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Note

Structure of the O-antigen of *Yersinia pseudotuberculosis* O:4a revised

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ABSTRACT

The O-specific polysaccharide was isolated by mild acid degradation of the lipopolysaccharide of *Yersinia pseudotuberculosis* O:4a and studied by NMR spectroscopy, including 2D ROESY and ^{1}H, ^{13}C HMBC experiments. The following structure of the pentasaccharide repeating unit of the polysaccharide was established, which differs from the structure reported earlier [Gorshkova, R. P. et al., *Bioorg. Khim.* **1983**, *9*, 1401–1407] in the linkage modes between the monosaccharides:

α-Tyvp
1
↓
3
→6)-α-D-Manp-(1→2)-α-D-Manp-(1→2)-β-D-Manp-(1→3)-α-D-GlcpNAc-(1 →

Yersinia pseudotuberculosis O:4a O-polysaccharide repeating unit

where Tyv stands for 3,6-dideoxy-D-*arabino*-hexose (tyvelose). The structure of the *Y. pseudotuberculosis* O:4a antigen resembles that of *Y. pseudotuberculosis* O:2c, which differs in the presence of abequose (3,6-dideoxy-D-*xylo*-hexose) in place of tyvelose only.

Strains of *Yersinia pseudotuberculosis*, a zoonotic pathogen causing acute and chronic gastrointestinal disorders in humans, are classified into 15 serovars, some of which are divided into subgroups.[1] The immunospecificity of these bacteria is determined by O-antigens, and the chemical structures of the O-antigens (O-specific polysaccharides) of a number of *Y. pseudotuberculosis* serovars have been established.[2,3] In early works, old methods were used for structural analysis of carbohydrates, which were not always reliable, and it seemed that complex polysaccharides studied >25 years ago required reinvestigation to confirm or to revise their structures. Recently, using high-resolution 2D NMR spectroscopy, we have determined the O-antigen structures of *Y. pseudotuberculosis* O:2a,[4] O:2b,[5] and O:4b[6] for the first time, and reinvestigated those of *Y. pseudotuberculosis* O:2c and O:3,[7] the O:2c structure being revised and the O:3 structure being confirmed. In this work, we reinvestigated the O-specific polysaccharide of *Y. pseudotuberculosis O:4a,* and revised the structure proposed earlier.[8]

Lipopolysaccharide was isolated from dried bacterial cells by the phenol–water procedure,[9] and degraded under mild acidic conditions (sodium acetate buffer, pH 4.5, 100 °C) to give a polysaccharide isolated by GPC on Sephadex G-50.

The ^{1}H and ^{13}C NMR (Fig. 1) spectra of the polysaccharide showed signals for anomeric atoms of five monosaccharides at δ_H 4.82–5.34 (H-1) and δ_C 98.6–103.8 (C-1), a methyl group of a 3,6-dideoxyhexose at δ_H 1.28 (H-6) and δ_C 18.1 (C-6), a methylene group of a 3,6-dideoxyhexose at δ_H 1.90, 2.07 (H-3); δ_C 34.8 (C-3), hydroxymethylene groups of Man and GalNAc at δ_C 62.0–66.3 (all C-6), and a nitrogen-bearing carbon of GalNAc at δ 50.4 (C-2). The signals for other sugar atoms were located in the regions of δ_H 3.40–4.33 and δ_C 66.6–79.7, and those for an N-acetyl group at δ_H 2.05; δ_C 23.5 (Me) and 175.3 (CO). These data are in agreement with published data,[8] which showed that the *Y. pseudotuberculosis* O:4a polysaccharide has a pentasaccharide repeating unit containing three residues of D-Man and one residue each of tyvelose (3,6-dideoxy-D-arabino-hexose, Tyv) and D-GalNAc.

The NMR spectra of the polysaccharide were fully assigned using 2D COSY, TOCSY and ^{1}H, ^{13}C HSQC experiments (Table 1). The spin systems for three mannose residues, designated as Man^{I}–Man^{III} according to their sequence in the repeating unit (see below), were identified by tracing connectivities from signals for H-1 and H-2 in the TOCSY spectrum. The spin system of Tyv was distinguished by correlations between H-1, methyl and meth-

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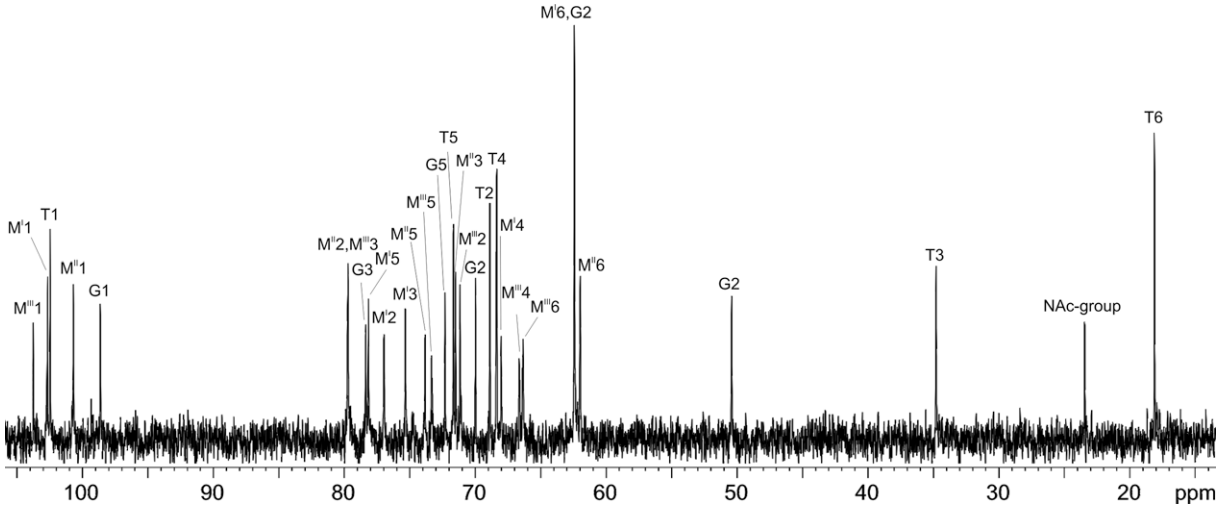

Figure 1. 125 MHz ^{13}C NMR spectrum of the O-specific polysaccharide of *Y. pseudotuberculosis* O:4a. The CO signal of NAc is not shown. M^{I}, M^{II}, and M^{III} stand for Man^{I}, Man^{II}, and Man^{III}; G and T for GalNAc and Tyv, respectively.

Table 1
^{1}H and ^{13}C NMR chemical shifts (δ, ppm) of the O-specific polysaccharide of *Y. pseudotuberculosis* O:4a

Residue	Nucleus	**1**	2	**3** (**3a**, **b**)	**4**	**5**	**6** (**6a**, **b**)
α-Tyv*p*-(1→	^{1}H	4.90	4.06	1.90, 2.07	3.64	3.84	1.28
	^{13}C	102.4	68.9	34.8	68.3	71.6	18.1
→3)-α-D-Gal*p*NAc-(1→	^{1}H	4.93	4.33	4.05	4.19	3.99	3.76, 3.76
	^{13}C	98.6	50.4	78.3	70.0	72.3	62.4
→2)-β-D-Man*p*^{I}-(1→	^{1}H	4.82	4.00	3.72	3.64	3.40	3.74, 3.93
	^{13}C	102.7	76.9	75.3	68.3	78.1	62.4
→2)-α-D-Man*p*^{II}-(1→	^{1}H	5.34	4.08	4.04	3.81	4.01	3.81, 3.81
	^{13}C	100.7	79.7	71.5	68.0	73.8	62.0
→3,6)-α-D-Man*p*^{III}-(1→	^{1}H	5.04	4.21	3.93	4.06	3.85	3.58, 4.12
	^{13}C	103.8	71.1	79.7	66.6	73.3	66.3

Chemical shifts for NAc are δ_H 2.05; δ_C 23.5 (Me) and 175.3 (CO).

ylene groups, and the identity of the 3,6-dideoxyhexose was confirmed by characteristic $^{3}J_{H,H}$ coupling constants. GalNAc was confirmed by a correlation between proton H-2 and a nitrogen-bearing carbon C-2 at δ 3.85/55.8.

A relatively small $J_{1,2}$ value of ~3 Hz indicated that GalNAc is α-linked. The α-linkage of Tyv, Man^{II} and Man^{III} and the β-linkage of Man^{I} were inferred by comparison of their ^{1}H and ^{13}C NMR chemical shifts with those of the corresponding free monosaccharides and glycosides.[10,11] The anomeric configurations were confirmed by a 2D ROESY experiment (Fig. 2), which showed a H-1,H-2 correlation as the only intraresidue correlation for the α-linked Tyv, Man^{II}, Man^{III}, and GalNAc, whereas strong H-1,H-3 and H-1,H-5 correlations were observed for the β-linked Man^{I}.

Relatively low-field positions of the signals for C-2 of Man^{I}, C-2 of Man^{II}, C-3 of Man^{III}, C-3 of GalNAc at δ *76.9–79.7,* and C-6 of Man^{III} at δ 66.3, as compared with their positions in the spectra of the corresponding unsubstituted monosaccharides,[10] revealed the glycosylation pattern in the repeating unit with Man^{III} at the branching point and Tyv in the lateral position. The terminal position of Tyv in the side chain was confirmed by a similarity of its ^{13}C NMR chemical shifts to those of terminal α-Tyv residue in synthetic oligosaccharides[11] and the O-specific polysaccharide of O:4b.[6]

The monosaccharide sequence in the repeating unit was analyzed by the ^{1}H, ^{13}C HMBC technique (Fig. 3). The following correlations between the anomeric protons and the linkage carbons were revealed: Tyv H-1, Man^{III} H-3; Man^{III} H-1, Man^{II} H-2; Man^{II} H-1, Man^{I} H-2 and Man^{I} H-1, GalNAc H-3 at δ_H/δ_C 4.90/79.7; 5.04/79.7; 5.34/76.9; 4.82/78.3, respectively. There were no cross-peaks for GalNAc H-1 in the HMBC spectrum, but the ROESY spectrum showed a clear correlation between GalNAc H-1 and Man^{III} H-6a at δ 4.93/3.58, which is in agreement with the Man^{III} C-6 chemical shift (see above). The ROESY spectrum (Fig. 2) also confirmed the sequence of the other constituent monosaccharides.

Therefore, the O-specific polysaccharide of *Y. pseudotuberculosis* O:4a has the structure shown in Structure 1. It differs from the structure proposed earlier[8] in the linkage modes between the monosaccharides. The only basis for combining serovars O:4a and O:4b in one serogroup is evidently the occurrence of a terminal α-Tyv residue since the remainders of their O-antigen repeating units are different.[6] On the other hand, the *Y. pseudotuberculosis* O:4a antigen shares the backbone structure with the *Y. pseudotuberculosis* O:2c antigen, and the only difference between the two and, accordingly, the basis for their classification into different O-serogroups is the occurrence of either tyvelose or abequose (3,6-dideoxy-D-*xylo*-hexose) in the lateral position, respectively.

1. Experimental

1.1. Bacterial strain, isolation, and degradation of the lipopolysaccharide

Wild-type strain of *Y. pseudotuberculosis* O:4a was kindly provided by Prof. M. Skurnik (Helsinki, Finland). Cultivation of bacteria was performed at 22 °C as described.[12] The lipopolysaccharide was isolated by the Westphal procedure.[9] A lipopolysaccharide sample (80 mg) was heated at 100 °C for 2 h in 0.1 M NaOAc buffer pH 4.5,

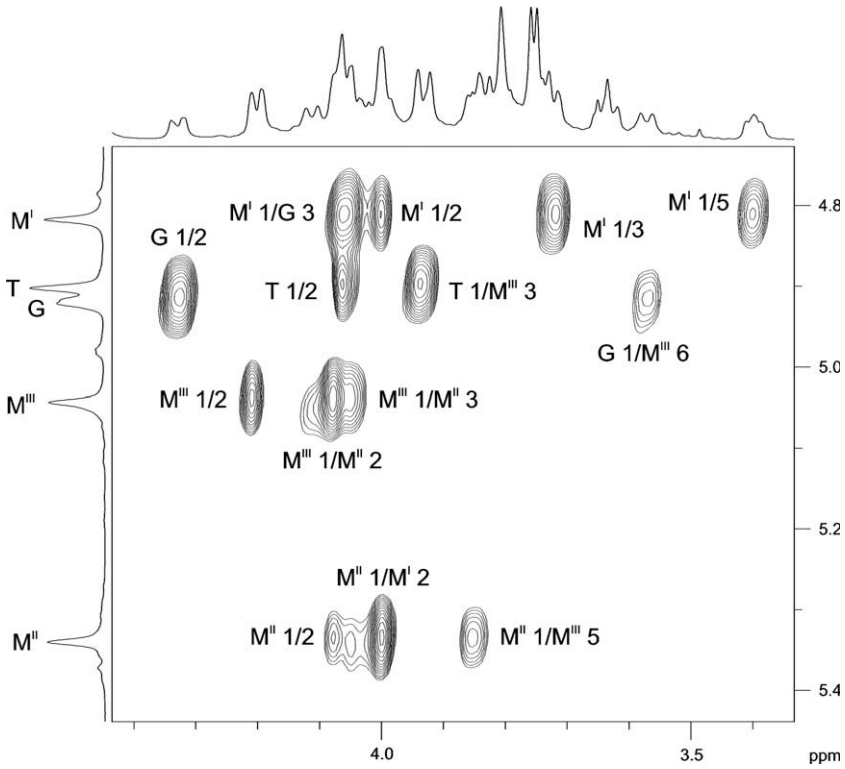

Figure 2. Part of a ROESY spectrum of the O-specific polysaccharide of *Y. pseudotuberculosis* O:4a. The corresponding parts of the ^{1}H NMR spectrum are displayed along the axes. For sugar abbreviations, see legend to Figure 1.

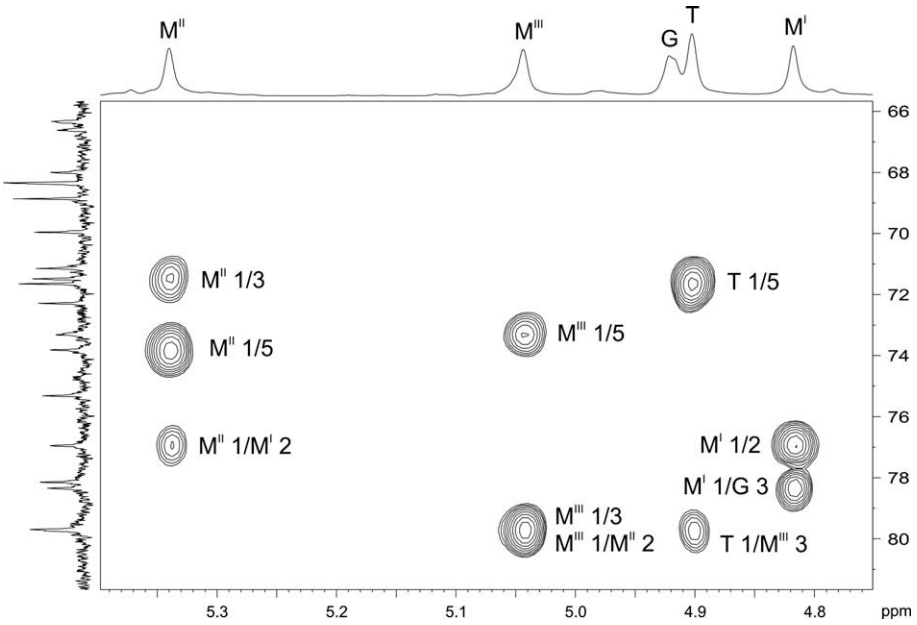

Figure 3. Part of a ^{1}H, ^{13}C HMBC spectrum of the O-specific polysaccharide of *Y. pseudotuberculosis* O:4a. The corresponding parts of the ^{1}H and ^{13}C NMR spectra are displayed along the horizontal and vertical axes, respectively. For sugar abbreviations, see legend to Figure 1.

α-Tyv*p*
1
↓
3
→6)-α-D-Man*p*^{III}-(1→2)-α-D-Man*p*^{II}-(1→2)-β-D-Man*p*^{I}-(1→3)-α-D-Gal*p*NAc-(1→

Structure 1. Revised structure of the O-specific polysaccharide of *Y. pseudotuberculosis* O:4a.

the precipitate separated by centrifugation (13,000*g*, 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). The yield of the O-specific polysaccharide was 9.5%.

1.2. NMR spectroscopy

An O-polysaccharide sample was deuterium exchanged by freeze drying twice from 99.9% D_2O, and then examined as a solution in 99.96% D_2O at 50 °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.[13]

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