluorescens* group based on 16S rRNA analysis.[1] As opposite to many other pseudomonads, lipopolysaccharide of *P. mandelii* has not been studied yet. In this work, we report on the structure of the O-polysaccharide of *P. mandelii* strain CYar1, which was isolated and re-grown from Siberian repeated cavern-lode ice.

The lipopolysaccharide was isolated from dried bacterial cells by the phenol–water procedure[2] and degraded with dilute acetic acid. The resultant high-molecular mass polysaccharide was isolated by GPC on Sephadex G-50. Its ^{1}H and ^{13}C NMR spectra showed a structural heterogeneity owing to an incomplete substitution (~50%) with, or a partial loss of, a lateral monosaccharide. Hydrolysis under milder conditions at pH 4.5 afforded a polysaccharide, in which incomplete oligosaccharide units were only minor.

Full acid hydrolysis of the polysaccharide followed by GLC of the alditol acetates revealed the presence of rhamnose, glucosamine, and glucose in the ratio ~2:0.6:0.4. GLC analysis of the acetylated (*S*)-2-octyl glycosides showed that Rha has the L configuration whereas Glc and GlcN have the D configuration. Further studies showed that the polysaccharide also includes a branched monosaccharide, 3,6-dideoxy-4-*C*-[(*S*)-1-hydroxyethyl]-D-*xylo*-hexose (yersiniose A, Yer). Glucose was not confirmed to be a constituent of the O-polysaccharide and could originate from the lipopolysaccharide core or/and a contaminating glucan.

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The ^{1}H NMR spectrum of the O-polysaccharide showed, inter alia, signals for four anomeric protons at δ 5.16, 5.07, 5.00, and 4.73, a CH_2 group at δ 1.80, four CH_3 groups at δ 1.33, 1.27, 1.16, and 1.14, and an *N*-acetyl group at δ 2.02. The ^{13}C NMR spectrum (Fig. 1) contained signals for four anomeric carbons at δ 103.8, 103.2, 101.8, and 99.2, a nitrogen-bearing carbon at δ 55.8, an $HOCH_2$–C group at δ 61.9, a C–CH_2–C group at δ 31.8, four CH_3–C groups at δ 18.1, 17.9, 16.5, and 13.9, other sugar carbons in the region δ 65.9–81.6, as well as an *N*-acetyl group at δ 23.7 (CH_3) and δ 176.0 (CO). Therefore, the O-polysaccharide has a tetrasaccharide repeating unit.

The ^{1}H and ^{13}C NMR signals for three sugar spin systems were assigned using 2D ^{1}H,^{1}H COSY, TOCSY, ROESY, ^{1}H,^{13}C HSQC, and HMBC experiments (Table 1). The TOCSY spectrum showed H-1/H-2, H-2/H-3, 4, 5, 6, and H-6/H-5, 4, 3 cross-peaks for each of two rhamnose residues (Rha^{I} and Rha^{II}), and H-1/H-2, 3, 4, 5 cross-peaks for GlcNAc. The signals within each spin system were assigned using the COSY spectrum. GlcN was confirmed by a correlation of H-2 to a nitrogen-bearing carbon (C-2) at δ 3.89/55.8 in the ^{1}H,^{13}C HSQC spectrum.

The forth sugar component (Yer) was represented by three isolated spin systems for H-1–H-3, H-5–H-6, and H-1′–H-2′. There, H-3 corresponded to a CH_2 group (δ_H 1.80, δ_C 31.8), and H-6 and H-2′ to a CH_3 group each (δ_H 1.14, δ_C 13.9; δ_H 1.16, δ_C 16.5). Protons of both CH_3 groups displayed correlations to a tertiary carbon signal (C-4) at δ_C 76.0, and those of the CH_2 group to carbons C-2 and C-5 in the ^{1}H,^{13}C HMBC spectrum (Fig. 2). Although the $J_{2,3ax}$ constant could not be measured exactly owing to a coincidence of the H-3ax and H-3eq signals at δ 1.80, it is evidently large (>9 Hz) as the H-2 signal is wide, and hence the H-2 proton is axial. The axial orientation of H-3 and H-5 followed from a strong NOE observed between these protons in the ROESY spectrum. Therefore, yersiniose has the *xylo* configuration, most likely, D-*xylo* configuration as in all other known Yer-containing bacterial polysaccharides. Analysis of the ^{13}C NMR chemical shifts indicated that the polysaccharide contains the (*S*)-1-hydroxyethyl isomer of yersiniose called yersiniose A (compare the chemical shifts δ 16.5 for C-2′ and 76.0 for C-4 in the O-polysaccharide with published data[3] δ 16.7 and 76.6 in yersiniose A and δ 18.0 and 77.4 in yersiniose B, respectively). To confirm the absolute configuration of Yer, the O-polysaccharide was subjected to Smith degradation to convert yersiniose to a 3,6-dideoxyhexose, which was identified as paratose (D-*ribo* isomer, Par) by GLC of the acetylated (*S*)-2-octyl glycoside.

The β configuration of GlcNAc was inferred from the H-1 chemical shift of δ 4.73 and a relatively large $J_{2,3}$ coupling constant of ~7 Hz. α-Rha^{I} and β-Rha^{II} were demonstrated by the C-5 chemical shifts of δ 70.4 and 73.5 (compare published data[4] δ 69.4 and 73.1 for α- and β-rhamnopyranose, respectively). Similarly, the C-5 chemical shift of δ 68.6 showed that Yer was α-linked (compare published data[3] δ 68.1 and 76.1 for methyl α- and β-yersiniopyranoside A, respectively). In the ROESY spectrum (Fig. 3), a strong H-1/H-5 correlation confirmed the β configuration of GlcNAc, strong H-1/H-3 and H-1/H-5 correlations corraborated the β configuration of Rha^{II}, and the lack of these correlations combined with the presence of strong H-1,H-2 correlations confirmed that Rha^{I} and Yer were α-linked.

Downfield displacements of the signals for C-3 of Rha^{I} and Rha^{II}, C-3 and C-4 of GlcNAc to δ 81.6, 81.6, 79.6, and 79.0, respectively, compared with their positions in the corresponding unsubstituted monosaccharides,[4] revealed the positions of glycosylation in the repeating unit. The monosaccharide sequence was established by the following interresidue correlations in the ROESY spectrum (Fig. 3): Rha^{I} H-1/Rha^{II} H-3, Rha^{II} H-1/GlcNAc H-3, GlcNAc H-1/Rha^{I} H-3, and Yer H-1/GlcNAc H-4. The pattern of sugar substitution and the sequence of the monosaccharides in the main chain were confirmed by the ^{1}H,^{13}C HMBC spectrum, which showed Rha^{I} H-1/Rha^{II} C-3, Rha^{II} H-1/GlcNAc C-3, and GlcNAc H-1/Rha^{I} C-3 correlations.

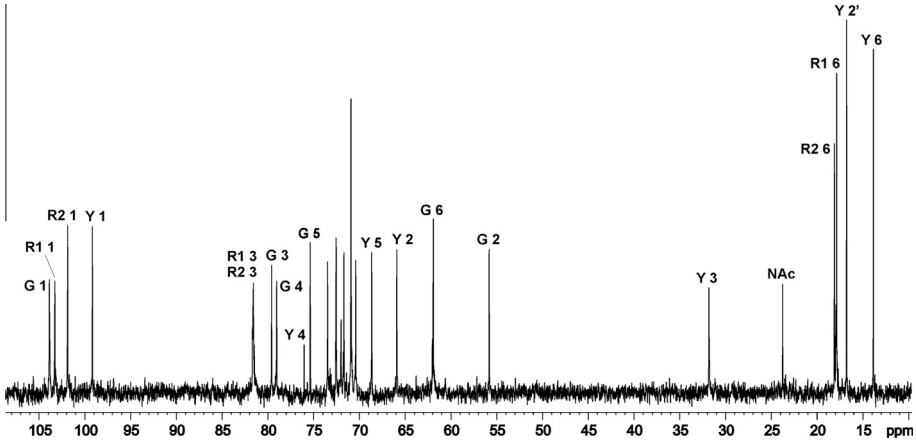

Figure 1. ^{13}C NMR spectrum of the O-polysaccharide from *P. mandelii*. R1, Rha^{I}; R2, Rha^{II}; G, GlcN; and Y, yersiniose A. Signal for the CO group is not shown.

Table 1
^{1}H and ^{13}C NMR chemical shifts (δ, ppm) of the O-polysaccharide from *P. mandelii*

Sugar residue	Nucleus	1	2	3	4	5	6	1′	2′
→3)-α-L-Rha*p*^{I}-(1→	^{1}H	5.00	4.28	3.91	3.51	3.83	1.27		
	^{13}C	103.2	70.9	81.6	72.0	70.4	18.1		
→3)-β-L-Rha*p*^{II}-(1→	^{1}H	5.07	4.12	3.55	4.34	3.39	1.33		
	^{13}C	101.8	71.7	81.6	72.5	73.5	17.9		
→3,4)-β-D-Glc*p*NAc-(1→	^{1}H	4.73	3.89	3.83	3.92	3.53	3.91		2.02
	^{13}C	103.8	55.8	79.6	79.0	75.3	61.9	176.0	23.7
α-Yer*p*-(1→	^{1}H	5.16	3.98	1.80		4.21	1.14	3.66	1.16
	^{13}C	99.2	65.9	31.8	76.0	68.6	13.9	70.9	16.5

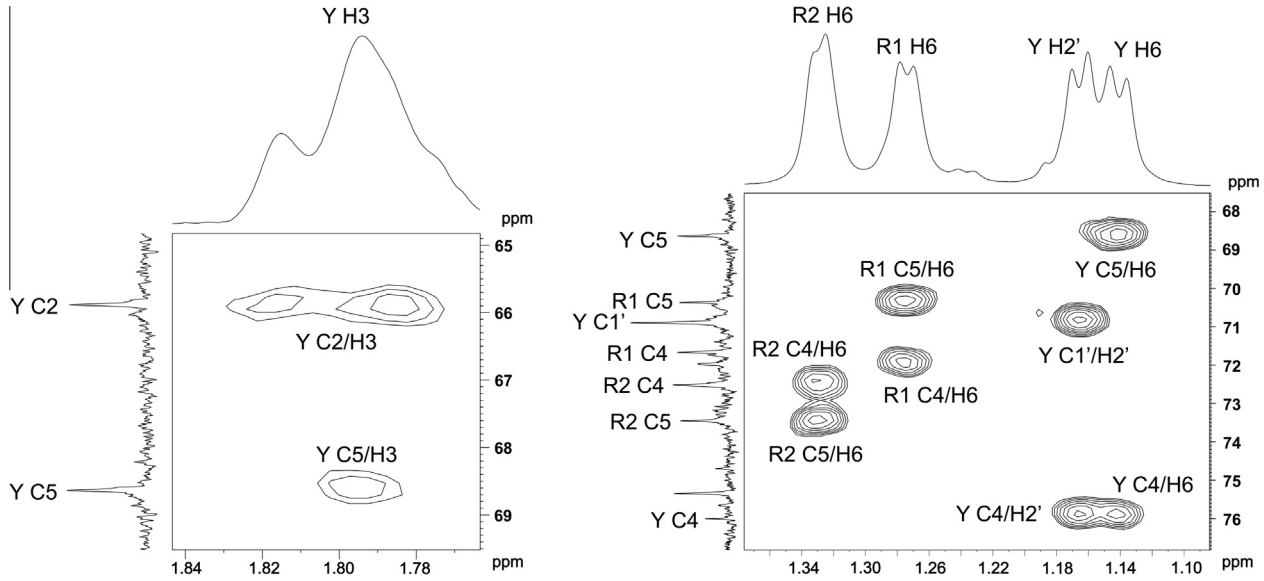

Figure 2. Parts of a $^{1}H,^{13}C$ HMBC spectrum of the O-polysaccharide from *P. mandelii*. R1, Rha^{I}; R2, Rha^{II}; Y, and yersiniose A.

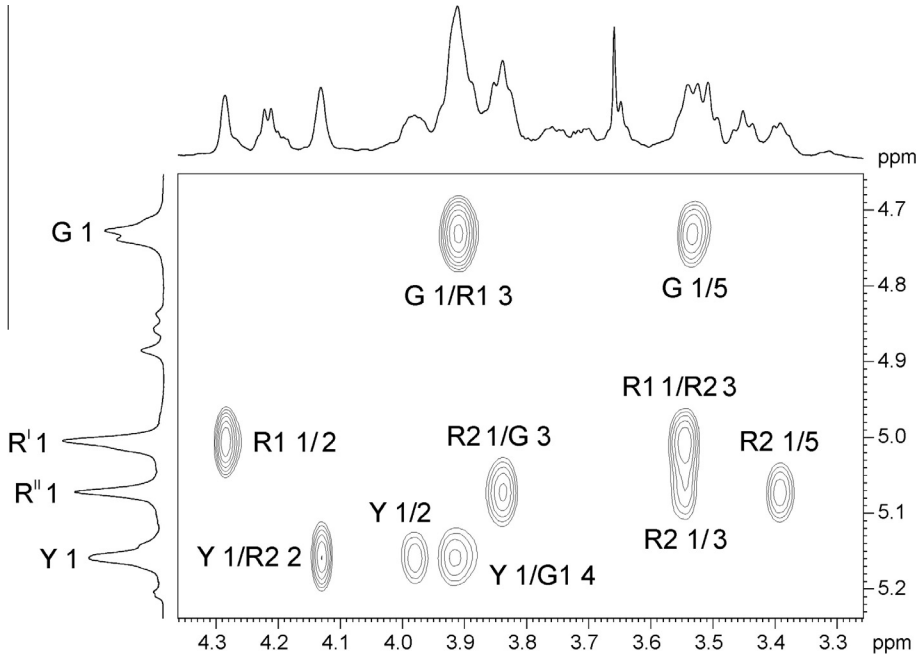

Figure 3. Part of a 2D ROESY spectrum of the O-polysaccharide from *P. mandelii*. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. R1, Rha^{I}; R2, Rha^{II}; G, GlcN; and Y, yersiniose A.

Therefore, the O-polysaccharide of *P. mandelii* has the following structure:

α-Yer*p*
1
↓
4
→3)-α-L-Rha*p*^{I}-(1→3)-β-L-Rha*p*^{II}-(1→3)-β-D-Glc*p*NAc-(1→

where Yer indicates 3,6-dideoxy-4-*C*-[(*S*)-1-hydroxyethyl]-D-*xylo*-hexose (yersiniose A). This sugar or its (*R*)-1-hydroxyethyl isomer (yersiniose B) has been reported as a component of lipopolysaccharides of *Yersinia*[5] and *Legionella*[6] species, *Burkholderia brasiliensis*,[7] *Budvicia aquatica*,[8] and *Vibrio fischeri*,[9] but, to our knowledge, none of them has been hitherto reported in lipopolysaccharides of pseudomonads.

1. Experimental

1.1. Bacterial strain and cultivation of bacteria

Samples of repeated cavern-lode ice were collected in the Mamontova Gora section located on the left bank area of the Aldan River (Central Yakutia, Siberia) in July 2009. The samples were transported from the sampling site in the frozen state and stored at −18 °C. The ice for isolation of microorganisms was recovered from the central core of the surface sterilized samples and exposed

to room temperature. *P. mandelii* strain CYar1 was isolated in December 2009.

Meltwater was used for the inoculation of solid culture medium (g/L): peptone 0.5, yeast extract 0.25, glucose 0.1, agar–agar 15, pH 7.0. Inoculated Petri dishes were incubated aerobically at 24 °C for 2 weeks. The biomass of *P. mandelii* CYar1 was obtained on liquid culture medium (g/L): peptone 5, yeast extract 3, pH 6.8–7.2, after constant shaking (150 rpm) at 28 °C for 16 h.

Preliminary identification of bacterial isolates was based on the 16S rRNA gene sequence data. The level of 16S rRNA sequence similarity was 99.0% between strain CYar1 and type strain of *P. mandelii*.

1.2. Isolation of the lipopolysaccharide and O-polysaccharide

Lipopolysaccharide was isolated from dried bacterial mass (3 g) by the phenol–water procedure[2] followed by dialysis of the extract without layer separation. After removal of insoluble contamination by centrifugation, the solution was freed from proteins and nucleic acids by treatment with cold (4 °C) aq 50% CCl_3CO_2H, the precipitate was removed by centrifugation, and the supernatant was dialyzed against distilled water and freeze-dried to yield a lipopolysaccharide preparation (84 mg).

A lipopolysaccharide sample (33 mg) was heated with 0.1 M NaOAc buffer pH 4.5 at 100 °C until precipitation of a lipid sediment, which was removed by centrifugation. The carbohydrate-containing supernatant was fractionated by GPC on a column (60 × 2.6 cm) of Sephadex G-50 Superfine (Amersham Biosciences, Sweden) in pyridinium acetate buffer (4 mL pyridine and 10 mL conc HOAc in 1 L water) and monitored using a differential refractometer (Knauer, Germany) to give a high-molecular-mass polysaccharide (4 mg).

1.3. Sugar analyses

For monosaccharide analysis, a polysaccharide sample (1 mg) was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h), dried under a stream of nitrogen, and reduced with $NaBH_4$ (10 mg) in water. After adding conc HOAc, evaporation and co-evaporation with 10% HOAc in MeOH (2 × 1 mL), the sample was acetylated with Ac_2O (0.5 mL, 100 °C, 20 min) and analyzed by GLC on a Agilent Technologies 7820A instrument with a HP-5 ms capillary column using a temperature gradient of 160 °C (3 min) to 290 °C at 7 °C min^{-1}.

To convert Yer to Par, a polysaccharide sample (3 mg) was oxidized with 0.1 M $NaIO_4$ (1 mL) in the dark for 72 h at 20 °C; after adding ethylene glycol (0.03 mL), the resultant modified polysaccharide was reduced with $NaBH_4$ (35 mg) and desalted on TSK HW-40 as above.

The absolute configurations of Par, Rha, and GlcN were determined by GLC of the acetylated (*S*)-2-octyl glycosides[10] under the same chromatographic conditions as in sugar analysis.

1.4. NMR spectroscopy

A polysaccharide sample was deuterium-exchanged by freeze-drying twice from 99.9% D_2O and then examined as a solution in 99.95% D_2O at 30 °C on an Avance II 600 NMR spectrometer (Bruker, Germany) using internal sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d_4 (δ_H 0.00) and acetone (δ_C 31.45) as references for calibration. 2D NMR spectra were obtained using standard Bruker software, and Bruker TopSpin 2.1 program was used to acquire and process the NMR data. Mixing times of 200 and 100 ms were used in TOCSY and ROESY experiments, respectively. Other NMR parameters were set essentially as described.[11]

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