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The structure of the carbohydrate backbone of the lipopolysaccharide of *Pectinatus frisingensis* strain VTT E-79104

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Abstract—The structure of the carbohydrate backbone of the lipopolysaccharide from *Pectinatus frisingensis* strain VTT E-79104 was analyzed using chemical degradations, NMR spectroscopy, mass spectrometry, and chemical methods. The LPS contains two major structural variants, differing in the presence or absence of an octasaccharide fragment. The largest structure of the carbohydrate backbone of the LPS, that could be deduced from experimental results, consists of 20 monosaccharides arranged in a nonrepetitive sequence:

β-Ara4N-(1-*P*-6)-α-GlcN-(1-4)-α-Kdo-(2-4)┐
α-Gal-(1-2)-α-Man-(1-4)-α-Man-(1-5)-α-Kdo-(2-6)-β-GlcN-(1-6)-α-GlcN1*P*
R-3)-α-Gal-(1-2)-α-Man-(1-6)┘└(3-2)-α-Kdo

where R is H or 4-O-Me-α-L-Fuc-(1-2)-4-O-Me-β-Hep-(1-3)-α-GlcNAc-(1-2)-β-Man-(1-3)-β-ManNAc-(1-4)-α-Gal-(1-4)-β-Hep-(1-3)-β-GalNAc-(1- where Hep is a residue of D-*glycero*-D-*galacto*-heptose; all monosaccharides have the D-configuration except for 4-*O*-Me-L-Fuc and L-Ara4N. This structure is architecturally similar to the oligosaccharide system reported previously in *P. frisingensis* VTT E-82164 LPS, but differs from the latter in composition and also in the size of the outer region.

Keywords: *Pectinatus frisingensis*; Lipopolysaccharide; Core; Lipid A

1. Introduction

Pectinatus cerevisiiphilus and *P. frisingensis* are strictly anaerobic bacteria spoiling bottled beer.[1–3] These bacteria, although being Gram-negative in Gram staining, are currently assigned to the *Sporomusa* sub-branch of the *Clostridium* subphylum of Gram-positive bacteria.[2] Furthermore, the cell envelope of *Pectinatus* bacteria exhibits characteristics of both Gram-negative and Gram-positive organisms, making it specially interesting to see what kind of LPS structures are present in such bacteria of intermediary character. Whereas type strains of *P. cerevisiiphilus* and *P. frisingensis* contain LPS with polymeric O-specific chains,[4] other strains exist whose LPS lack O chains and exhibit in gel electrophoresis two distinct bands. A strain of the latter type, *P. frisingensis* VTT E-82164 was recently shown to contain a large nonrepetitive oligosaccharide structure involving an

Abbreviations: LPS, lipopolysaccharide; Kdo, 3-deoxy-D-*manno*-octulosonic acid; Hep, D-*glycero*-D-*galacto*-heptose; Ara4N, 4-amino-4-deoxy-L-arabinose, *P*, phosphate; HPAEC, high-performance anion-exchange chromatography; ESIMS, electrospray ionization mass spec-trometry.

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inner core that partly carries an additional complex decasaccharide. The inner core region was devoid of heptose and contained an additional Kdo residue outside the region of linkage to lipid A.[5] Here we present the results of structural analysis of the LPS from another strain of the O-chain-lacking type, *P. frisingensis* VTT E-79104, which shares a general architecture of LPS with *P. frisingensis* VTT E-82164.

2. Experimental

P. frisingensis VTT E-79104 was obtained from the culture collection of VTT Biotechnology, Espoo, Finland. All experiments were performed as described,[5] except that NMR spectra were recorded on a Varian UNITY INOVA 600 spectrometer, and more harsh conditions were used for alkaline deacylation (120 °C, 24 h) because of high stability of the N-acetyl groups.

For the isolation of 4-*O*-methyl-L-fucose, the LPS (100 mg) was hydrolyzed with 3 M TFA (100 °C, 3 h), dried, treated with charcoal, filtered, and the products separated by ascending paper chromatography on Whatman 1 paper in 5:5:1:3 pyridine–EtOAc–AcOH–water. The fastest moving band, detected by silver nitrate visualization on small strip, was eluted with water, and separated by HPLC on a C18 column (9 × 250 mm, Aqua, Phenomenex) in water at 3 mL/min. Fractions of 1 min were collected, the first 20 fractions dried and analyzed by ^{1}H NMR spectroscopy to find pure 4-*O*-methyl-L-fucose in fractions 10–12; yield 0.4 mg, $[\alpha]_D$ − 46 (*c* 0.05, water); lit.[6] $[\alpha]_D$ − 76 (*c* 1.12, water).

3. Results and discussion

Screening of *Pectinatus* strains revealed that the LPS from certain strains exhibit only two distinct bands on PAGE with no polymeric O chains (Fig. 1). This indicates the presence of two structurally distinct LPS populations, as was recently shown[4] in *P. frisingensis* VTT E-82164. After structure determination of one such LPS (VTT E-82164),[5] we used the same approaches to the structural analysis of the strain VTT E-79104.

The LPS was deacylated with 4 M KOH. Deacylation at 100 °C for 16 h gave two products, differing by the presence of an N-acetyl group. More severe conditions (120 °C, 24 h) led to a complete removal of all acyl groups. The mixture was separated by gel-chromatography and then HPAEC to give products **1** and **2** and 4-amino-4-deoxy-β-L-arabinopyranosyl phosphate (Ara4N*P*); the structure of the latter compound was confirmed by NMR spectroscopy and optical rotation measurements ($[\alpha]_D$ + 37 (*c* 0.1, water)). Ara4N*P* originated from two places in the LPS: O-4 of the GlcN residue **B** in lipid **A** part, and O-6 of GlcN W. The structure of **1** was determined using NMR spectroscopy (Table 1) and confirmed by mass spectral determination of the molecular mass peak of 2052.7 Da ($Hex_5HexN_3Kdo_3P_1$). Since most of the procedures and logic of conclusions was exactly the same as used for the analysis of the LPS from VTT E-82164, we will describe here only the points that were significantly different. The oligosaccharide **1** had most of its structure identical to that of the respective oligosaccharide from strain E-82164, and differed by the absence of the **K**–**J** (Scheme 1) fragment and by the substitution of the Man G with α-Gal at position 2 instead of the substitution with α-Glc at position 3.[5]

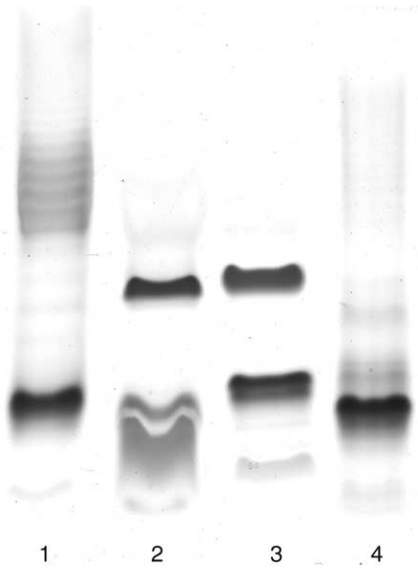


Figure 1. Deoxycholate PAGE separation of the LPSs. Lane 1, *P. frisingensis* type strain, lane 2, VTT E-79104, lane 3 VTT E-82164, lane 4, *P. cerevisiiphilus* type strain.

The ^{1}H NMR spectrum of oligosaccharide **2** had strong overlap of the anomeric signals and the monosaccharide residues situated close to the nonreducing end gave two series of signals. Although it had the expected mass of 3418.2 Da ($dHex_1Hex_7Hep_2HexN_6Kdo_3P_1Me_2$) and one could find duplicated spin systems of all expected monosaccharides, the spectra were not completely interpreted and the reason for duplication of the signals was not found. Thus, its structure presented on a Scheme 1 is tentative. We suppose that strong alkaline treatment had caused racemization of some chiral center, a situation already found in the alkaline deacylation of the LPS from *P. frisigensis* VTT-82164, which resulted in the inversion of the anomeric configuration of the glucuronic acid residue.[5]

Acid hydrolysis of the LPS with 2% acetic acid gave one large oligosaccharide **4**, separated from the rest of the products by gel-chromatography on Sephadex G-50 column, and smaller oligosaccharides **3**, **5**, **6**, which were reduced with $NaBH_4$ and separated by HPAEC.

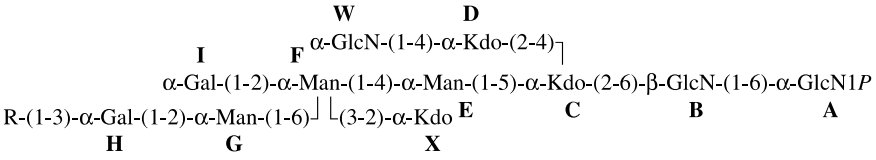
Table 1. NMR data for oligosaccharides **1** and **4**

Unit, compound	Nucleus	**1**	**2** (**3a**)	**3** (**3e**)	**4**	**5**	**6**	**7** (**6b**)	**8** (**7b**)	**8b** (Me)
α-GlcN1*P* **A**, **1**	^{1}H	5.74	3.46	3.93	3.60	3.86	4.28			
	^{13}C	92.6	55.1	70.4	70.5	73.8	70.2			
β-GlcN **B**, **1**	^{1}H	4.88	3.15	3.66	3.55	3.65	3.59	3.59		
	^{13}C	100.5	56.8	73.3	70.9	75.7	62.4			
α-Kdo **C**, **1**	^{1}H		2.05	2.10	4.20	4.34	3.63	3.75	3.62	3.93
	^{13}C			35.2	70.8	72.9	73.7	70.3	64.6	
α-Kdo **C**, **4**	^{1}H		1.92	2.09	4.15	4.15	3.91	3.79	3.64	3.82
	^{13}C	178.2	97.6	35.4	67.0	77.2	72.4	70.1	64.2	
α-Kdo **D**, **1**	^{1}H		1.97	2.58	3.97	4.28	3.79	4.01	3.87	3.98
	^{13}C			34.8	78.4	66.2	72.7	71.0	63.8	
α-GlcN **W**, **1**	^{1}H	5.17	3.43	3.92	3.43	3.77	3.79	3.92		
	^{13}C	98.5	55.0	70.5	70.8	74.4	61.9			
α-Kdo **X**, **1**	^{1}H		1.91	2.15	4.13	4.07	3.80	3.92	3.73	3.89
	^{13}C			35.4	66.5	67.4	73.3	72.0	63.1	
α Man **E**, **1**	^{1}H	5.19	4.13	4.06	3.81	4.24	3.79			
	^{13}C	100.4	71.6	72.3	75.5	71.4	61.9			
α-Man **E**, **4**	^{1}H	5.07	4.06	4.06	3.84	4.12				
	^{13}C	102.7	72.0	72.0	76.1	73.0	62.2			
α Man **F**, **1**	^{1}H	5.66	4.23	3.92	4.08	3.75	3.64	4.02		
	^{13}C	100.9	79.9	75.8	65.7	74.3	66.7			
α-Man **F**, **4**	^{1}H	5.54	4.10	3.94	3.84	3.85	3.73	4.01		
	^{13}C	101.3	81.1	71.8	68.0	73.3	67.3			
α-Man **G**, **1**	^{1}H	5.23	3.96	3.97	3.76	3.75	3.80	4.00		
	^{13}C	99.2	81.0	70.3	68.3	73.6	62.9			
α-Man **G**, **4**	^{1}H	5.25	3.99	3.93	3.79	3.70	3.79	3.91		
	^{13}C	99.5	81.0	72.0	68.2	73.9	62.0			
α-Gal **H**, **1**	^{1}H	5.14	3.83	3.93	4.00	4.13	3.75	3.75		
	^{13}C	102.4	69.9	70.4	70.3	72.4	62.4			
α-Gal **H**, **4**	^{1}H	5.12	3.90	3.99	4.22	3.73	3.73			
	^{13}C	102.7	69.1	80.0	70.5	72.1	62.3			
α-Gal **I**, **1**	^{1}H	5.24	3.80	3.90	4.05	4.06	3.73	3.73		
	^{13}C	101.9	69.5	70.5	70.1	72.2	61.7			
α-Gal **I**, **4**	^{1}H	5.15	3.84	3.86	4.02	4.09	3.74	3.74		
	^{13}C	102.4	69.9	70.7	70.4	72.5	62.2			
β-GalNAc **Q**, **4**	^{1}H	4.72	4.08	3.92	4.07	3.72	3.79	3.79		
	^{13}C	104.0	52.8	81.0	69.2	75.6	62.0			
β-Hep **Z**, **4**	^{1}H	4.52	3.57	3.72	4.15	3.62	4.02	3.64	3.78	
	^{13}C	106.4	71.6	73.3	77.2	74.5	69.2	64.0		
α-Gal **Y**, **4**	^{1}H	5.11	3.83	4.03	4.26	4.42	3.67	3.81		
	^{13}C	101.4	70.0	70.5	77.7	71.5	61.4			
β-ManNAc **V**, **4**	^{1}H	4.96	4.83	4.04	3.46	3.43	3.83	3.93		
	^{13}C	101.0	50.8	78.3	66.4	77.5	61.6			
α-Man **K**, **4**	^{1}H	4.84	3.86	3.73	3.71	3.44	3.79	3.97		
	^{13}C	97.5	78.0	74.5	68.3	78.0	62.1			
α-GlcNAc **P**, **4**	^{1}H	5.12	3.84	4.17	3.64	4.13	3.73	3.95		
	^{13}C	99.2	54.7	77.3	69.2	71.5	61.1			
4-*O*-Me-β-Hep **T, 4**	^{1}H	4.65	3.54	3.91	3.80	3.55	3.81	3.71	3.82	3.62
	^{13}C	101.3	77.8	75.4	79.9	74.3	69.9	64.2		62.7
4-*O*-Me-α-Fuc **S, 4**	^{1}H	5.18	3.72	3.65	3.40	4.23	1.26			3.57
	^{13}C	100.6	69.6	71.1	83.8	67.8	16.6			63.1

4-Amino-4-deoxy-L-arabinose has been also isolated after gel chromatography and purified by paper chromatography in 5:5:1:3 pyridine–ethyl acetate–acetic acid–water and characterized on the form of its hydrochloride, $[\alpha]_D + 38$ (*c* 0.2, water) (lit.[7] +49.6, *c* 0.8, D_2O).

The structure of product **3** included a fragment of structure **1** with Kdo-ol (**C**) at the reducing end. It was analyzed by NMR spectroscopy; data not shown, but they are almost identical to the data for the respective monosaccharides in the oligosaccharide **4** except for the galactose residue **H**, which is terminal in **3** and 3-substituted in **4** (Table 1). The structure was confirmed by methylation analysis:[8] partially methylated alditol acetates derived from 2-, 4-, and 2,6-substituted Man, and terminal Gal were identified by GC–MS analysis. ESIMS allowed to estimate the mass for **3** as 1048 Da in accordance with the suggested structure.

Oligosaccharide **4** contained all elements of structure **3** and an additional linear octasaccharide sequence. In its NMR spectra, spin systems for 4-*O*-methyl-α-fucopyranose (**S**), 4-*O*-methyl-D-*glycero*-β-D-*galacto*-heptopyranose (**T**), α-GlcNAc (**P**), β-Man (**K**), β-ManNAc (**V**), D-*glycero*-β-D-*galacto*-heptopyranose (**Z**), β-GalNAc (**Q**) were identified on the basis of signal position, coupling constants, and intraresidual NOEs. The β-*galacto*-configuration of both heptose residues was evident from the coupling constants. The nonmethylated heptose gave an alditol acetate having identical retention time on GC with that of L-*glycero*-D-*manno*-heptitol acetate, indicating its D-*glycero*-D-*galacto*-configuration

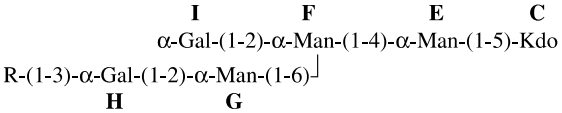


1, R = H;

2, R =

S T P K
4-O-Me-α-L-Fuc-(1-2)-4-O-Me-β-Hep-(1-3)-α-GlcN-(1-2)-β-Man-(1-

V U Z Q
-3)-β-ManN-(1-4)-α-Gal-(1-4)-β-Hep-(1-3)-β-GalN-(1-



3, R = H (C = Kdo-ol)

4, R =

S T P K
4-O-Me-α-L-Fuc-(1-2)-4-O-Me-β-Hep-(1-3)-α-GlcNAc-(1-2)-β-Man-(1-

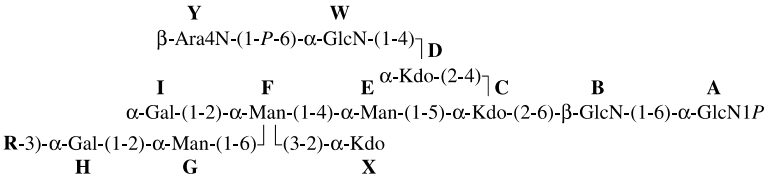
V U Z Q
-3)-β-ManNAc-(1-4)-α-Gal-(1-4)-β-Hep-(1-3)-β-GalNAc-(1-

β-Ara4N-(1-*P*-6)-α-GlcN-(1-4)-Kdo-ol **5**
Y W D

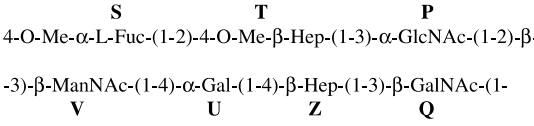
P-6)-α-GlcN-(1-4)-Kdo-ol **6**
W D

Scheme 1. Structures of the isolated oligosaccharides and deduced structures of the carbohydrate backbone of the LPS from *P. frisingensis*.

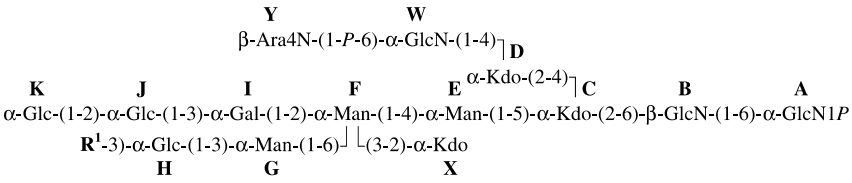
Proposed structure for the carbohydrate backbone of P. *frisingensis* VTT E-79104 LPS:



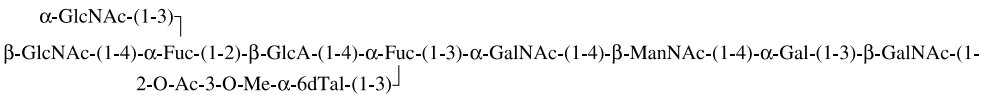
where R is H or



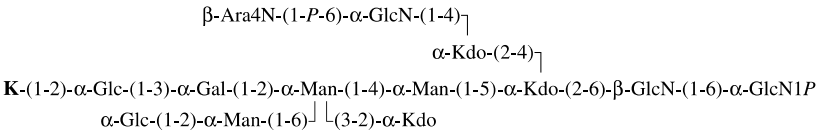
P. frisingensis VTT E-82164



where $\mathbf{R^1}$ is H or



P. frisingensis VTT E-79100



where **K** = α-Glc or H

Scheme 1 (*continued*)

(or enantiomeric), because L-*glycero*-D-*manno*-heptose and D-*glycero*-D-*galacto*-heptose give the same alditol upon reduction. The C-6 signal of the 4-*O*-methylated heptose residue **T** had a similar position to that of residue **Z** (Table 1), as well as to that reported (70 ppm) for D-*glycero*-β-D-*galacto*-heptopyranose in the polysaccharide from *Chromobacterium violaceum*.[9] The position of the C-6 signal in heptopyranoses is sensitive to the configuration at C-6, and we thus conclude that residue **T** has also the D-*glycero*-β-D-*galacto*-configuration. Other components of oligosaccharide **4** were also identified by GC–MS. The position of methyl groups in 2-*O*-Me-Hep and 4-*O*-Me-Fuc was deduced from the fragmentation in mass spectra of the respective 1*d*-alditols and NMR data.

The sequence of monosaccharides in **4** was obtained from NOE data (Fig. 2): correlations S1T1, S1T2, T1P3, P1K1, P1K2, K1P5, K1V3, V1U4, U1Z4, Z1Q3, Q1H3,

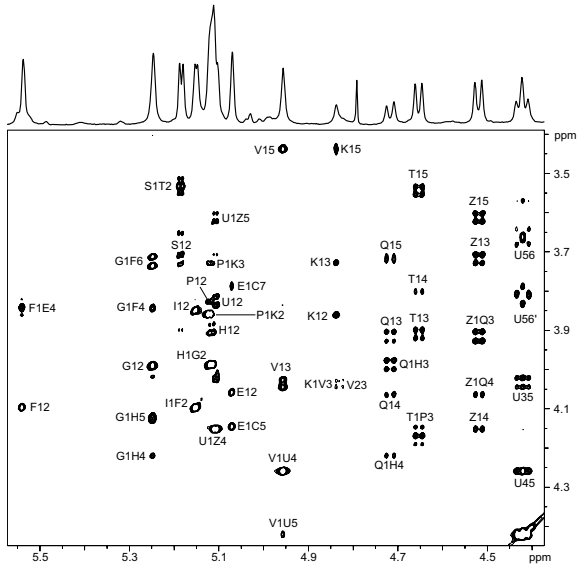


Figure 2. Fragment of the NOESY spectrum of oligosaccharide **4**, showing correlations from anomeric protons.

H1G1, H1G2, H1H5, G1F6, I1F1, I1F2, F1I5, F1E4, F1E3, E1C5, E1C7 were observed. All respective HMBC correlations from anomeric protons to trans-glycosidic carbon atoms were also identified. HMBC and NOE correlations between methyl groups and **S**4 and **T**4 signals were used to identify methyl groups positions.

Oligosaccharide **4** showed an expected mass of 2541.2 Da in ESIMS analysis. For the sequence analysis, MS/MS spectra of **4** were obtained. From several primary fragments, the base ion with m/z 1493 was selected, corresponding to $[M-H_2O]^+$ of the following sequence:

S **T** **P** **K** **V** **U** **Z** **Q**

4-*O*-Me-α-Fuc--4-*O*-Me-β-Hep--α-GlcNAc--β-Man--β-ManNAc--α-Gal--β-Hep--β-GalNAc-

Its secondary fragmentation showed the loss of the glycosyl-cations of the fragment **S** (ion at m/z 1333), **S**–**T** (1127), **S**–**T**–**P** (924), **S**–**T**–**P**–**K** (762), **S**–**T**–**P**–**K**–**V** (558), **S**–**T**–**P**–**K**–**V**–**U** (396), and **S**–**T**–**P**–**K**–**V**–**U**–**Z** (203) in agreement with the proposed structure.

The structure of **4** was further confirmed by methylation analysis using the Ciucanu–Kerek procedure, where the following monosaccharides were identified by GC–MS as alditol acetates: terminal Fuc, 2- and 4-substituted Hep, 2-, 4-, and 2,6-substituted Man, terminal, 3-, and 4-substituted Gal, and 3-substituted residues of GlcNAc, GalNAc, and ManNAc.

The structure of oligosaccharide **5** was the same as of the previously identified product obtained from the *P. frisingensis* strain E-82164;[5] oligosaccharide **6** was a smaller product lacking an Ara4N residue probably due to hydrolysis. Another series of small oligosaccharides included a residue of 2,7-anhydro-Kdo with the same substituents as in **5** and **6**.

The absolute configuration of all hexoses, hexosamines, and D-*glycero*-D-*galacto*-heptose was estimated as D by GC of the acetylated 2-butyl glycosides obtained with optically pure 2-butanol. A sample of synthetic D-*glycero*-D-*galacto*-heptose was obtained as a gift from Dr. M. B. Perry, NRC Canada). The absolute configurations of 4-*O*-Me-L-Fuc was determined by optical rotation measurement of the sample of free monosaccharide isolated after hydrolysis of the LPS with subsequent paper chromatography and HPLC purification. Attempts to deduce the configuration of 4-*O*-methyl-D-*glycero*-D-*galacto*-heptose from the analysis of the ^{13}C NMR chemical shifts[10] was unsuccessful because of the presence of too many factors influencing the chemical shifts in an unknown way (methylation, substitution at O-2, lack of good reference data). Thus, its configuration is presented tentatively.

CE–MS analysis of the O-deacylated LPS showed the presence of a largest structure of 4392 Da ($dHex_1Hex_7Hep_2HexNAc_6Ara4N_2Kdo_3P_3Me_2C13{:}0(3\text{-}OH)_2$) in agreement with the proposed structure. All Pectinatus LPSs studied so far contain 13:0(3-OH) fatty acid as the major lipid A component.[11]

Combined together, the results of the structural analysis of the isolated oligosaccharides led to the structure of the carbohydrate backbone of the *P. frisingensis* 79104 LPS presented on Scheme 1. Its inner part, including the residues **A**–**G**, **W**, **Y** is identical to that of the LPS from *P. frisingensis* E-82164 and E-79100 (Scheme 1). There is some further similarity in adjacent residues, but the fragment **S**–**T**–**P**–**K**–**V**–**U**–**Z**–**Q** is completely different from the structures of two other strains. The absence of substituent at O-4 of β-GlcN residue **B** in the lipid A portion agrees with the previously reported presence of Ara4N1*P* at this place, which is removed in alkaline conditions and thus is not present in the oligosaccharides **1** and **2**.

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