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Note

Structural studies of the O-antigens of *Yersinia pseudotuberculosis* O:2a and mutants thereof with impaired 6-deoxy-D-*manno*-heptose biosynthesis pathway

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Abstract—The full structure of the long- and short-chain O-antigen of *Yersinia pseudotuberculosis* O:2a containing two uncommon deoxy sugars, abequose and 6-deoxy-D-*manno*-heptose (6dmanHep), was established, for the first time, by sugar analysis, NMR spectroscopy, and high-resolution ESIMS. Similar structural studies were also performed on two O:2a mutants with single disruption of 6dmanHep synthesis pathway genes each, which synthesize modified long-chain (*dmhA* mutant) and short-chain (both *dmhA* and *dmhB* mutants) O-antigens with 6dmanHep replaced by its putative biosynthetic precursor, D-*glycero*-D-*manno*-heptose.

Keywords: *Yersinia pseudotuberculosis*; O-Antigen; Bacterial polysaccharide structure; Single gene inactivation; Sugar biosynthesis pathway; 6-Deoxy-D-*manno*-heptose

A zoonotic pathogen *Yersinia pseudotuberculosis* causes a broad range of acute and chronic gastrointestinal disorders and is the recent ancestor of *Yersinia pestis*, the cause of plague.[1] Based on the O-antigens, strains of *Y. pseudotuberculosis* are currently serotyped into 15 serovars, some of which are divided into subgroups.[2] Structures of the O-antigens, which represent O-polysaccharide chains of the lipopolysaccharides (LPS), have been established for most serovars 1–7.[3,4] But for some of them, including *Y. pseudotuberculosis* O:2a, only tentative structures have been proposed based on incomplete chemical data[5] and genetic data.[6,7] Recently, the structure of the core, a linker oligosaccharide region between the O-antigen and lipid A, was studied in *Y. pseudotuberculosis*[8,9] and was found to be similar to that in *Y. pestis*.[10] In this work, we have elucidated, for the first time, the full structure of the carbohydrate portion of the LPS of *Y. pseudotuberculosis* O:2a and confirmed the earlier proposed tentative structure of the O:2a antigen.

A number of *Y. pseudotuberculosis* O-polysaccharides are distinguished by the presence of rare deoxy sugars, such as 3,6-dideoxyhexoses, 6-deoxy-D-*manno*-heptose (6dmanHep), and 4-*C*-(1-hydroxyethyl)-3,6-dideoxy-D-*xylo*-hexoses.[3–5] The biosynthesis pathways have been elucidated for 3,6-dideoxyhexoses,[11,12] and based on O-antigen genetic sequence data, a pathway has been proposed tentatively for 6dmanHep.[13] In studies of the latter, two mutants have been derived from *Y. pseudotuberculosis* O:2a by nonpolar inactivation of single genes putatively involved in the consecutive conversion of GDP-D-*glycero*-D-*manno*-heptose (GDP-manHep) into GDP-6-deoxy-4-keto-D-*lyxo*-heptose and GDP-6dmanHep catalyzed by a 4,6-dehydratase (DmhA) and a reductase (DmhB), respectively.[14] It has also been demonstrated that both mutants produce a modified O-antigen, in which 6dmanHep is replaced by its putative

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biosynthetic precursor, D-*glycero*-D-*manno*-heptose (manHep). In this paper, we also report on the full-structure elucidation of the carbohydrate portions of the LPS of *dmhA* and *dmhB* mutants.

The LPS was isolated from bacterial cells by the phenol–water procedure[15] and purified as described.[14] The LPS samples were degraded under mild acid conditions, using either sodium acetate buffer pH 4.5 or 1% HOAc, and the following GPC on Sephadex G-50 afforded polysaccharides (PS_{NaOAc} and PS_{HOAc}) from both the wild type and *dmhA* mutant, as well as oligosaccharides (OS_{NaOAc} and OS_{HOAc}, respectively) from all three strains studied.

Sugar analysis of the polysaccharides by GLC of the alditol acetates derived after full acid hydrolysis (1 M CF_3CO_2H, 120 °C, 1 h) revealed galactose, 6dmanHep, and GlcNAc as the major components in wild type or galactose, manHep, and GlcNAc in *dmhA* mutant. Abequose (Abe, 3,6-dideoxy-D-*xylo*-hexose) reported as another major sugar component of the O:2a antigen was partially destroyed under the hydrolysis conditions used, and its content was about 20% of the expected value. Glucose and L-*glycero*-D-*manno*-heptose derived from the LPS core region[8–10] were detected as well. Similar analysis of the oligosaccharides revealed the same monosaccharides, including 6dmanHep in the wild type and manHep in both mutants, with a significant relative increase of the contents of the core components.

The ^1H (not shown) and ^{13}C NMR (Fig. 1, bottom panel) spectra of the wild-type PS_{NaOAc} showed signals for anomeric atoms of four monosaccharides at δ_H 4.76–5.43; δ_C 100.9–104.1, methyl groups of GlcNAc and Abe at δ_H 2.05 and 1.20; δ_C 24.0 and 17.3, respectively, methylene groups of Abe (H-3 and C-3) and 6dmanHep (H-6 and C-6) at δ_H 1.76–2.14; δ_C 34.5 and 35.0, hydroxymethylene groups of Gal, GlcNAc (both C-6), and 6dmanHep (C-7) at δ_C 59.8–62.4, a nitrogen-bearing carbon (C-2 of GlcNAc) at δ 56.0, and other signals at δ_H 3.42–4.38 and δ_C 65.2–82.1. The ^1H (not shown) and ^{13}C NMR (Fig. 1, top panel) spectra of the *dmhA*-mutant PS_{NaOAc} were similar, except that no signals for the methylene group of 6dmanHep were present, and the signal for C-7 of heptose shifted from δ_C 59.8 to 63.2. Therefore, the PS_{NaOAc} from both strains have tetrasaccharide repeats (O-units) containing one residue each of the major monosaccharides identified in sugar analysis.

The NMR spectra of the PS_{NaOAc} were fully assigned using 2D COSY, TOCSY, ROESY, and ^1H,^{13}C HSQC experiments (Table 1), and spin systems for all the sugar residues were identified. A relatively large $J_{1,2}$ coupling constant of 7 Hz showed that GlcNAc is β-linked, whereas relatively small $J_{1,2}$ values of <3 Hz indicated that Gal and Abe are α-linked. Comparison of the ^1H and ^{13}C chemical shifts of 6dmanHep and manHep with those of the corresponding free monosaccharides[16,17] demonstrated that both sugar residues are β-linked. The anomeric configurations were confirmed by a 2D ROESY experiment, which showed an H-1,H-2 correlation for α-linked Gal and Abe, and H-1,H-3 and H-1,H-5 correlations for β-linked GlcNAc, 6dmanHep, and manHep.

Downfield displacements of the signals for C-3 of GlcNAc and 6dmanHep, C-3 and C-4 of Gal to δ 77.6–82.1 revealed the glycosylation pattern in the O-unit of the PS_{NaOAc} of wild-type, Gal being at the branching point and Abe the terminal residue of the side chain. Similarly, substitution of GlcNAc and manHep at position 3 and Gal at positions 3 and 4 was confirmed in the *dmhA*-mutant PS_{NaOAc}.

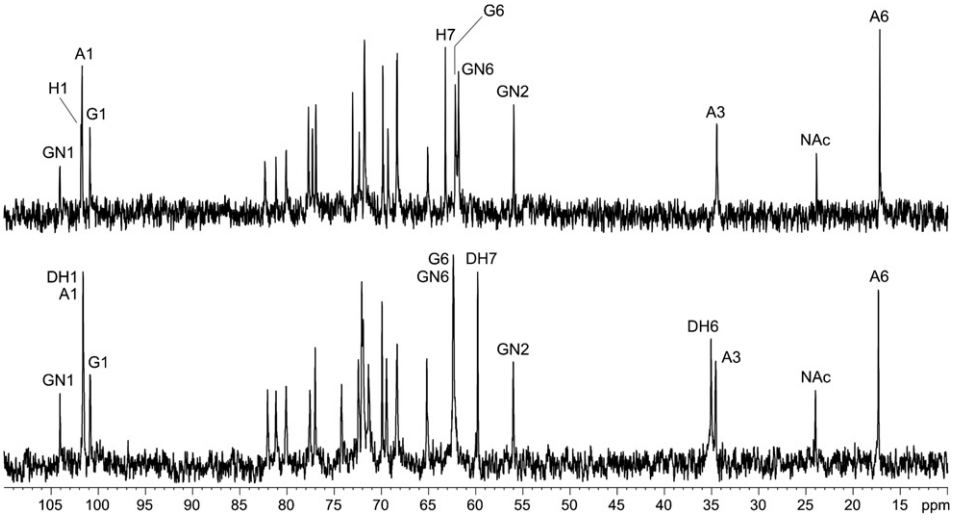

Figure 1. 125-MHz ^{13}C NMR spectra of the PS_{NaOAc} from *Y. pseudotuberculosis* O:2a wild-type strain (bottom) and *dmhA* mutant (top). Numbers refer to carbons in sugar residues denoted as follows: G, galactose; A, abequose; DH, 6-deoxy-D-*manno*-heptose; H, D-*glycero*-D-*manno*-heptose; GN, GlcNAc.

Table 1. ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) related to internal acetone (δ_H 2.225, δ_C 31.45). Chemical shifts for NAc are δ_H 2.05 and δ_C 23.9–24.0

Residue	Nucleus	1	2	3	4	5	6	7
Y. pseudotuberculosis O:2a wild-type PS_{NaOAc}								
→3)-β-D-Glc*p*NAc-(1→	^{1}H	4.76	3.86	3.80	3.70	3.51	3.75, 3.95	
	^{13}C	104.1	56.0	81.2	72.4	77.0	62.4	
→ 3,4)-α- D-Gal*p*-(1→	^{1}H	5.43	3.96	3.93	4.38	3.93	3.76, 3.76	
	^{13}C	100.9	69.4	80.1	77.6	72.1	62.4	
→3)-β-D-6dmanHep*p*-(1→	^{1}H	5.01	4.20	3.67	3.64	3.42	1.76, 2.14	3.77, 3.79
	^{13}C	101.6	72.0	82.1	71.4	74.2	35.0	59.8
α-Abe *p*-(1→	^{1}H	5.16	4.04	2.00, 2.09	3.89	4.20	1.20	
	^{13}C	101.6	65.2	34.5	69.9	68.3	17.3	
Y. pseudotuberculosis O:2a dmhA-mutant PS_{NaOAc}								
→3)-β-D-Glc*p*NAc-(1→	^{1}H	4.75	3.86	3.77	3.73	3.51	3.78, 3.96	
	^{13}C	104.0	55.9	81.2	72.3	76.9	62.1	
→3,4)-α-D-Gal*p*-(1→	^{1}H	5.43	3.94	3.92	4.42	3.91	3.72, 3.82	
	^{13}C	100.8	69.3	80.1	77.3	71.8	61.8	
→3)-D-β-D-manHep*p*-(1→	^{1}H	5.00	4.19	3.70	3.88	3.48	4.08	3.73, 3.83
	^{13}C	101.7	71.8	82.3	68.4	77.7	73.0	63.2
α-Abe*p*-(1→	^{1}H	5.15	4.04	2.00, 2.10	3.89	4.19	1.21	
	^{13}C	101.6	65.1	34.4	69.8	68.3	17.2	

The monosaccharide sequence in the O-units was established based on the following interresidue correlations between anomeric protons and protons at the linkage carbons in the ROESY spectrum: Abe H-1,6dmanHep H-3 and 6dmanHep H-1,Gal H-4 (in wild type) or Abe H-1,manHep H-3 and manHep H-1,Gal H-4 (in *dmhA* mutant), Gal H-1,GlcNAc H-3 and GlcNAc H-1,Gal H-3 (in both). Therefore, the PS_{NaOAc} from *Y. pseudotuberculosis* O:2a wild-type strain and the *dmhA* mutant represent long-chain O-antigens with the O-units having the structures **1** and **2** shown in Chart 1.

Similar NMR-spectroscopic studies of PS_{HOAc} from both strains (data not shown) demonstrated that they have two types of O-repeats. One is a tetrasaccharide containing Abe and having the same structures as the O-unit in PS_{NaOAc}, and the other is a trisaccharide that lacks Abe. The latter represents 30–50% of the O-repeats. This finding is not unexpected in light of the well-known lability of the glycosidic linkage of 3,6-dideoxyhexoses toward acids.

The OS_{NaOAc} and OS_{HOAc} from all three strains were analyzed by high-resolution negative-ion ESI FT-ICR MS (Fig. 2) taking into account the known *Y. pseudotuberculosis* LPS core structure[8,9] shown in Chart 1. The major OS_{HOAc} compounds consisted of the LPS cores with one O-unit attached. The oligosaccharides differed from each other in substitution of manHep with Gal (Δm 30 u) in the core and the presence or absence of abequose (Δm 130 u) in the O-unit, which was partially cleaved upon mild acid hydrolysis of the LPS. Yet another difference of Δm 254 u reflected alternatives for the lateral residues of D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko) and 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) in the core. The lateral Kdo, but not Ko, split off from the oligosaccharide upon mild acid hydrolysis, the cleavage being accompanied by dehydration of the Kdo residue at the reducing end of the core.[10] Such manHep/Gal and Ko/Kdo monosaccharide variations are characteristic of the LPS core of both *Y. pseudotuberculosis*[9] and *Y. pestis*.[10]

Mass spectra of the OS_{HOAc} from the wild-type (Fig. 2, top panel) and *dmhA* mutant (Fig. 2, middle panel) showed similar patterns, but all the compounds in the latter had molecular masses higher by 16 u. This increase evidently resulted from the replacement of 6dmanHep with manHep, which is in accord with the *dmhA*-mutant long-chain O-antigen structure **2**. Minor peaks for the ions with higher masses by 16 u were also observed in the mass spectrum of the wild-type OS_{HOAc}, which may be accounted for by the incorporation of manHep in place of 6dmanHep to a minor degree in the wild type, too.

The mass spectrum of the *dmhB*-mutant OS_{HOAc} (Fig. 2, bottom panel) showed a different pattern. As the spectrum of the *dmhA*-mutant OS_{HOAc}, it contained peaks for the full-O-unit-containing oligosaccharides with (2091.69 u and 2061.69 u) and without (1837.63 u and 1807.63 u) lateral Ko but these peaks were minor, whereas the major peaks corresponded to the compounds with an incomplete O-unit (1515.50 u and 1485.50 u) and without any O-unit (1150.36 u and 1120.36 u). Peaks corresponding to the core lacking an O-unit were only minor in the wild-type strain and were totally absent from the *dmhA* mutant. Remarkably, the content in Ko-containing compounds was significantly lower in the *dmhB* mutant than in the wild type and *dmhA* mutant. The data of the *dmhB*-mutant OS_{HOAc} confirmed that the substitution of lateral manHep for Gal occurs in the LPS core region and demonstrated the monosaccharide sequence in the biological O-unit.

wild-type PS_{NaOAc}

α-Abe*p*-(1→3)-β-D-6dmanHep*p*
1
↓
4
→3)-α-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→ **1**

dmhA-mutant PS_{NaOAc}

α-Abe*p*-(1→3)-D-β-D-manHep*p*
1
↓
4
→3)-α-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→ **2**

wild-type and mutant OS_{NaOAc}

Sug2-(1→7)-L-α-D-manHep*p* β-D-Glc*p* Sug1
1 1 2
↓ ↓ ↓
7 4 4
R→3)-L-α-D-manHep*p*-(1→3)-L-α-D-manHep*p*-(1→5)-Kdo **3**, **4**

Sug1 = α-Kdo*p* or α-Ko*p*; Sug2 = D-α-D-manHep*p* or β-D-Gal

O-unit in wild-type OS_{NaOAc}

R = α-Abe*p*-(1→3)-β-D-6dmanHep*p*-(1→4)-α-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→ (**3**)

O-unit in *dmhA*- and *dmhB*-mutant OS_{NaOAc}

R = α-Abe*p*-(1→3)-D-β-D-manHep*p*-(1→4)-α-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→ (**4**)

Chart 1. Structures of the O-units in PS_{NaOAc} (**1**, **2**) and OS_{NaOAc} (**3**, **4**) from *Y. pseudotuberculosis* O:2a wild-type strain (**1**, **3**), *dmhA* mutant (**2**, **4**), and *dmhB* mutant (**4**). The LPS core structure has been reported earlier;[8–10] phosphoethanolamine in the core is not shown.

The mass spectra of the OS_{NaOAc} from the three strains (not shown) were in agreement with the data of the OS_{HOAc}, and in particular confirmed all the differences between the oligosaccharides from various strains discussed above. As expected, the OS_{NaOAc} contained no Abe-lacking compounds. Therefore, the major compounds with the full O-unit (short-chain O-antigen) present have structures **3** (from wild type) and **4** (from both mutants) as shown in Chart 1. In addition, the OS_{NaOAc} included minor Ko-containing oligosaccharides bearing phosphoethanolamine (ion peaks at 2198.70 u and 2168.69 u in wild type and 2214.70 u and 2184.69 u in both mutants). In *Y. pestis*, which possesses the same core carbohydrate backbone structure as *Y. pseudotuberculosis*,[8–10] phosphoethanolamine occupies position 7 of the lateral Ko residue.[18]

In summary, the LPS of *Y. pseudotuberculosis* O:2a, including the biological O-unit structure established in this work, confirmed a tentative structure proposed earlier based on methylation analysis before and after cleavage of Abe[5] and comparison of the O-antigen gene cluster sequence data with those of other serovars for which the O-antigen structures had been unambiguously established.[6,7] The O-antigen of the *dmhA* mutant with the impaired 6dmanHep biosynthesis pathway has the same structure except that 6dmanHep is replaced with its putative biosynthetic precursor manHep. The *dmhB* mutant produces no significant amount of the long-chain O-antigen, but it does produce a short-chain O-antigen identical to that of the *dmhA* mutant. The degree of core capping by the O-unit is high in the wild type and in the *dmhA* mutant, but it hardly exceeds 50% in the *dmhB* mutant. The last strain is also distinguished by the ability to ligate both the complete and incomplete O-unit to the core. The biological implications of the O-antigen modification in *Y. pseudotuberculosis* O:2a have been reported elsewhere.[14]

1. Experimental

1.1. Bacterial strains, isolation and degradation of lipopolysaccharides

The wild-type strain of *Y. pseudotuberculosis* O:2a was kindly provided by Professor M. Skurnik (Helsinki, Fin-

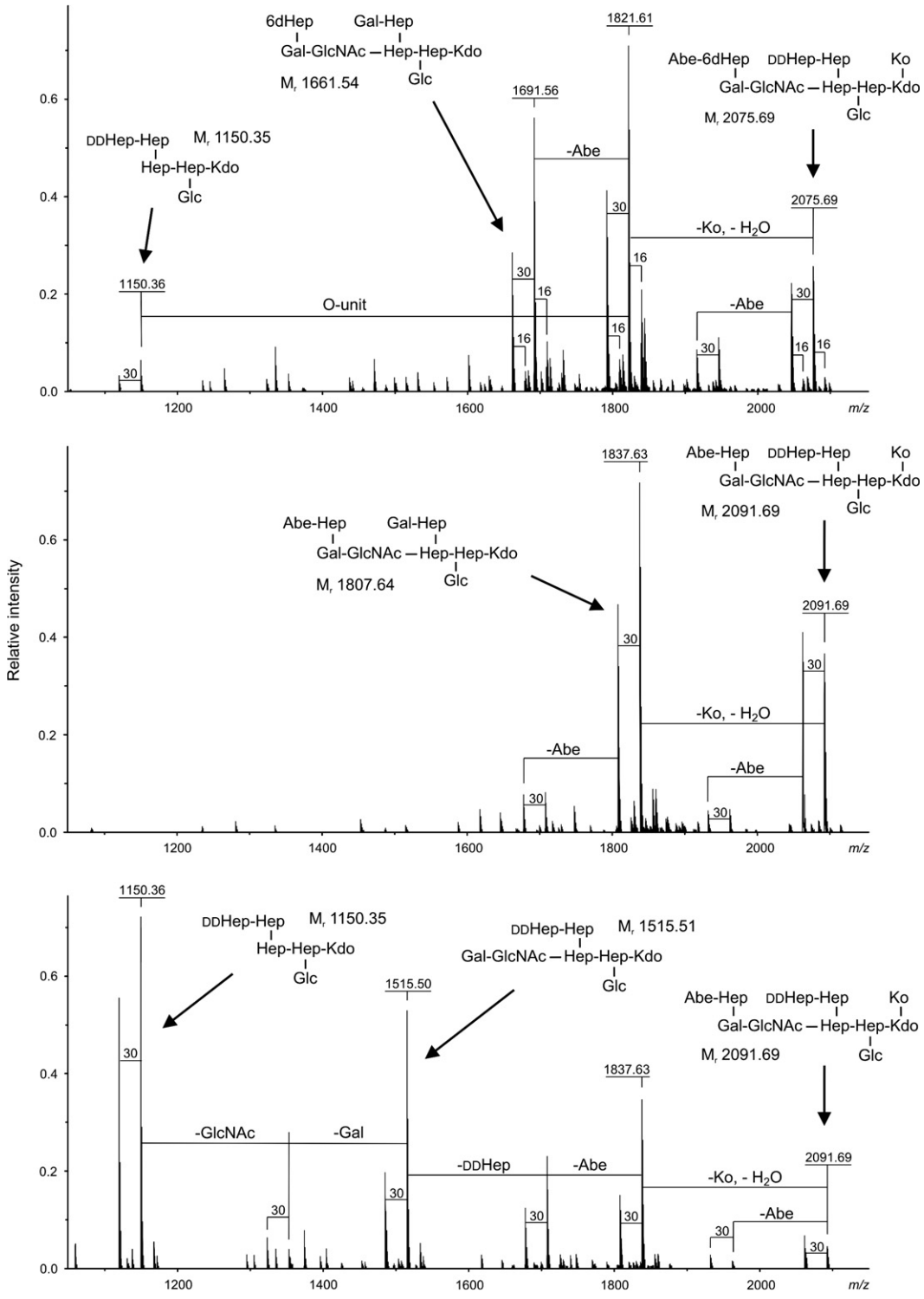

Figure 2. Charge-deconvoluted negative-ion ESI FT-ICR MS mass spectra of the OS_{HOAc} derived from wild-type strain (top), *dmhA* mutant (middle), and *dmhB* mutant (bottom). The Q-voltage was set to 20 V. The mass difference of 30 u corresponds to the substitution of Gal by manHep in the core; the mass difference of 16 u in the mass spectrum of wild-type strain is due to the replacement of 6dmanHep by manHep in the O-unit.

land). Generation of mutants, cultivation of bacteria, and isolation of the LPS were performed as described.[14] A portion of the LPS (30 mg from each strain) was heated at 100 °C for 2 h in 0.1 M NaOAc buffer pH 4.5, 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) to give PS_{NaOAc} (from wild-type strain and *dmhA* mutant) and OS_{NaOAc} (from all strains).

The precipitate was heated with aqueous 1% HOAc at 100 °C for 2 h, the residue removed by centrifugation, and the supernatant fractionated on Sephadex G-50 as above to afford the corresponding PS_{HOAc} and OS_{HOAc}. The following yields (%) of the fractions obtained were wild-type PS_{NaOAc} 6, OS_{NaOAc} 4, PS_{HOAc} 8, OS_{HOAc} 8; *dhmA*-mutant PS_{NaOAc} 4, OS_{NaOAc} 8, PS_{HOAc} 6, OS_{HOAc} 8; *dmhB*-mutant OS_{NaOAc} 9, OS_{HOAc} 8.

1.2. Sugar analysis

The samples (0.5 mg each) were hydrolysed with 1 M CF_3CO_2H (120 °C, 2 h), the acid was removed by a stream of nitrogen, and the monosaccharides were conventionally reduced with $NaBH_4$, acetylated with Ac_2O in pyridine, and analysed by GLC on a Hewlett–Packard 5880 chromatograph and by GLC–MS on a Hewlett–Packard HP 5989A instrument (USA) using a HP-5 ms capillary column and a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C min^{-1}.

1.3. NMR spectroscopy

The samples were deuterium-exchanged by freeze-drying twice from 99.90% D_2O and then examined as solutions in 99.96% D_2O at 40 °C on a Bruker DRX-500 NMR spectrometer (Germany) using internal acetone (δ_H 2.225, δ_C 31.45) as the reference. 2D NMR spectra were obtained using standard Bruker software, and the 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.[19]

1.4. Mass spectrometry

Negative-ion electrospray ionization ion-cyclotron resonance Fourier transform mass spectrometry (ESI FT-ICR MS) was performed on a hybrid Apex Qe instrument (Bruker Daltonics, USA) equipped with an actively shielded 7 T magnet and an Appollo II Dual ESI/MALDI ion source. The mass spectra were acquired using standard experimental sequences as provided by the manufacturer. Mass scale was calibrated externally with Re-LPS of known structure. Samples (~10 ng μL^{-1}) were dissolved in a 50:50:0.001 (v/v/v) mixture of 2-propanol, water, and triethylamine pH ~8.5 and sprayed at a flow rate of 2 μL min^{-1}. The drying gas temperature was set to 200 °C. The Q-voltage in the Q-cell was set to 20 V for OS_{HOAc} and 4 V for OS_{NaOAc}. The spectra showing several charge states for each component were evaluated, including charge deconvolution, using DATAANALYSIS software version 3.4 (Bruker Daltonics, USA), and mass numbers given in the charge-deconvoluted spectra refer to the monoisotopic molecular masses.

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