Structure of the O-polysaccharide of *Yersinia frederiksenii* H56-36/81 (serotype O:60) containing 4-deoxy-D-*arabino*-hexose

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Aiming at creation of the chemical basis for serotyping of strains of *Yersinia* spp., we studied the O-specific polysaccharide (OPS) of *Yersinia frederiksenii* H56-36/81 belonging to serotype O:60. A branched polysaccharide was obtained by mild alkaline degradation of the lipopolysaccharide (LPS) isolated from bacterial cells by the phenol—water extraction and found to contain D-ribose, L-rhamnose, and 4-deoxy-D-*arabino*-hexose (D-ara4dHex). The last monosaccharide occurs rarely in nature and earlier has been found only in OPS of *Citrobacter* spp. Mild acid hydrolysis of the LPS resulted in cleavage of the D-ara4dHex side-chains to give a linear polysaccharide. Structure of the hexasaccharide repeating unit of the OPS was established by studies of both isolated polysaccharides by 1D and 2D ^{1}H and ^{13}C NMR spectroscopy.

Key words: *Yersinia frederiksenii*, *Citrobacter,* O-specific polysaccharide, O-antigen, lipopolysaccharide, 4-deoxy-D-*arabino*-hexose.

Currently, the genus *Yersinia* comprises 18 species, three of which (*Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*) are pathogenic for humans. They are studied intensively, whereas the remaining species, which are considered as opportunistic pathogens, have been investigated significantly poorer.[1,2] One of these species, *Yersinia frederiksenii*, was separated from *Y. enterocolitica* in 1980.[3] Its representatives are primarily isolated from fresh water, sewage, soil, fish, foods (milk, sandwiches, fruits, and vegetables), wild rodents, domestic animals (cattle, pigs, *etc*.), as well as healthy and sick humans.[1]

The O-specific polysaccharide (OPS) called O-antigen is a part of the lipopolysaccharide (LPS) on the cell surface of Gram-negative bacteria. It consists of oligosaccharide repeats (O-units) usually containing two to eight monosaccharide residues. The O-antigen is one of the most variable cell surface constituents providing the basis for typing of bacterial strains. Based on the O-antigens, strains of a zoonotic pathogen *Yersinia pseudotuberculosis* have been classified into 15 serotypes,[4] and the OPS structures have been elucidated for most of them (see Bacterial Carbohydrate Structure Database at http://csdb.glycoscience.ru/bacterial/). Other *Yersinia* species have been investigated scarcely in respect to the OPS structure.

In this work, we studied the OPS of *Y. frederiksenii* H56-36/81 belonging to serotype O:60 and found it to contain a 4-deoxy-D-*arabino*-hexose residue that occurs rarely in nature. The structure of the branched hexasaccharide repeating unit of the OPS was established. Based on the data obtained, the structure of the OPS of *Citrobacter braakii* PCM 1531 (serogroup O6) that had been determined earlier[5] was revised.

Experimental

Y. frederiksenii H56-36/81 strain (serotype O:60) was from the collection of Max von Pettenkofer Institute for Hygiene and Clinical Medicine, Ludwig Maximilian University (Münich, Germany). Bacteria were grown using a New Brunswick Scientific fermenter at 28 °C and pH 7.1 for 7 h in constantly aerated liquid medium (2 L) composed of tryptone (25 g L^{-1}), yeast extract (10 g L^{-1}), K_2HPO_4 (3.3 g L^{-1}), KH_2PO_4 (2.7 g L^{-1}), $MgSO_4$ (0.5 g L^{-1}), $Na_2S_2O_3$ (0.6 g L^{-1}), $ZnSO_4$ (1.0 g L^{-1}), $MnSO_4$ (1.0 g L^{-1}), $CuSO_4$ (2.0 g L^{-1}), and $FeSO_4$ (1.0 g L^{-1}).

Bacterial cells were extracted with hot aqueous phenol, the aqueous layer was dialyzed, and LPS was purified by treatment with DNAase, RNAse, and Proteinase K[7] followed by dialysis against deionized water and ultracentrifugation (105 000 *g*, 4 h). Lipopolysaccharide was detected by silver stained polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.[8]

An LPS sample (40 mg) in 12.5% aqueous ammonia was heated at 60 °C for 14 h. After evaporation, a residue was dissolved in water (2 mL) and a precipitate was removed by centrifugation (14000 *g*, 40 min). An OPS sample (19 mg) was isolated by gel-permeation chromatography on a column (74×1.6 cm) of Sephadex G-50 Superfine (Amersham Biosciences, Sweden) in 0.05 *M* pyridinium acetate buffer (pH 4.25) at flow rate

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0.4 mL min^{-1}; elution was monitored using a differential refractometer (Knauer, Germany).

An LPS sample (20 mg) was treated with 2% HOAc for 6 h at 100 °C, the precipitate was removed by centrifugation and the supernatant was fractionated by Sephadex G-50 chromatography as described above to give a modified polysaccharide (MPS) (9 mg).

An OPS sample (2 mg) was hydrolyzed with 0.5 *M* CF_3CO_2H (100 °C, 1 h). The hydrolysate was divided into two equal parts and evaporated to dryness with a stream of air. One part was reduced with $NaBH_4$ in 5% aqueous ammonia (10 mg mL^{-1}, 0.2 mL, 20 °C, 16 h) and acetylated with an Ac_2O—pyridine mixture (1 : 1, 0.2 mL, 20 °C, 16 h). The derived alditol acetates were analyzed by GLC on an Agilent 7820 chromatograph (Interlab, Russia) equipped with an HP-5ms column (Agilent) using a temperature program of 160 °C (1 min) to 290 °C at heating rate of 7 °C min^{-1}. 4-Deoxy-D-*arabino*-hexose from the OPS of *Citrobacter braakii* PCM 1531 (O6)[5] was used as an authentic sample. The other part of the hydrolysate was heated (120 °C, 16 h) with (*S*)-2-octanol (0.1 mL) containing anhydrous CF_3CO_2H (15 μL), an excess of 2-octanol was removed with a stream of air, the residue was acetylated, and analyzed by GLC as described above.

^1H and ^{13}C NMR spectra were recorded on an Avance II 600 MHz spectrometer (Bruker, Germany). Prior to measurements, samples were freeze-dried from 99.9% D_2O and studied in 99.95% D_2O at 30 °C using internal sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d_4 (δ_H 0, δ_C −1.6) as reference for calibration. 2D NMR spectra were obtained using standard Bruker software and Bruker TopSpin 2.1 program was employed to process the data. A spin-lock time of 60 ms and a mixing time of 200 ms were used in TOCSY and ROESY experiments, respectively. A 60-ms delay was used for evolution of long-range coupling to optimize the ^1H—^{13}C HMBC experiment (for coupling constant $J_{H,C}$ = 8 Hz).

Results and Discussion

Lipopolysaccharide was isolated from cells of *Y. frederiksenii* H56-36/81 (serotype O:60) by extraction with hot aqueous phenol. To enable analysis of the OPS structure, the LPS was O-deacylated by treatment with 12.5% aqueous ammonia. Monosaccharide analysis of the OPS by GLC of the acetylated aditols derived after full acid hydrolysis revealed rhamnose, ribose, and ara4dHex, which were identified by comparison with the authentic samples. The GLC analysis of the acetylated (*S*)-2-octyl glycosides[9] showed that rhamnose has the L configuration and ribose has the D configuration. The D configuration of ara4dHex was determined by analysis of glycosylation effects in the ^{13}C NMR spectrum of the OPS[10] (see below).

Mild acid hydrolysis of the LPS resulted in a modified polysaccharide (MPS), which lacked the ara4dHex residues. This finding indicated that the residue(s) of ara4dHex is located in a side chain(s) of the OPS.

The ^1H NMR spectrum of the MPS (Table 1) showed signals for four anomeric protons at δ 4.98—5.34 (all broadened singlets) and three CH_3—C groups at δ 1.29—1.32 (H(6) Rha, all doublets, $J_{5,6}$ = 6 Hz). The ^{13}C NMR spectrum of the MPS (see Table 1) displayed signals for four anomeric carbons at δ 100.8—108.2, three $\underline{C}H_3$—C groups at δ 17.9—18.2 (C(6) Rha), and one HO$\underline{C}H_2$—C group at δ 63.0 (C(5) Rib). These data demonstrated that a tetrasaccharide repeating unit of the MPS contains one residue of D-Rib and three residues of L-Rha. The ^1H and ^{13}C NMR spectra of the OPS (see Table 1) showed additional signals for two ara4dHex residues including those for two anomeric atoms at δ_H 4.96 and 5.04, δ_C 101.1 and 101.7, two C—$\underline{C}H_2$—C groups (H(4) and C(4) ara4dHex) at δ_H 1.53—1.78, δ_C 29.4 and 29.8, and two HO$\underline{C}H_2$—C groups (C(6) ara4dHex) at δ_C 65.5 (2 C).

Signals in the ^1H and ^{13}C NMR spectra of both polysaccharides were assigned using 2D ^1H—^1H COSY, ^1H—^1H TOCSY, ^1H—^1H ROESY, ^1H—^{13}C HSQC, and ^1H—^{13}C HMBC experiments, and spin-systems for all sugar residues were identified (see Table 1). A comparison

Table 1. ^1H and ^{13}C NMR chemical shifts (δ) of the polysaccharides from *Y. frederiksenii* H56-36/81

Monosaccharide residue	H(1)	H(2)	H(3)	H(4)	H(5)	H(6)	C(1)	C(2)	C(3)	C(4)	C(5)	C(6)
						MPS						
→2)-β-D-Rib*f*-(1→ (**A**)	5.34	4.21	4.43	4.06	3.71, 3.87	—	108.2	82.0	71.1	83.9	63.0	—
→3)-α-L-Rha*p*-(1→ (**B**)	5.06	4.23	3.92	3.53	3.90	1.32	103.2	71.0	79.9	72.4	70.5	17.9[a]
→3)-α-L-Rha*p*-(1→ (**C**)	4.98	4.17	3.86	3.56	3.79	1.29	103.2	71.1	79.3	72.6	70.7	17.9
→2)-α-L-Rha*p*-(1→ (**D**)	5.14	4.10	3.93	3.52	3.77	1.31	100.8	79.3	71.2	73.5	70.7	18.0[a]
						OPS						
→2)-β-D-Rib*f*-(1→ (**A**)	5.27	4.18	4.48	4.06	3.73, 3.88	—	108.0	82.5	71.1	83.4	62.6	—
→3,4)-α-L-Rha*p*-(1→ (**B**)	5.05	4.28	4.02	3.74	3.96	1.36	103.0	71.0	80.6	78.9	69.3	18.5[b]
→3)-α-L-Rha*p*-(1→ (**C**)	5.26	4.17	3.87	3.57	3.74	1.28	102.2	71.1	79.3	72.6	70.6	18.0
→2,3)-α-L-Rha*p*-(1→ (**D**)	5.09	4.38	4.03	3.63	3.81	1.35	101.5	77.4	81.8	72.7	70.8	18.2[b]
β-D-ara4dHex*p*-(1→ (**E**)	4.96	3.68	4.11	1.53, 1.76	3.92	3.64	101.1	69.7	69.3	29.8	73.1	65.5
β-D-ara4dHex*p*-(1→ (**F**)	5.04	3.73	4.11	1.53, 1.78	3.97	3.66	101.7	70.1	69.3	29.4	73.0	65.5

[a,b] Assignment could be interchanged.

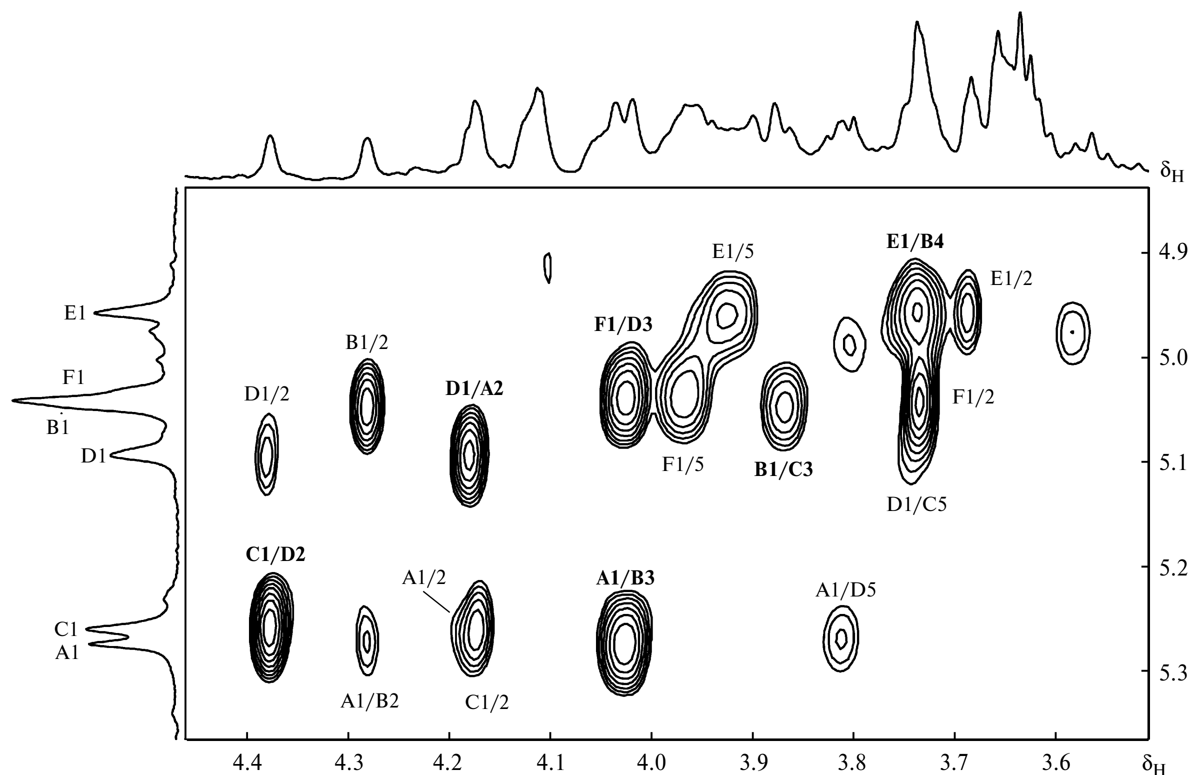


Fig. 1. Fragment of $^1H-^1H$ ROESY spectrum of the OPS. The corresponding parts of the ^1H NMR spectrum are shown along the axes. Numbers refer to protons in monosaccharide residues denoted by letters as shown in Fig. 3 and Table 1. Interresidue correlation peaks are annotated in bold type.

of ^{13}C NMR chemical shifts of the monosaccharide residues (see Table 1) with the published data[11] indicated that the Rib residue is in the β-furanose form and all Rha residues are in the α-pyranose form. The β configuration of both ara4dHex residues was inferred by an intraresidue H(1)/H(5) correlation, which was revealed by the $^1H-^1H$ ROESY experiment (Fig. 1). No such correlation would be observed in case of the α configuration of this monosaccharide (Fig. 2).

Sequence and linkage analyses were performed using the $^1H-^1H$ ROESY (see Fig. 1) and $^1H-^{13}C$ HMBC experiments, which showed the interresidue correlations of the anomeric protons with the protons at the linkage carbons or with the linkage carbons, respectively (Table 2). The glycosylation patterns in the repeating units of the polysaccharides were confirmed by low-field positions of the ^{13}C NMR signals for the substituted carbons of β-Rib*f* (C(2)) at δ 82.0—82.5 and α-Rha*p* (C(2)—C(4)) at δ 77.4—81.8 (see Table 1) as compared with their positions in the corresponding non-substituted monosaccharides at δ 76.0 and 71.1—73.3,[11] respectively.

A comparison of the ^{13}C NMR spectra of the MPS and OPS showed that attachment of β-ara4dHex **F** at position 3 of the L-Rha **D** residue caused the displacements of the signals for the C(3) and C(4) atoms of Rha **D** by $\Delta\delta$ = = +10.6 and –0.8 ppm (α- and β-effects of glycosylation, respectively[10]). Such values are characteristic for different absolute configurations of the monosaccharides linked in this manner and, therefore, ara4dHex **F** has the D configuration (the expected α- and β-effects of glycosylation would be[10] $\Delta\delta$ = +8.6±1.5 and –0.6±0.6 ppm in

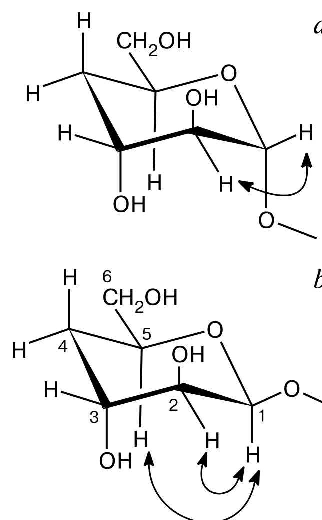


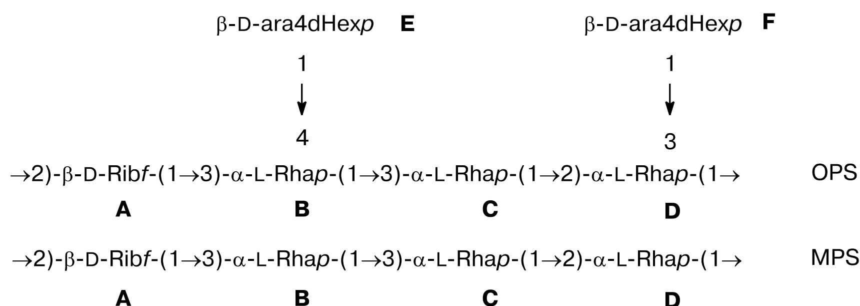
Fig. 2. Conformation of the α- (*a*) and β-anomers (*b*) of 4-deoxy-D-*arabino*-hexopyranose. Spatial contacts between protons revealed by the $^1H-^1H$ ROESY experiment are shown by arrows.

Table 2. Correlations for the anomeric protons in the 2D $^{1}H—^{1}H$ ROESY and $^{1}H—^{13}C$ HMBC experiments for the polysaccharides from *Y. frederiksenii* H56-36/81

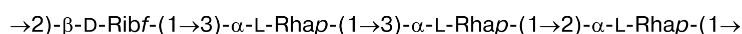
Monosaccharide residue	$\delta_{H(1)}$	Correlations to atoms in sugar residue (δ)	
		ROESY	HMBC
		MPS	
→2)-β-D-Rib*f*-(1→ (**A**)	5.34	**B** (3.92, H(3)), **A** (4.21, H(2))	**B** (79.9, C(3)), **A** (71.1, C(3)), (83.9, C(4))
→3)-α-L-Rha*p*-(1→ (**B**)	5.06	**C** (3.86, H(3)), **B** (4.23, H(2))	**C** (79.3, C(3)), **B** (79.9, C(3)), (70.5, C(5))
→3)-α-L-Rha*p*-(1→ (**C**)	4.98	**D** (4.10, H(2))	**D** (79.3, C(2)), **C** (79.3, C(3)), (70.7, C(5))
→2)-α-L-Rha*p*-(1→ (**D**)	5.14	**A** (4.21, H(2))	**A** (82.0, C(2)), **D** (79.3, C(2)), (71.2, C(3)), (70.7, C(5))
		OPS	
→2)-β-D-Rib*f*-(1→ (**A**)	5.27	**B** (4.28, H(2)), (4.02, H(3)), **D** (3.81, H(5)), **A** (4.18, H(2))	—
→3,4)-α-L-Rha*p*-(1→ (**B**)	5.05	**C** (3.87, H(3)), **B** (4.28, H(2))	—
→3)-α-L-Rha*p*-(1→ (**C**)	5.26	**D** (4.38, H(2)), **C** (4.17, H(2))	—
→2,3)-α-L-Rha*p*-(1→ (**D**)	5.09	**A** (4.18, H(2)), **C** (3.74, H(5)), **D** (4.38, H(2))	—
β-D-ara4dHex*p*-(1→ (**E**)	4.96	**B** (3.74, H(4)), **E** (3.68, H(2)), (3.92, H(5))	—
β-D-ara4dHex*p*-(1→ (**F**)	5.04	**D** (4.03, H(3)), **F** (3.73, H(2)), (3.97, H(5))	—

β-D-ara4dHex-(1→3)-L-Rha disaccharide or $\Delta\delta = +5.8\pm2.1$ and -1.7 ± 0.2 ppm in β-L-ara4dHex-(1→3)-L-Rha disaccharide, respectively). For β-ara4dHex **E** linked to L-Rha **B** at position 4, differences between the α- and β-effects of glycosylation in disaccharides with a different combination of enantiomers of the constituent monosaccharides are insufficient for the unambiguous inferring of their relative absolute configuration. Therefore, the D configuration of ara4dHex **E** was not confirmed experimentally and was taken the same as that of D-ara4dHex **F**.

Y. frederiksenii H56-36/81 (O:60)



Klebsiella pneumoniae O7



Citrobacter braakii PCM 1531 (O6)

Fig. 3. Structures of O-polysaccharide (OPS) and modified polysaccharide (MPS) of *Y. frederiksenii* H56-36/81 (serotype O:60), OPS of *Klebsiella pneumoniae* O7,[12] and revised structure of the OPS of *Citrobacter braakii* PCM 1531 (O6).

Based on these data, it was concluded that the OPS and MPS have the structures shown in Fig. 3. Interestingly, the MPS that represents the main chain of the OPS of *Y. frederiksenii* H56-36/81 has the same structure as the linear OPS of *Klebsiella pneumoniae* O7 studied earlier[12] (see Fig. 3).

A peculiar feature of the OPS of *Y. frederiksenii* H56-36/81 is the presence of 4-deoxy-D-*arabino*-hexose. Earlier, this monosaccharide has been identified only in the OPS of several strains of *Citrobacter* spp.[5,13,14]. In all these strains except for *Citrobacter braakii* PCM 1531 (O6),[5] the ara4dHex residues have been reported to be β-linked as in *Y. frederiksenii* H56-36/81 studied in this work. A comparison of the ^{1}H and ^{13}C NMR chemical shifts of the OPS of *C. braakii* PCM 1531 (O6)[5] and *Y. frederiksenii* H56-36/81 (see Table 1) showed that the anomeric configuration of ara4dHex in both OPS is the same and, hence, that in the OPS of *C. braakii* proposed earlier must be revised from α to β as shown in Fig. 3 (the wrong conclusion on the α-configuration[5] was based on the presence of an H(1)/H(2) correlation in the ^{1}H—^{1}H ROESY spectrum, which would be observed for both anomers, whereas indicative is only the presence or absence of the H(1)/H(5) correlation, see Fig. 2).

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