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Structure of the exopolysaccharide produced by *Enterobacter amnigenus*

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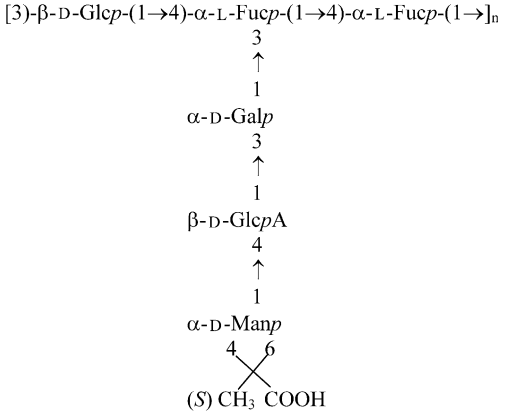
Abstract—The bacterial species *Enterobacter amnigenus* was isolated from sugar beets harvested in Finland. It produced an exopolysaccharide rich in L-fucose, which gave viscous water solutions. Its primary structure was determined mainly by NMR spectroscopy and ESIMS of oligosaccharides and a polysaccharide with decreased molecular weight, obtained by Smith degradation of the O-deacetylated native polymer.



Keywords: *Enterobacter amnigenus*; L-fucose; Polysaccharide structure; NMR spectroscopy; Mass spectrometry

1. Introduction

Some microbial polysaccharides, in addition to their thickening, gelling or emulsifying properties, offer an alternative source to some rare sugars, thus offering a

Abbreviations: L-4dThr, 4-deoxy-L-threitol; ISV, ionspray voltage; OR, orifice potential.

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controllable way to manufacture required amount of unusual monosaccharides such as L-fucose (6-deoxy-L-galactose). L-Fucose and its oligosaccharides have potential applications in the medical field due to their anticancer and anti-inflammatory effects. Polysaccharides rich in L-fucose can be also used as skin moisturising agents in the cosmetic industry. The chemical synthesis of L-fucose is laborious and suffers from low yield; direct extraction from brown algae is costly and subject to seasonal variation.[1]

Fucose is present in a wide variety of organisms. In mammals, fucose-containing glycans have important roles in blood transfusion reactions, selectin-mediated leucocyte-endothelial adhesion, host–microbe interactions and numerous ontogenic events including signaling events by the Notch receptor family. Alterations in the expression of fucosylated oligosaccharides have also been observed in several pathological processes, including cancer and atherosclerosis.[2]

The major pathway for the biosynthesis of L-fucose in prokaryotic cells involves conversion of GDP-D-mannose to GDP-L-fucose, which in turn is the fucosyl donor for polysaccharides, glycoproteins and glycolipids.[3]

In a former survey, the slime production of 600 microorganisms from sugar beets collected from different parts of Finland was studied.[4] One hundred and seventy of them produced exopolysaccharides (EPS), of which 35% were heteropolysaccharides, although previously it was believed that 95% of polysaccharides produced by sugar beet spoilage organisms were homopolysaccharides, like levan or dextran. One of the isolates was identified as *Enterobacter amnigenus*, which produced, at the optimum temperature of 30 °C, a heteropolymer containing D-glucose, D-galactose, L-fucose and D-mannose.

In this report, the determination of the primary structure of *E. amnigenus* EPS is described.

2. Experimental

2.1. Bacterial culture and EPS purification

EPS production was carried out in a 10 dm^3 Biostat® E bioreactor (B. Braun Biotech International, Germany) in 6 dm^3 growth vol. Growth medium consisted of 10 g/L yeast extract (LAB M, MC1), 20 g/L bactopeptone (LAB M, MC24) and 80 g/L (inoculum 40 g/L) sucrose (BHD), 11.8 g/L $K_2HPO_4{\cdot}3H_2O$ (Merck), 3 g/L KH_2PO_4 (Riedel-de Haen) and 2 g/L $MgSO_4{\cdot}7H_2O$ (Merck). The inoculum was prepared in 250 mL shake flasks using 50 mL growth vol. Growth medium was inoculated with *E. amnigenus BPT 165* (laboratory collection in Helsinki University of Technology). Shake flask cultures were grown 6 h in a Certomat® HT (B. Braun Biotech International, Germany) shaker cabin at 32 °C and 150 rpm. The inoculum (300 mL) was added to the bioreactor. Aeration rate (1 vvm), growth temperature, foam level, dissolved oxygen tension (DOT) and pH were measured and/or controlled by the bioreactor control unit. Broth viscosity was measured using a Brookfield DVII+ viscometer with a small sample adapter. Cultivation was stopped after viscosity had reached its maximum. Proteins from the cultivation broth were hydrolysed using alkaline protease (Alcalase, Novo). Treatment with this protease resulted in removal of most of the acetyl groups. Therefore, the EPS was O-deacetylated completely with 0.01 M NaOH at room temperature for 5 h[5] and subsequent structural determinations were performed on this material. Diafiltration against RO water (Pellicon Mini with Biomax 1000, Millipore Inc.) was used to remove amino acids, cultivation metabolites and impurities to get the crude polysaccharide. The polymer was precipitated in 5 vol of cold acetone, and after solvent evaporation, it was recovered by lyophilisation.

2.2. Analytical procedures

Analytical GLC was performed on a Perkin Elmer Autosystem XL gas chromatograph equipped with a flame ionisation detector and an SP2330 capillary column (Supelco, 30 m), using He as the carrier gas. The following temperature programs were used: for alditol acetates, 200–245 °C at 4 °C/min; for methylated alditol acetates, 150–250 °C at 4 °C/min. GLC–MS analyses were carried out on a Hewlett–Packard 5890 gas chromatograph coupled to a Hewlett–Packard 5971 mass selective detector. Separation of trimethylsilylated methyl glycosides and of trimethylsilylated (+)-2-butyl glycosides was obtained on a HP1 column (Hewlett–Packard, 50 m).

2.3. Composition analysis

Hydrolysis of the EPS was carried out with 2 M trifluoroacetic acid at 125 °C for 1 h, while hydrolysis of oligosaccharides was performed at 100 °C for 6 h. Alditol acetates were prepared as previously described.[6] Methanolysis was performed with 1 M HCl in MeOH (Supelco) at 85 °C for 18 h according to Dudman et al.[7] Trimethylsilyl derivatives were obtained incubating the mixture of methyl glycosides with the Sylon HTP kit (3:1:9 HMDS + TMCS + pyridine, Supelco) at room temperature for 1 h. The products were dried under a stream of N_2, dissolved in *n*-hexane and centrifuged to remove insoluble materials. The clear supernatant was subjected to GLC analysis. The absolute configuration of the sugar residues was established via GLC analysis of the derived trimethylsilylated (+)-2-butyl glycosides.[8,9]

2.4. Methylation analysis and related experiments

Methylation of polysaccharide was performed according to the modified Hakomori method[10] using potassium methylsulfinyl-methanide.[11] Reduction of the methyl ester groups was achieved with LiAlD4.[12] Oligosaccharides were methylated according to Dell.[13] The permethylated samples were hydrolysed, derivatised into alditol acetates and analysed by GC–MS. Molar ratio values were corrected by use of effective carbon-response factors.[14]

2.5. Smith degradation

The O-deacetylated polysaccharide (51 mg) was treated with $NaIO_4$ at 4 °C for 7 days.[15,16] After reduction of the aldehyde groups with $NaBH_4$ and dialysis, the sample was treated with 0.05 M TFA at room temperature for 7 days. The soln was then evaporated to dryness; the dried residue was dissolved in 3 mL of water and subjected to gel filtration chromatography on two Biogel P2 columns in series (1.6 cm id × 90 cm). Water was used as eluent. One fraction (# 47), eluting as a single sharp peak at about 35 h, contained only one component, as revealed by ESIMS analysis. All the fractions belonging to that peak were then pooled together (the sample was named **SD-4**), and subjected to ESIMS, NMR spectroscopy and methylation analysis.

At the void vol, and adjacent to it, three overlapping peaks eluted; assuming that the hydrolysis was not complete, their fractions were pooled and hydrolysed again with 0.05 M TFA for 8 days at room temperature. The soln was taken to dryness and separated again on the same chromatographic system. The chromatogram showed a low shoulder at the void vol and two partially overlapping peaks after it, the most intense of which and with a higher MW was isolated. ESIMS showed that it contained two oligosaccharides, **SD-5** and **SD-6**. The latter was purified by repeated size exclusion chromatography on a Biogel P10 column (1.6 cm id × 90 cm) using water as eluent. **SD-6** was then analysed by NMR spectroscopy.

2.6. Electrospray mass spectrometry

Mass spectra were recorded on a API-I PE SCIEX quadrupole mass spectrometer equipped with an articulated ion spray connected to a syringe pump for the injection of the samples. The instrument was calibrated using a polypropylene glycol mixture (3.3×10^{-5} M polypropylene glycol $M_n = 425$, 1×10^{-4} M polypropylene glycol $M_n = 1000$ and 2×10^{-4} M polypropylene glycol $M_n = 2000$), 0.1% MeCN and 2 mM ammonium formate in 50% aq MeOH.

Oligosaccharides were dissolved in 50% aq MeCN, 0.13×10^{-3} M ammonium acetate. The spectra were recorded in the positive ion mode, using an injection flow rate of 5 μL/min, with a ionspray voltage (ISV) of 5000 V and an orifice potential (OR) of 50 V, and using a step size of 0.1 amu. When fragmentation was needed, the OR was raised to a higher voltage value.

2.7. Preparation of an EPS sample for NMR spectroscopy

The native and O-deacetylated EPS formed water solutions characterised by rather high viscosity. For this reason, the O-deacetylated EPS was selectively degraded by Smith degradation prior to NMR analysis. After $NaIO_4$ oxidation using a molar ratio of $NaIO_4$/repeating unit of 0.05 and an incubation time of 1 h, the sample was processed as described.[15,16] The degraded polymer was then evaporated to dryness, dissolved in 3.5 mL of $NaNO_3$ 0.05 M and subjected to gel filtration chromatography on a Sephacryl S-400 column. The sample eluted as a single broad peak, of which only the central part was used for NMR spectroscopic analysis (sample **SD-EPS**), after desalting.

2.8. NMR spectroscopy

Samples to be analysed were exchanged three times with 99.9% D_2O by lyophilisation and finally dissolved in 0.7 mL 99.96% D_2O. Spectra were acquired at 50 °C on a VARIAN^{UNITY} INOVA spectrometer operating at 500 MHz (^1H). 2D experiments were performed using standard VARIAN pulse sequences and pulsed field gradients for coherence selection when appropriate. TOCSY spectra were acquired for each sample with three mixing times: 30, 80 and 140 ms. The ROESY and NOESY spectra used mixing times of 400 and 100 ms, respectively.

3. Results and discussion

3.1. Composition and methylation analysis of the EPS

The EPS was hydrolysed and the neutral sugars were converted to alditol acetates; GLC analysis gave fucose, mannose, galactose and glucose in the molar ratio 1.5:0.2:1.1:1.0. GLC analysis of the trimethylsilyl methyl glycosides revealed the presence of glucuronic acid, besides the neutral sugars listed above. The absolute configuration was found to be L for the fucose residues and D for all the other sugars.

Methylation analysis showed the presence of 4-substituted fucopyranose, 3,4-disubstituted fucopyranose, 3-substituted glucopyranose, 3-substituted galactopyranose and 4,6-disubstituted mannopyranose in the molar ratio 0.83:1.00:0.96:0.63:0.45.

3.2. Oligosaccharide SD-4

SD-4 obtained by Smith degradation of the EPS, was subjected to ESIMS analysis. The molecular ions at *m*/*z* 594.3, 599.2 and 615.2 corresponded to the ammonium, sodium and potassium adducts, respectively, of an oligosaccharide composed of two hexose residues, one fucose residue and 4-deoxy-threitol. The latter residue derived from oxidation of the 4-substituted fucose and therefore, had the L absolute configuration. The sample was subjected to fragmentation by increasing the orifice potential. The fragments identified (Table 1) indicated the sequence Hex-Fuc-L-4dThr. Since no fragment Hex-Hex was detected and considering that the 4-deoxy-threitol could only originate from the 4-substituted fucose, it followed that the other Hex residue is linked to the Fuc residue to give a branched tetrasaccharide.

Methylation analysis of **SD-4** gave terminal glucose, terminal galactose and 3,4-disubstituted fucose in the molar ratio 1.24:1.00:0.39. The low relative molar ratio of the 3,4-disubstituted fucose might be ascribed to its labile character with respect to the hexoses.

Table 1. Assignment of the ions obtained upon fragmentation of **SD-4**

Ions (*m*/*z*)	Proposed sequence
162.8	$[Hex]^+$
253.1	$[Fuc\text{-}4dThr + H]^+$
309.2	$[Hex\text{-}Fuc]^+$
415.2	$[Hex\text{-}Fuc\text{-}4dThr + H]^+$
437.0	$[Hex\text{-}Fuc\text{-}4dThr + Na]^+$
493.2	$[Hex\text{-}(Hex)\text{-}Fuc\text{-}(-H_2O) + Na]^+$
599.2	$[Hex\text{-}(Hex)\text{-}Fuc\text{-}4dThr + Na]^+$
615.3	$[Hex\text{-}(Hex)\text{-}Fuc\text{-}4dThr + K]^+$

3.3. NMR analysis of oligosaccharide SD-4

SD-4 was subjected to 1D- and 2D NMR analysis. The ^1H NMR spectrum (Fig. 1A) showed three signals in the anomeric region attributed to two α- and one β-anomeric protons at 5.36, 5.10 and 4.58 ppm, respectively. The latter signal partially overlapped with the HOD

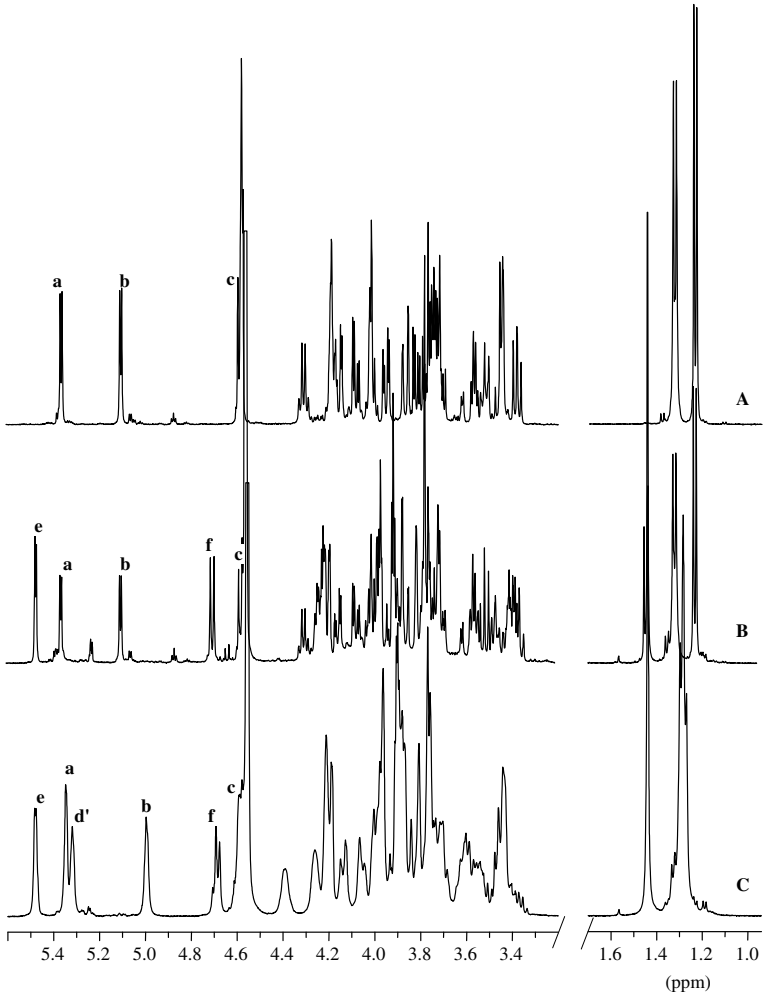

Figure 1. ^1H NMR spectrum of the oligosaccharides **SD-4** (A) and **SD-6** (B), and of the reduced molecular weight polysaccharide **SD-EPS** (C) recorded at 50 °C.

Table 2. ^{1}H and ^{13}C NMR chemical shifts of **SD-4**

Residue	Nucleus	Chemical shift (ppm)					
		1	2	3	4	5	6, 6′
Gal(α1- (**a**)	^{1}H	5.36	3.81	3.94	4.01	4.18	3.76, 3.72
	^{13}C	100.35	69.66	70.28	70.43	72.21	62.31
3,4)Fuc(α1- (**b**)	^{1}H	5.10	4.07	4.15	4.18	4.30	1.32
	^{13}C	100.96	69.82	73.68	79.83	68.43	16.29
Glc(β1- (**c**)	^{1}H	4.58	3.37	3.51	3.43	3.45	3.86–3.72
	^{13}C	103.90	74.66	76.45	70.39	76.97	61.72
4dThr (**d**)	^{1}H	3.77, 3.70	3.56	4.00	1.23		
	^{13}C	62.54	84.42	68.07	18.82		

The chemical shifts are given relative to internal acetone (2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C).

signal. They were named **a** to **c** starting from the signal resonating at the highest field value. The spin system of 4-deoxy-L-threitol was named **d**. In the high field region of the spectrum, there were two $-CH_3$ signals at 1.32 and 1.23 ppm.

The assignment of most of the proton chemical shifts for each spin system was achieved by inspecting the COSY and the TOCSY plots. The assignment of the ^{13}C resonances was obtained from inspection of the HSQC plot. The chemical shifts for each spin system are reported in Table 2. On the basis of the chemical shift values,[17,18] residue **a** was assigned to α-galactose, and residue **b** to α-fucose. Residues **c** and **d** were assigned, respectively, to β-glucose and 4-deoxy-L-threitol, based on the complete magnetisation transfer through their spin system in TOCSY spectra. Inspection of the HMBC plot confirmed some of the chemical shifts found for each sugar residue and showed interesting

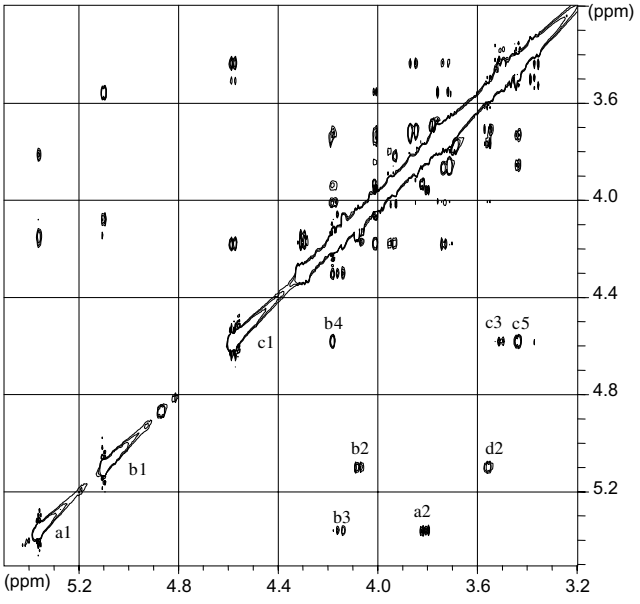

Figure 2. Part of a ROESY spectrum of the oligosaccharide **SD-4** recorded at 50 °C. For symbols used, see structure (**1**).

c **b** **d**
β-D-Glc*p*-(1→4)-α-L-Fuc*p*-(1→2)-L-4dThr
3
↑
a 1 **(1)**
α-D-Gal*p*

Chart 1. Proposed structure for oligosaccharide **SD-4**.

inter-residue connectivities. In particular, H-1 of residue **b** connected to the signal at 84.42 ppm belonging to C-2 of **d** (see structure **1**); H-1 of residue **c** connected to C-4 of residue **b**, thus establishing the sequence **c** → **b** → **d**. Regarding residue **a**, its ^{13}C chemical shifts indicated that none of its carbon atoms, except C-1 was involved in a glycosidic linkage, while in the HMBC plot its H-1 showed an inter-residue connection with C-3 of **b**. The ROESY spectrum in Figure 2 confirmed these sequence and linkage information. NOE contacts between H-4 of residue **b** and H-5 and H-6 of α-fucose (data not shown in Figure 2) proved that the spin system assignment of residues **a** and **b** to α-galactose and α-fucose based on the chemical shift of their anomeric protons was correct. All the data collected showed that **SD-4** has the primary structure **1** (Chart 1).

3.4. Oligosaccharides SD-5 and SD-6

The ESI mass spectrum showed four peaks at *m*/*z* 775.4, 797.3,1007.3 and 1029.4, that corresponded to the proton and sodium adducts of two different oligosaccharides of 774.4 and 1006.3 molecular weight. The former corresponded to an oligosaccharide composed of two Hex, one Fuc, one HexA and one 4-deoxy-L-threitol and was named **SD-5**. The latter contained the same residues as **SD-5** with, in addition, one more Hex and a 1-carboxyethylidene substituent and it was named **SD-6**. Therefore, the peak obtained by gel filtration chromatography was a mixture of two different oligosaccharides. The oligosaccharides were permethylated and subjected to ESIMS. The spectrum showed prominent ammonium adducts of two molecular species at *m*/*z* 966.5 (100%) and 1226.6 (30%), which corresponded to

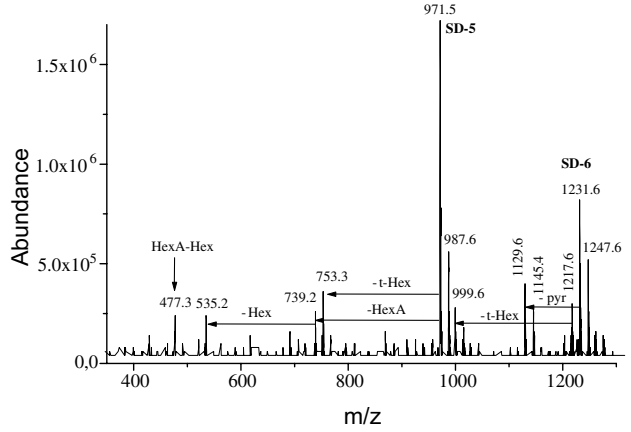

Figure 3. Mass spectrum of permethylated **SD-5** + **SD-6** recorded in the positive mode with OR = 180 V. Loss of fragments is indicated. t-Hex = terminal hexose residue; pyr = 1-carboxyethylidene.

the permethylated oligomers **SD-5** and **SD-6**. Moreover, the ammonium adduct at *m*/*z* 1124.7 (15%) was attributed to oligomer **SD-6** without the 1-carboxyethylidene methyl ester substituent, and with a dehydrated Hex at the non-reducing terminus. To obtain sequence information, spectra were recorded at OR 180 V (Fig. 3). Under these experimental conditions, the prominent ions observed corresponded to the sodium and potassium adducts of the two methylated oligomers and of the deacetalated **SD-6**. From the molecular ion at *m*/*z* 971.5, attributed to $[M+Na^+]$ of **SD-5**, fragmentation from the non-reducing end was observed: the loss of 232 amu corresponding to a t-HexA followed by a loss of 204 amu corresponding to a linked Hex residue. The same molecular ion lost also a fragment of 218 amu corresponding to a terminal hexose residue suggesting that the oligosaccharide **SD-5** is branched. No fragmentation was observed for **SD-6**, except for the peak at *m*/*z* 1217.6, probably caused by undermethylation, that lost a terminal hexose residue (−218). A fragment at *m*/*z* 477.3, corresponding to the dimer HexA-Hex, was also observed.

These data showed that oligosaccharides **SD-5** is branched and has a terminal non-reducing HexA, while **SD-6** has a terminal non-reducing Hex bearing a 1-carboxyethylidene substituent. Both of these oligosaccharides originated from incomplete diol oxidation of the O-deacetylated polymer.

The mixture of permethylated oligosaccharides was subjected to methylation analysis and the results are reported in Table 3. Two new derivatives of glucose, both C-6 dideuterated, were obtained from carboxyl reduction of the glucuronic acid: 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylglucitol. The former derived from a terminal non-reducing glucuronic acid, while the latter arose from a 4-substituted glucuronic acid. Therefore, it was deduced that, in the polymer, the glucuronic acid is 4-

Table 3. Methylation analysis of the mixture of **SD-5** + **SD-6**

Partially methylated alditol acetates[a]	*t*	Molar ratio	
		I	II
2,3,4,6-Glc	0.80	1.1	1.1
2-Fuc	0.93	1.4	1.1
2,4,6-Gal	1.00	1.0	1.0
2,3,4-Glc[b]	1.03		0.6
2,3-Glc[b]	1.24		0.3
2,3-Man	1.25	0.2	0.2

Retention times are relative to 2,4,6-Gal. Molar ratio values were corrected by use of effective carbon-response factors.[10] I, methylated oligomers; II carboxy-reduced, methylated oligomers.
[a] 2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, etc.
[b] C-6 dideuterated according to GLC/MS.

β-D-Glc*p*-(1→4)-α-L-Fuc*p*-(1→2)-L-4dThr
3
↑
1
α-D-Gal*p* **(2)**
3
↑
1
β-D-Glc*p*A

Chart 2. Proposed structure for oligosaccharide **SD-5**.

c **b** **d**
β-D-Glc*p*-(1→4)-α-L-Fuc*p*-(1→2)-L-4dThr
3
↑
1
α-D-Gal*p* **a** **(3)**
3
↑
1
β-D-Glc*p*A **f**
4
↑
1
α-D-Man*p* **e**
4 6
CH$_3$ COOH

Chart 3. Proposed structure for oligosaccharide **SD-6**.

substituted. At the same time, comparison with the methylation analysis data of **SD-4** indicated that galactose is in the side chain, while the glucose residue is in the main chain. The primary structures of oligosaccharides **SD-5** and **SD-6** are reported in formula **2** (Chart 2) and **3** (Chart 3), respectively.

3.5. NMR analysis of oligosaccharide SD-6 and of SD-EPS

The mixture of **SD-5** and **SD-6** was subjected to gel filtration chromatography on Biogel P10, yielding pure **SD-6**. The ^{1}H NMR spectrum of **SD-6** is shown in Figure 1B. In the anomeric region, besides the signals due

Table 4. ^{1}H and ^{13}C NMR chemical shifts of **SD-6**

Residue	Nucleus	Chemical shift (ppm)					
		1	2	3	4	5	6, 6′
Man(α1- (**e**)	^{1}H	5.47	3.92	3.89	4.18	3.80	3.99, 3.85
	^{13}C	100.30	68.72[a]	69.17[a]	72.23	63.58	65.85
Pyr[b]	^{1}H	—	—	1.44	—	—	—
	^{13}C	176.80	101.48	26.06	—	—	—
-3)Gal(α1- (**a**)	^{1}H	5.36	3.97	3.96	4.20	4.23	3.73, 3.69
	^{13}C	100.94	68.64	80.31	70.26	71.90	62.47[c]
-3,4)Fuc(α1- (**b**)	^{1}H	5.10	4.07	4.15	4.22	4.30	1.32
	^{13}C	100.26	69.80	73.64	79.13	68.39	16.34
-4)GlcA(β1- (**f**)	^{1}H	4.70	3.46	3.77	3.77	3.76	—
	^{13}C	104.64	74.18	77.5[d]	78.13[d]	76.96	175.73
Glc(β1- (**c**)	^{1}H	4.57	3.38	3.51	3.35	3.41	3.88, 3.55
	^{13}C	103.58	74.71	76.36	70.73	77.5	62.50[c]
-2)-L-4dThr (**d**)	^{1}H	3.76, 3.70	3.55	4.00	1.23	—	—
	^{13}C	62.53	84.41	68.07	18.81	—	—

The chemical shifts are given relative to internal acetone (2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C).
[a] May be interchanged.
[b] Pyr = 1-carboxyethylidene.
[c] May be interchanged.
[d] May be interchanged.

to α-Gal (**a**), α-Fuc (**b**) and β-Glc (**c**) at 5.36, 5.10 and 4.57 ppm already present in the spectrum of **SD-4**, two other resonances are present at 5.47 (**e**) and 4.70 (**f**) ppm; these signals were assigned to α-Man and β-GlcA. The structure of **SD-6** was also determined by inspection of the COSY, TOCSY and HSQC spectra, which led to the assignment of all the ^{1}H and ^{13}C resonances (Table 4). The ROESY spectrum in Figure 4 confirmed the monosaccharide sequence and linkage position deduced from the structure of **SD-4**, the methylation analysis and the ESIMS fragmentation. The ^{13}C signal of the methyl group of the pyruvate 4,6-*O*-linked to mannose was found at 26.06 ppm, consistent with the *S* absolute configuration.[19]

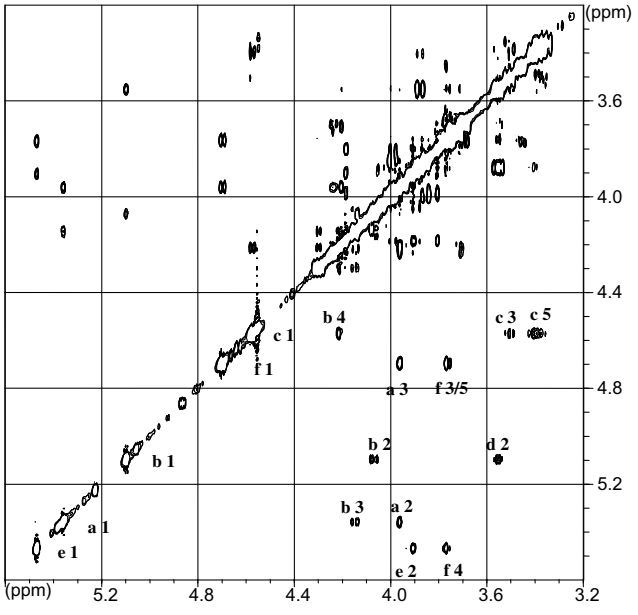

Figure 4. Part of a ROESY spectrum of the oligosaccharide **SD-6** recorded at 50 °C.

Given the monosaccharide composition and the methylation analysis data of the polysaccharide, structure **3** represents the repeating unit of the EPS from *E. amnigenus*, threitol being the remnant of the 4-substituted fucose residue. The only missing parameter for the complete elucidation of the EPS primary structure is the anomeric configuration of the fucose linked to glucose. In order to get this information, we analysed the NMR spectra of the reduced molecular weight **SD-EPS** which, compared to the native samples, gave

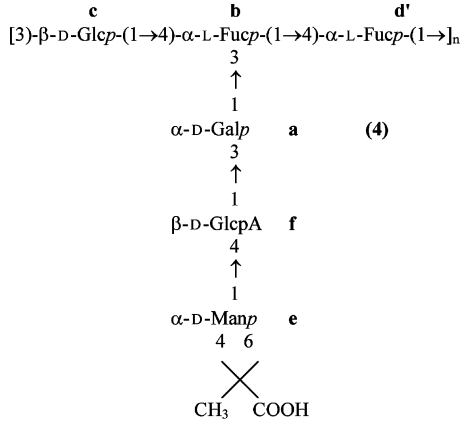

Chart 4. Structure of the *E. amnigenus* EPS repeating unit.

Table 5. ^{1}H and ^{13}C NMR chemical shifts of **SD-EPS**

Residue	Nucleus	Chemical shift (ppm)					
		1	2	3	4	5	6, 6′
Man(α1- (**e**)	^{1}H	5.48	3.92	3.92	4.19	3.82	4.01, 3.87
	^{13}C	100.19	68.71[a]	69.16[a]	72.22	63.57	65.85
Pyr[b]	^{1}H	—	—	1.44			
	^{13}C	176.80	101.48	26.06			
-3)Gal(α1- (**a**)	^{1}H	5.35	3.97	3.97	4.21	4.26	3.74, 3.70
	^{13}C	100.33	68.62	80.57	70.30	71.87	62.46
-4)Fuc(α1- (**d′**)	^{1}H	5.32	3.88	4.00	3.88	4.39	1.27
	^{13}C	100.19	69.16	69.88	80.87	68.18	16.09
-3,4)Fuc(α1- (**b**)	^{1}H	5.00	4.06	4.13	4.22	4.58	1.29
	^{13}C	101.24	69.99	73.66	79.36	68.48	16.27
-4)GlcA(β1- (**f**)	^{1}H	4.69	3.46	3.76	3.77	3.76	—
	^{13}C	104.74	74.17	77.42	77.96	76.97	175.71
-3)Glc(β1- (**c**)	^{1}H	4.59	3.59	3.62	3.44	3.41	3.88, 3.55
	^{13}C	103.51	75.39	82.76	77.42	77.42	62.33

The chemical shifts are given relative to internal acetone (2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C).
[a] May be interchanged.
[b] Pyr = 1-carboxyethylidene.

narrower signals. Its ^{1}H NMR spectrum in D_2O at 50 °C is shown in Figure 1C. In the anomeric region, six signals are discernible, that one of β-glucose being partially overlapped with the HOD resonance. The signal at 5.32 ppm (**d′**) is the only one not present in the spectrum of **SD-6**, establishing, therefore, the configuration α for the 4-linked fucose residue, as shown in structure **4** (Chart 4). In Table 5 we report the complete NMR assignment obtained for **SD-4** and **SD-6**, and in Figure 5 the NOESY spectrum which showed all the inter-glycosidic NOE contacts expected on the basis of the proposed structure of the EPS.

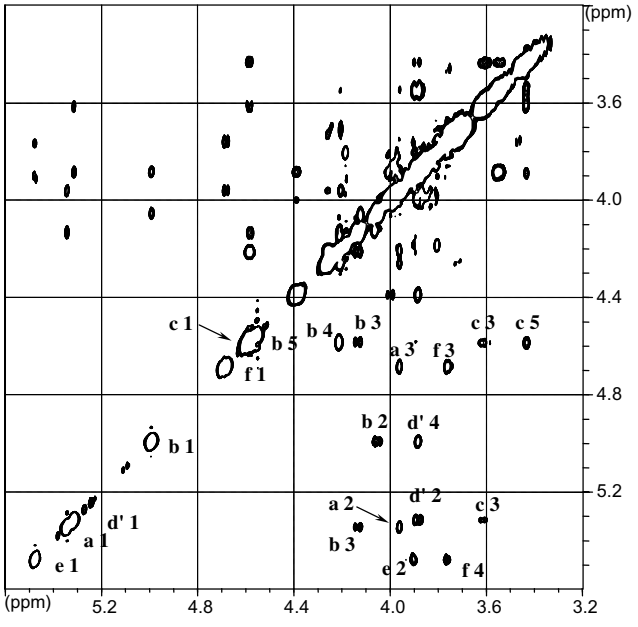

Figure 5. Part of a NOESY spectrum of the reduced molecular weight polysaccharide **SD-EPS** recorded at 50 °C.

4. Conclusions

It was established that the EPS produced by the bacterial species *E. amnigenus*, isolated from sugar beets, has the primary structure **4** (Chart 4), which is very similar to that of other two bacterial polymers. Clavan produced by *Clavibacter michiganensis* subsp. *insidiosus*[20] has an identical backbone, but a shorter side chain composed only of a pyruvylated galactose residue. The EPS produced by the environmental *Pseudomonas* sp. strain 1.15 has a repeating unit identical in size and differing only by a pyruvylated galactose in place of a pyruvylated mannose.[18] Pyruvate ketals are very common substituents in EPS from Gram-negative bacteria[21] and the end of the side chain in *E. amnigenus* EPS (**f** and **e** in structure **4**) is similar to xanthan from *Xanthomonas campestris*.

E. amnigenus is a fast producer of polysaccharide (about 5–7 times faster than *Clavibacter*) which could be utilised also for its thickening, gelling or emulsifying properties and, along with its repeating oligosaccharide unit, could have potential applications in the medical field, for example, as constituent in a plasma expander solution or as galenic excipient.[22] In fact, it is an acute problem to find new high molecular weight molecules for intravenous medical use which can be purified from pyrogenic impurities by easy and cheap processes, do not have any negative pharmacological[23] or toxic[24] effect on the host and do not interfere with other concomitant medication or diagnostic bioanalysis.[25] Finally,

this polysaccharide could be exploited for its skin moisturising properties in the cosmetic industry.

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