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Structural studies on the R-type lipopolysaccharide of *Aeromonas hydrophila*

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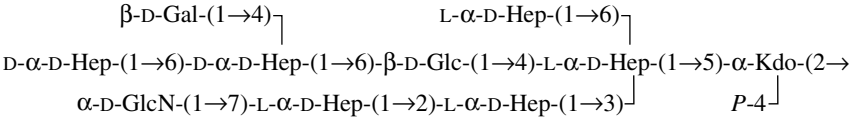
Abstract—A rough strain of *Aeromonas hydrophila*, AH-901, has an R-type lipopolysaccharide with the complete core. The following core structure was established by chemical degradations followed by sugar and methylation analyses along with ESIMS and NMR spectroscopy:



where D-α-D-Hep and L-α-D-Hep stand for D-*glycero*- and L-*glycero*-α-D-*manno*-heptose, respectively; Kdo stands for 3-deoxy-D-*manno*-oct-2-ulosonic acid; all monosaccharides are in the pyranose form; the degree of substitution with β-D-Gal is ~50%. Lipid A of the lipopolysaccharide has a 1,4′-bisphosphorylated β-D-GlcN-(1→6)-α-D-GlcN disaccharide backbone with both phosphate groups substituted with 4-amino-4-deoxyarabinose residues.
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Keywords: Lipopolysaccharide; Core structure; Lipid A backbone; *Aeromonas hydrophila*

1. Introduction

The ubiquitous water-borne bacteria of the genus *Aeromonas* are significant pathogens of poikilothermic animals, including amphibians, fish and reptiles.[1] They cause diseases that pose a serious problem for aquaculture, such as furunculosis in salmonid fish and motile aeromonad septicaemia in other freshwater species. Mesophilic aeromonads, including *A. hydrophila* (HG1 and HG3), *A. veronii* biovar sobria (HG8/10) and *A. caviae* (HG4), are most frequently associated with gastroenteritis and cause also bacteremia and septicemia in healthy and immunocompromised humans.[2,3] Being involved in adhesion to epithelial cells,[4] resistance to nonimmune serum[5] and virulence,[6] the lipopolysaccharide (LPS) plays an important role in pathogenesis of *Aeromonas* infections.

Recently, we have elucidated the structure of the O-polysaccharide (O-antigen) of the LPS of *A. hydrophila* O:34.[7] Strains of this serogroup are most common

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among mesophilic *Aeromonas* species,[8] accounting for 26.4% of all isolates, and have been documented as an important cause of infections in humans.[9,10] In addition to clinical specimens,[10] serogroup O:34 strains have been recovered from moribund fish.[11] In this paper, we report on the elucidation of the LPS core structure of *A. hydrophila* O:34 using a mutant strain, AH-901. This strain is unable to express the O:34-antigen owing to a miniTn5 Km-1 transposon insertion in *manC*, a gene of GDP-D-mannose pathway in the O:34 *wb* gene cluster. Since mannose is a component of the O:34-antigen and is not found in the LPS core, AH-901 has an R-type LPS with the complete core.

2. Results and discussion

The R-form LPS phenotype of *A. hydrophila* AH-901 was confirmed by SDS-PAGE (Fig. 1). The LPS was isolated from dried cells by the phenol–water procedure and degraded with dilute acetic acid to give an oligosaccharide mixture (**1**), which was isolated by GPC on Sephadex G-50. Mixture **1** showed complex ^{1}H and ^{13}C NMR spectra owing to the occurrence of a Kdo residue at the reducing end in multiple forms, including 4,7- or/and 4,8-anhydro forms, which influenced NMR signals of all monosaccharide residues. This heterogeneity resulted from partial elimination of the phosphate group from Kdo 4-phosphate present in the initial LPS. In addition, the spectra were complicated by the presence of a hexose in a nonstoichiometric amount. These conclusions followed from the ESIMS, which showed the presence of compounds **1a** and **1b** with molecular masses 1696.5 and 1858.6 Da, respectively (for structures of compounds **1**, see Fig. 2). Further studies confirmed this conclusion and showed that the additional hexose in the

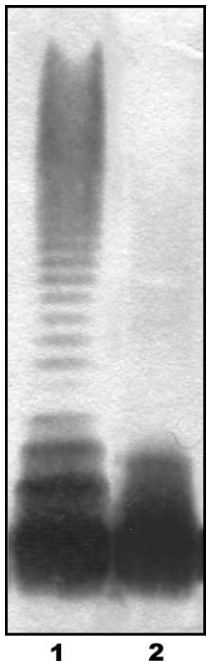

Figure 1. Silver-stained polyacrylamide gel electrophoresis of the LPS from *A. hydrophila* AH-3 wild type (lane 1) and *A. hydrophila* AH-901 mutant strain derived from AH-3 (lane 2).

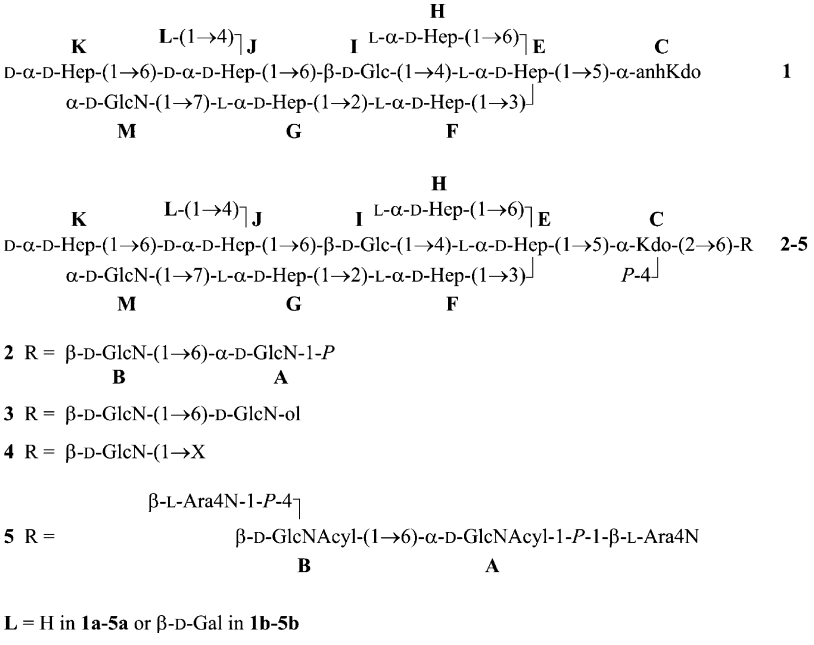

Figure 2. Structures of products of mild acid hydrolysis (**1**), strong alkaline degradation (**2**–**4**) and mild hydrazinolysis (**5**) from the LPS of *A. hydrophila*. All monosaccharides are in the pyranose form; D-α-D-Hep and L-α-D-Hep stand for D-*glycero*- and L-*glycero*-α-D-*manno*-heptose, respectively, anhKdo stands for an anhydro form of Kdo, GlcN-ol for 2-amino-2-deoxyglucitol, Acyl for 3-hydroxymyristoyl, and X for a GlcN **A** remainder of unknown structure.

b-series is galactose. The ^{1}H NMR spectrum of **1** showed neither signal for *N*-acetyl groups at $\delta \sim 2$ ppm, nor another *N*-acyl groups, and, hence, amino sugar(s) that are present in the core are not acylated.

Mixture **1** was N-acetylated and subjected to monosaccharide and methylation analyses. GC of the alditol acetates derived after full acid hydrolysis revealed Glc, Gal, GlcN, D-*glycero*- and L-*glycero*-D-*manno*-heptose (DDHep and LDHep) in the ratios 1:0.4:1:2.2:4.1, respectively. Upon the procedure, a part of LDHep was converted into a 1,6-anhydroheptitol derivative (compare published data[12]), which had a retention time 0.96 related to glucitol. It was assumed that the acetylated 1,6-anhydroheptitol has the same GC detector response factor as the acetylated heptitol (LDHep-ol), and the LDHep content was estimated as the total of both derivatives.

Methylation of N-acetylated **1**, followed by full acid hydrolysis and GC–MS analysis of the partially methylated monosaccharides as the acetylated alditols-1-*d*, resulted in identification of terminal Gal (**L**), 6-substituted Glc (**I**), terminal GlcNAc (**M**), terminal DDHep (**K**), 6-substituted (from **1a**) and 4,6-disubstituted (from **1b**) DDHep (**J**), terminal LDHep (**H**), 2-substituted (**F**), 7-substituted (**G**) and 3,4,6-trisubstituted (**E**) LDHep. Isomeric monosaccharides (Glc/Gal and DDHep/LDHep) were discriminated based on GC retention times of their derivatives.

In addition to the monosaccharides, acid hydrolysis of non-N-acetylated **1** gave a GlcN → Hep disaccharide, which was isolated using cation-exchange chromatography. Deamination of the disaccharide with nitrous acid, followed by borohydride reduction, afforded 2,5-anhydromannitol and LDHep-ol, which were identified by GC of the acetylated derivatives. Therefore, GlcN (**M**) in the core is attached to a LDHep residue (**G**, see below).

The LPS was N,O-deacylated by treatment with KOH in the presence of $NaBH_4$, and the products were fractionated by high-performance anion-exchange chromatography (HPAEC) on CarboPac PA1. No sharp peak was observed, only multiple smears of irregular shape. Further studies showed that the reason for the highly complex pattern was degradation of the lipid A moiety owing to substitution of both phosphate groups in the 1,4′-bisphosphorylated GlcN-(1 → 6)-GlcN (**B** → **A**) disaccharide backbone with 4-amino-4-deoxyarabinose (Ara4N). Under alkaline conditions, Ara4N 1-phosphate was eliminated from GlcN **B** and either Ara4N 1-phosphate or Ara4N from GlcN **A**, that is, phosphate is distributed between leaving Ara4N and retaining GlcN **A**. The products that retained 1-phosphate at GlcN **A** (e.g., **2**) were stable under alkaline conditions, whereas in other products GlcN **A** was either reduced with borohydride or destroyed to give **3** and **4**, respectively (Fig. 2). A deeper destruction, which affected both GlcN **A** and GlcN **B**, was observed when *Proteus* LPS having a similar lipid A backbone was subjected to strong alkaline degradation in the absence of borohydride.[13]

The LPS was O-deacylated by hydrazinolysis, and the products were analysed by ESIMS. The mass spectrum the O-deacylated LPS showed peaks of double- and triple-charged molecular ions for two compounds with the molecular masses 3153.0 and 2991.0 Da (**5a** and **5b**, respectively; for structures of compounds **5** see Fig. 2). This finding confirmed the composition of the core moiety and indicated that the lipid A moiety in **5** contains two N-linked 3-hydroxymyristoyl groups and two phosphate groups, which are both substituted with Ara4N. The last feature accounts for the behaviour of the LPS under the alkaline conditions described above.

ESIMS showed that there are two series of the strong alkaline degradation products **2**–**4** that differ in the absence (**a**-series) or presence (**b**-series) of the terminal galactose residue **L** (Table 1). **2b** as the individual compound and **2a** admixed with **2b** were isolated by preparative HPAEC on CarboPac PA1 and studied by 2D NMR spectroscopy.

The ^{1}H and ^{13}C NMR spectra of **2a** and **2b** (Table 2) were completely assigned using ^{1}H,^{1}H COSY, TOCSY, NOESY (Fig. 3), ^{1}H,^{13}C HSQC (Fig. 4), HMBC and HSQC-TOCSY experiments according to the published methodology.[14] Briefly, tracing connectivities in the TOCSY spectrum for the anomeric and some other protons standing apart from the main group of overlapping signals at δ 3.2–4.3 revealed spin systems of all 12 sugar constituents in each compound. When connectivities could not be further traced owing to interruption of the polarisation transfer, for example, from H-4 to H-5 of Gal or from H-5 to H-6 of Hep and Kdo, the correlations between the neighbouring protons were observed in the NOESY spectrum. The COSY spectrum

Table 1. ESIMS data of the strong alkaline degradation products **2**–**4**[a]

Compound	Lipid A backbone structure	L	Molecular mass (Da)
2a	β-D-GlcN-(1 → 6)-α-D-GlcN-1-*P*	H	2195.3
2b	β-D-GlcN-(1 → 6)-α-D-GlcN-1-*P*	β-D-Gal	2357.6
3a	β-D-GlcN-(1 → 6)-D-GlcN-ol	H	2118.0
3b	β-D-GlcN-(1 → 6)-D-GlcN-ol	β-D-Gal	2280.1
4a	β-D-GlcN-(1 → X	H	2058.7
4b	β-D-GlcN-(1 → X	β-D-Gal	2220.8

[a]For full structures of **2**–**4** see Figure 2.

Table 2. ^{1}H and ^{13}C NMR data of **2** (δ)[a]

Unit	Nucleus	Atom								
		1	2 (3ax)	3 (3eq)	4	5	6	7 (6b)	8a (7b)	8b
GlcN **A**	^{1}H	5.72	3.43	3.93	3.48	4.15	3.90	4.28		
	^{13}C	91.9	54.6	70.0	70.3	73.3	69.5			
GlcN **B**	^{1}H	4.93	3.10	3.68	3.55	3.67	3.62	3.62		
	^{13}C	99.7	56.3	72.7	70.5	75.1	62.1			
Kdo **C**	^{1}H		1.98	2.28	4.60	4.28	3.73	3.77	3.66	3.96
	^{13}C		100.2	35.0	70.4	70.8	72.6	69.5	64.2	
LDHep **E**	^{1}H	5.22	4.19	3.96	4.28	4.29	4.15	4.07	4.14	
	^{13}C	99.4	70.4	72.8	74.0	73.2	78.2	61.8		
LDHep **F**	^{1}H	5.74	4.16	3.90	3.89	3.64	4.11	3.69	3.78	
	^{13}C	99.0	80.4	70.4	67.2	71.9	69.1	63.7		
LDHep **G**	^{1}H	5.14	4.03	3.90	3.77	3.85	4.25	3.75	3.95	
	^{13}C	101.8	71.1	71.1	66.7	72.7	69.4	69.8		
LDHep **H**	^{1}H	5.44	4.11	3.89	3.88	3.83	4.02	3.72	3.80	
	^{13}C	99.2	71.1	71.2	66.9	72.6	69.6	63.5		
Glc **I**	^{1}H	4.57	3.49	3.46	3.63	3.52	3.81	4.05		
	^{13}C	103.3	73.9	77.7	70.0	74.8	65.3			
DDHep **J** (**2a**)	^{1}H	4.95	4.10	3.90	3.80	3.79	4.14	3.81	3.91	
	^{13}C	99.7	70.2	71.8	67.8	70.2	76.9	60.9		
DDHep **J** (**2b**)	^{1}H	4.97	4.17	3.90	3.98	4.04	4.31	3.83	3.98	
	^{13}C	99.4	69.8	70.3	77.5	70.2	75.4	60.6		
DDHep **K**	^{1}H	5.04	3.93	3.85	3.79	3.98	4.02	3.74	3.81	
	^{13}C	98.3	70.6	71.3	67.7	73.7	72.7	62.6		
Gal **L**	^{1}H	4.49	3.57	3.68	3.95	3.77	3.78	3.83		
	^{13}C	103.5	71.4	73.1	69.1	76.1	61.6			
GlcN **M**	^{1}H	5.09	3.43	3.89	3.59	3.67	3.82	3.89		
	^{13}C	95.3	54.7	70.6	69.9	73.8	61.1			

[a]Chemical shifts of units **A**–**I** and **K**–**M** are given for **2a** and are essentially the same in **2b**.

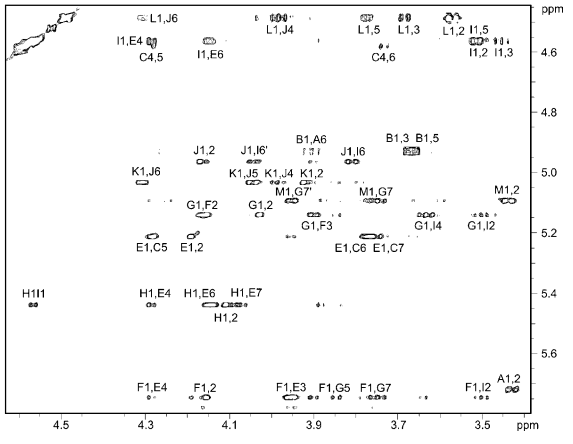

Figure 3. Part of a NOESY spectrum showing correlations for anomeric protons of compound **2b**. Arabic numerals refer to protons in sugar residues denoted by letters as shown in Table 2 and Figure 2.

enabled differentiation between protons within each spin system.

Monosaccharide residues were identified based on the ^{1}H and ^{13}C NMR chemical shifts and $^{3}J_{H,H}$ coupling constants of sugar-ring protons, which were in agreement with published data of the respective pyranosides.[15,16] GlcN was distinguished from Glc by correlation of the proton at the nitrogen-bearing carbon (H-2) to the corresponding carbon (C-2) at δ 3.43/54.7. The position of C-6 of Hep **H** at δ 69.6, Hep **F** at δ 69.1 and Hep **K** at δ 72.7 indicated the L-*glycero*-D-*manno* configuration of units **H** and **F** (LDHep) and the D-*glycero*-D-*manno* configuration of unit **K** (DDHep).[17] Three other heptose residues were substituted at either O-6 (units **E** and **J**) or O-7 (unit **G**), and the C-6 chemical shift could not be used for determination of the configuration at C-6. However, Hep **G** was identified as LDHep by isolation and cleavage of a GlcN → Hep disaccharide, and Hep **E** and Hep **J** were identified as LDHep and DDHep, respectively, based on methylation analysis data (see above). The NMR spectra of **3** and **4** were similar to those of **2**, except for the signals of the lipid A backbone.

The monosaccharide sequence in **2** was determined by NOESY (Fig. 3) and HMBC experiments, which revealed the respective transglycosidic correlations (Table 3). In addition to intense cross-peaks between the anomeric protons and protons at the linkage carbons, the NOESY spectrum showed a number of other, medium to strong cross-peaks between protons of sugar residues that are not linked to each other, including Hep **G** H-1,Hep **J** H-2; Hep **G** H-1,Hep **I** H-2; Hep **G** H-1,Hep **I** H-4 and Hep **J** H-1,Hep **F** H-2 cross-peaks. These data indicated that the core oligosaccharide has a tightly packed spatial structure.

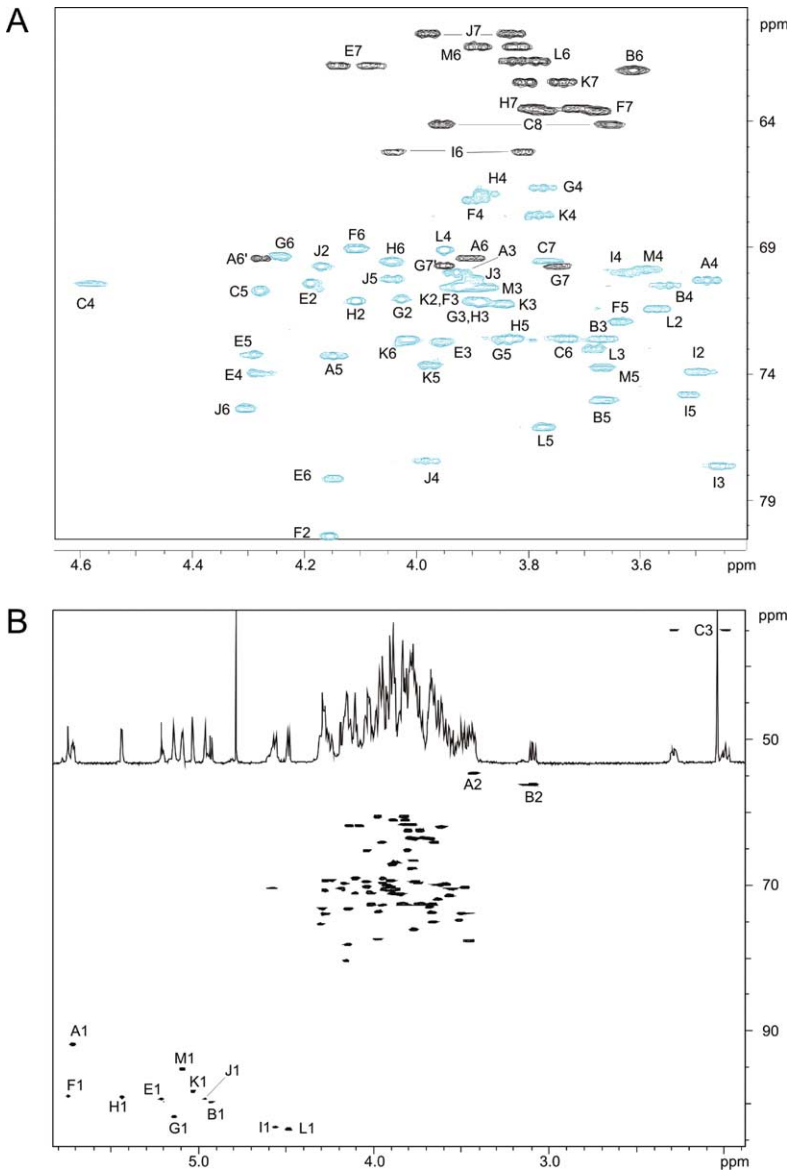

Figure 4. A part of the 2D $^{1}H,^{13}C$ HSQC spectrum of **2b** showing correlations for nonanomeric oxygen bearing carbons (A) and the full ^{1}H NMR and 2D $^{1}H,^{13}C$ HSQC spectra of **2b** (B). In part A correlations for CH and CH_2 groups are shown in blue and black, respectively. Arabic numerals refer to atoms in sugar residues denoted by letters as shown in Table 2 and Figure 2.

Table 3. Homonuclear (NOESY) and heteronuclear ($^{1}H,^{13}C$ HMBC) interresidue connectivities for the anomeric protons in **2a**

H-1 of sugar residue	Correlations	
	NOE	HMBC
GlcN **B**	**A** H-6	**A** C-6
L-D-Hep **E**	**C** H-5, **C** H-6, **C** H-7	**C** C-5
L-D-Hep **F**	**E** H-3, **E** H-4, **G** H-5, **G** H-7, **I** H-2	**E** C-3
L-D-Hep **G**	**F** H-2, **F** H-3, **I** H-2, **I** H-4	**F** C-2
L-D-Hep **H**	**E** H-4, **E** H-6, **E** H-7, **I** H-1	**E** C-6
Glc **I**	**E** H-4, **E** H-6	**E** C-4
D-D-Hep **J**	**I** H-6	**I** C-6
D-D-Hep **K**	**J** H-4, **J** H-5, **J** H-6	**J** C-6
Gal **L**	**J** H-4, **J** H-6	**J** C-4
GlcN **M**	**G** H-7	**G** C-7

The ^{31}P NMR spectrum of **2** showed signals for two phosphate groups at δ 4.2 and 3.7. The position of these groups at O-1 of GlcN **A** and O-4 of Kdo **C** was determined by a 2D ^{1}H, ^{31}P HMQC spectrum, which showed GlcN **A** H-1,P-1 and Kdo **C** H-4,P-4 correlations at δ 5.72/4.2 and 4.60/3.7, respectively.

Based on the data obtained, it was concluded that compounds **2**–**4** have structures depicted in Figure 2. These data, combined with the data of mild acid degradation products **1**, enabled determination of the full structure of the LPS core of *A. hydrophila* AH-901 as shown in Figure 5. It shows both similarities to and differences from the structure of the LPS core of *A. hydrophila* A6 reported earlier.[18]

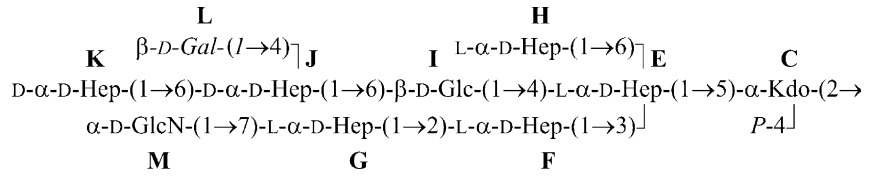

Figure 5. Structure of the LPS core of *A. hydrophila*. All monosaccharides are in the pyranose form; the galactose residue **L** present in a non-stoichiometric amount is shown in italics.

3. Experimental

3.1. Bacterial strain and growth

A. hydrophila mutant strain AH-901 was derived from the wild type AH-3 (serogroup O:34) after miniTn5Km1 mutagenesis.[19] AH-901 possesses a unique miniTn5 transposon inserted in a gene named *manC*, which showed high levels of both predicted amino acid identity and similarity to different GDP-D-mannose pyrophosphorylases catalysing synthesis of GDP-D-mannose from D-mannose 1-phosphate. Since mannose is one of the sugar components of the O34-antigen of *A. hydrophila*,[7] the mutation in this gene implies production of an R-type LPS. This is in agreement with the R-LPS phenotype of mutant strain AH-901. Bacteria were routinely grown on tryptic-soy-broth at 30 °C,[20] that is, under conditions resulting in a better yield of the LPS compared to cultivation on agar.[21]

3.2. Isolation of the lipopolysaccharide

Bacterial cells (6 g dried weight) were digested with DNAse, RNAse (24 h, 1 mg g^{-1} each) and Proteinase K (36 h, 1 mg g^{-1}) in water (60 mL) at 37 °C with stirring; the suspension was dialysed against distilled water and freeze-dried. Digested cells were extracted with aq 45% phenol at 68 °C,[22] the extract was dialysed against tape water without separation of the layers and, after removal of residual cells by centrifugation, freeze-dried to give the LPS (320 mg).

3.3. O-deacylation of the lipopolysaccharides

The LPS (20 mg) was treated with anhyd hydrazine (1 mL) for 1 h at 50 °C. A slightly higher temperature of hydrazinolysis than recommended (37 °C[23]) was applied to achieve full O-deacylation of the lipid moiety. The cooled mixture was poured into cooled acetone (200 mL) under stirring, the precipitated material was collected by centrifugation, dissolved in water and lyophilised to yield O-deacylated LPS (15 mg).

3.4. Mild acid degradation of the lipopolysaccharides

The LPS of each strain (50 mg) was treated with aq 2% HOAc (100 °C, 2 h), the precipitate was removed by centrifugation, and the supernatant was fractionated by GPC on a column (80 × 2.5 cm) of Sephadex G-50 (S) (Amersham Biosciences, Sweden) using pyridinium acetate buffer (4 mL pyridine and 10 mL concd HOAc in 1 L water) as the eluant and a Waters differential refractometer (USA) for monitoring to give core oligosaccharides **1** (15 mg) and a monosaccharide fraction, which was not studied further.

3.5. Partial acid hydrolysis and deamination

Mixture **1** was hydrolysed with 3 M CF_3CO_2H (100 °C, 3 h), and the products were absorbed on a column (5 × 0.5 cm) of Dowex 50 × 8 (H^+). Neutral compounds were eluted with water, and then a GlcN → LD-Hep disaccharide was eluted with 5% aq ammonia. The disaccharide was deaminated with 5% aq $NaNO_2$ in 10% aq AcOH (25 °C, 1 h), desalted using a Dowex 50 × 8 (H^+) resin, lyophilised, reduced with $NaBH_4$ in water (25 °C, 30 min), the excess of $NaBH_4$ was destroyed with concd AcOH, boric acid was removed by co-evaporation with MeOH (2 × 1 mL), the products were acetylated with Ac_2O (0.5 mL, 100 °C, 20 min), dried and analysed by GC–MS on an HP Ultra 1 column (25 m × 0.3 mm) using a Varian Saturn 2000 instrument (USA) equipped with an ion-trap MS detector.

3.6. Strong alkaline treatment of the lipopolysaccharides

The LPS (100 mg) was deacylated with 4 M KOH (4 mL) in the presence of $NaBH_4$ (20 mg) at 100 °C for 16 h. After workup,[23] the products were fractionated by HPAEC on a CarboPac PA1 column (Dionex, USA; 250 × 9 mm) using a linear gradient of 0.1 → 0.8 M NaOAc in 0.1 M NaOH at flow rate 3 mL min^{-1} for 1 h. Fractions were combined into four pools and desalted by GPC on a column (1.6 × 80 cm) of Sephadex G-15.

3.7. Monosaccharide and methylation analyses

Mixture **1** was N-acetylated with Ac_2O (0.1 mL) in satd aq $NaHCO_3$ (1 mL) in the presence of an excess of solid $NaHCO_3$ with stirring for 30 min, and the products were isolated by GPC on Sephadex G-15.

For monosaccharide analysis,[24] a sample (1 mg) was hydrolysed with 4 M CF_3CO_2H (100 °C, 2 h), dried under stream of nitrogen and reduced with $NaBH_4$. After adding concd AcOH and MeOH (2 × 1 mL), the

sample was dried, acetylated with Ac_2O (0.5 mL, 100 °C, 20 min), dried and analysed by GC–MS as above.

Methylation was performed according to the procedure of Ciucanu and Kerek.[25] The methylated substance was recovered by extraction using a chloroform–water system, and the partially methylated monosaccharides were released by hydrolysis with 3 M CF_3CO_2H (100 °C, 2 h), reduced with $NaBD_4$ to the corresponding alditols-1-*d*, acetylated and analysed by GC–MS as above.

3.8. NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded using a Varian UNITY/Inova 500 spectrometer (USA) in D_2O solutions at 25 °C with acetone standard (δ 2.225 for ^1H and 31.5 for ^{13}C) using standard pulse sequences COSY, TOCSY (mixing time 120 ms), NOESY (mixing time 300 ms), ^1H,^{13}C HSQC, gHMBC (optimised for 5 Hz coupling constant) and HSQC–TOCSY (mixing time 80 ms). Spectra were assigned with the help of a computer program PRONTO.[26]

3.9. Mass spectrometry

ESIMS was performed in the negative-ion mode using a Micromass Quattro spectrometer with direct injection in aq 50% acetonitrile with 0.2% HCO_2H at a flow rate of 15 $\mu L\,min^{-1}$.

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