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Note

Reinvestigation of the O-antigens of *Yersinia pseudotuberculosis*: revision of the O2c and confirmation of the O3 antigen structures

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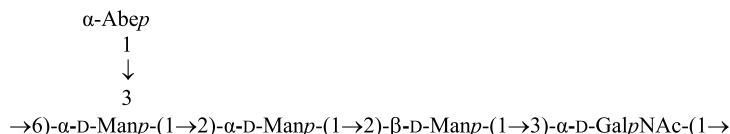
O-Antigen

Bacterial polysaccharide structure

Abequose

ABSTRACT

Structures of the O-antigens of *Yersinia pseudotuberculosis* O2c and O3 were reinvestigated by NMR spectroscopy, including 2D ¹H, ¹H COSY, TOCSY, ROESY, ¹H, ¹³C HSQC, and HMBC experiments. The following revised structure of the O2c tetrasaccharide repeating unit was established, which differs from the structure proposed earlier in the glycosylation pattern of the mannose residue at the branching point:



where Abe stands for 3,6-dideoxy-D-xylo-hexose. The structure of the *Y. pseudotuberculosis* O3 antigen reported earlier was confirmed.

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Yersinia pseudotuberculosis is an enteric bacterium that causes a broad range of acute and chronic gastrointestinal disorders.¹ The species is antigenically heterogeneous and, based on the O-antigen, strains of *Y. pseudotuberculosis* are classified into 15 serovars, some of which are divided to subgroups.² Structures of the O-antigen polysaccharides of a number of serovars have been either established by older methods^{3,4} or proposed tentatively based on incomplete chemical data⁵ and genetic data.^{6,7} Recently, we have confirmed the O-antigen structure of *Y. pseudotuberculosis* O2a.⁸ In this work, we reinvestigated the O-antigens of *Y. pseudotuberculosis* O2c⁹ and O3,^{10,11} whose structures have been established earlier. As a result, the structure of the former was revised and that of the latter confirmed.

The lipopolysaccharides were isolated from bacterial cells of *Y. pseudotuberculosis* O2c and O3 by the phenol–water procedure¹² and degraded under mild acidic conditions (NaOAc buffer pH 4.5, 100 °C) to give polysaccharides isolated by GPC on Sephadex G-50.

According to the published data,⁹ the O2c polysaccharide contains Man, GalNAc, and 3,6-dideoxy-D-xylo-hexose (abequose,

Abe). The ¹H and ¹³C NMR spectra of the polysaccharide showed signals for anomeric atoms of five monosaccharides at δ_H 4.82–5.34; δ_C 98.6–103.9, methyl groups of GalNAc and Abe at δ_H 2.04 and 1.18; δ_C 23.5 and 16.9, respectively, a methylene group of Abe (H-3 and C-3) at δ_H 2.00, 2.07; δ_C 34.4, four hydroxymethylene groups (C-6 of Man and GalNAc) at δ_C 61.8–66.2, a nitrogen-bearing carbon (C-2 of GalNAc) at δ 50.5, and other signals at δ_H 3.40–4.32 and δ_C 65.1–80.6. Therefore, the polysaccharide has a pentasaccharide repeating unit.

The ¹H and ¹³C NMR spectra were fully assigned using 2D COSY, TOCSY, ROESY, ¹H, ¹³C HSQC, and HMBC experiments, and spin systems for all sugar residues were identified (Table 1). The spin system of abequose was identified by the correlations with the high-field signals for H-6 (δ_H 1.18) and H-3 (δ_H 2.00, 2.07) in the COSY and TOCSY spectra. The spin system of GalNAc was distinguished by a 4.32/50.5 correlation of the H-2 proton to the nitrogen-bearing carbon in the HSQC experiment. The three mannose residues (Man^I–Man^{III}) were characterized by low $J_{2,3}$ coupling constant values typical of this sugar. The ¹H and ¹³C chemical shifts were consistent with the α configuration of abequose, GalNAc and two mannose residues (Man^I and Man^{II}), whereas the third mannose residue (Man^{III}) was found to be β -linked. The chemical

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Table 1¹H and ¹³C NMR chemical shifts (δ, ppm) for *Y. pseudotuberculosis* O2c and O3 polysaccharides related to internal acetone (δ_H 2.225, δ_C 31.45)

Residue	Nucleus	1	2	3 (3a,3b)	4	5	6 (6a,6b)
O2c polysaccharide (1)							
α-A bep-(1→	¹ H	5.10	4.02	2.00, 2.07	3.88	4.16	1.18
	¹³ C	101.9	65.1	34.4	69.8	68.3	16.9
→3,6)-α-D-Manp ^I -(1→	¹ H	5.03	4.22	3.94	4.14	3.86	3.55, 4.12
	¹³ C	103.9	71.4	80.6	66.4	73.2	66.2
→2)-α-D-Manp ^{II} -(1→	¹ H	5.34	4.08	4.05	3.84	4.05	3.80, 3.82
	¹³ C	100.7	80.0	71.5	68.4	73.8	61.8
→2)-β-D-Manp ^{III} -(1→	¹ H	4.82	3.99	3.74	3.62	3.40	3.77, 3.91
	¹³ C	102.8	77.0	75.3	68.3	78.2	62.5
→3)-α-D-GalpNAc-(1→	¹ H	4.90	4.32	4.06	4.17	4.00	3.71
	¹³ C	98.6	50.5	78.5	70.0	72.4	62.4
O3 polysaccharide (2)							
β-Parp-(1→	¹ H	4.46	3.58	1.50, 2.34	3.40	3.39	1.32
	¹³ C	106.8	70.1	39.7	77.4	71.2	18.9
→2)-α-D-Manp-(1→	¹ H	5.49	4.05	4.05	3.81	3.81	3.79, 3.91
	¹³ C	100.9	80.3	71.5	68.5	74.6	62.3
→3,4)-α-L-Fucp-(1→	¹ H	5.09	3.94	4.08	4.11	4.18	1.31
	¹³ C	102.3	70.3	74.5	80.7	69.7	16.8
→3)-α-D-GalpNAc-(1→	¹ H	5.12	4.34	4.02	4.04	4.07	3.74
	¹³ C	101.3	50.9	76.8	70.3	72.7	62.7

Chemical shifts for NAc are δ_H 2.04 and δ_C 23.5, 175.5.

shifts for abequeose were close to those of the non-substituted monosaccharide¹³ and indicated the terminal position of this sugar.

Downfield displacements of the signals for C-3 of GalNAc to δ 78.5, C-2 of Man^{II} to δ 80.0, C-2 of Man^{III} to δ 77.0, and C-3 and C-6 of Man^I to δ 80.6 and 66.2, respectively, revealed the glycosylation pattern in the O-unit with the disubstituted Man^I at the branching point and Abe as the terminal residue of the side chain.

The monosaccharide sequence in the O-unit was established by the following inter-residue correlations between anomeric protons and protons at the linkage carbons in the ROESY spectrum (Fig. 1): Abe H-1, Man^I H-3 at δ 5.10/3.94, GalNAc H-1, Man^I H-6a at δ 4.90/3.55, Man^I H-1, Man^{II} H-2 at δ 5.03/4.08, Man^{II} H-1, Man^{III} H-2 at δ 5.34/3.99, and Man^{III} H-1, GalNAc H-3 at δ 4.82/4.06. In addition, a Man^{II} H-1, Man^I H-5 correlation supported the α-Man^I-(1→2)-Man^{II} fragment. The sugar sequence was further confirmed by the ¹H, ¹³C

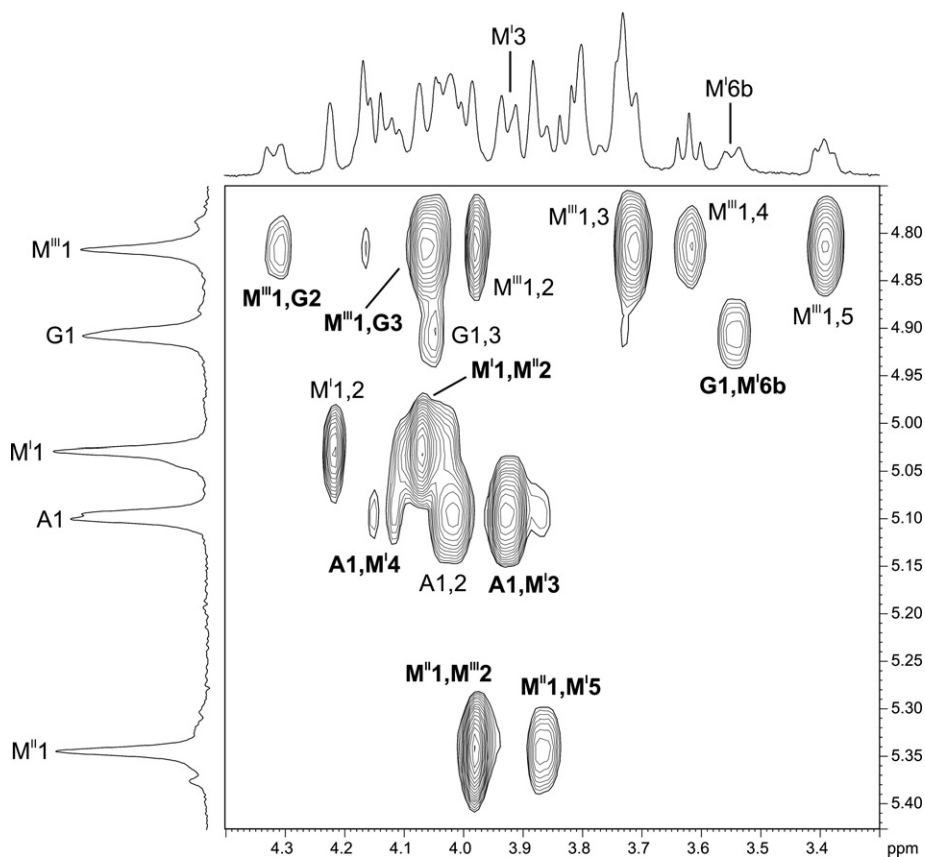


Figure 1. Part of a 2D ROESY spectrum of the O-polysaccharide from *Y. pseudotuberculosis* O2c. Correlations of the Abe H-1 and GalNAc H-1 signals with the isolated signals of Man^I H-3 and Man^I H-6b, respectively, confirmed unambiguously the arrangement of the monosaccharide residues at the branching point. M^I, M^{II}, and M^{III} stand for Man^I, Man^{II}, and Man^{III}, respectively; G stands for GalNAc and A for abequeose. Inter-residue correlations are shown in boldface.

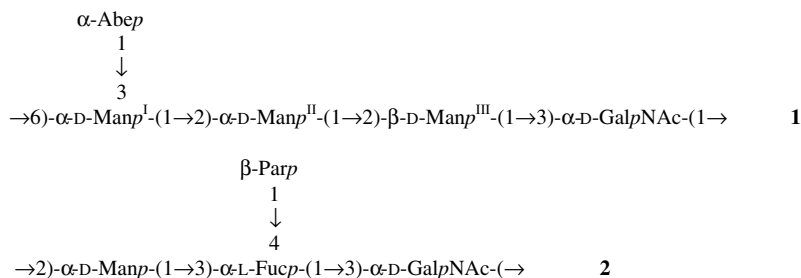


Chart 1. Structures of the O-polysaccharides of *Y. pseudotuberculosis* O2c (**1**) and O3 (**2**). Abe stands for abequeose (3,6-dideoxy-D-xylo-hexose) and Par for paratose (3,6-dideoxy-D-ribo-hexose).

HMBC spectrum, which showed cross-peaks between anomeric carbons and protons at the linkage carbons, as well as between anomeric protons and the linkage carbons, of the neighboring sugar residue. Particularly, it revealed Abe C-1,Man^I H-3 and Abe H-1,Man^I C-3 correlations, which demonstrated unambiguously the (1→3)-linkage between these two monosaccharides.

Therefore, the O-antigen of *Y. pseudotuberculosis* O2c has the structure **1** shown in Chart 1. It differs from the earlier proposed structure⁹ in the sites of attachment of Abe and GalNAc to the Man residue at the branching point.

A similar study of the O-polysaccharide from *Y. pseudotuberculosis* O3, including the full unambiguous assignment of the ¹H and ¹³C NMR chemical shifts (Table 1), confirmed the structure **2** established earlier,^{10,11} which is shown in Chart 1.

1. Experimental

1.1. Bacterial strains, isolation, and degradation of lipopolysaccharides

Wild-type strains of *Y. pseudotuberculosis* O2c and O3 were kindly provided by Professor M. Skurnik (Helsinki, Finland). Cultivation of bacteria was performed at 22 °C as described.¹⁴ The lipopolysaccharides were isolated by the Westphal procedure.¹² A portion of the O2c lipopolysaccharide (20 mg) was heated at 100 °C for 2 h in 0.1 M NaOAc buffer pH 4.5, the precipitate separated by centrifugation (13,000g, 20 min), and the supernatant fractionated on a column (56 × 2.6 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer pH 4.5 with monitoring using a differential refractometer (Knauer, Germany) to give poly- and oligosaccharide fractions. A sample of the O3 lipopolysaccharide (50 mg) was degraded under the same conditions. The yield of the polysaccharides from the O2c and O3 lipopolysaccharides was 15% and 3%, respectively.

1.2. NMR spectroscopy

The samples were deuterium-exchanged by freeze-drying twice from 99.90% D₂O and then examined as solutions in 99.96% D₂O at

40 °C on a Bruker DRX-500 NMR spectrometer (Germany) using internal acetone (δ_H 2.225, δ_C 31.45) as reference. 2D NMR spectra were obtained using standard Bruker software, and Bruker xwinnmr 2.6 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 earlier.¹⁵

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