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Structural studies of a neutral polysaccharide produced by *Alcaligenes latus*

Yasuhiro Nohata [a], Jun-ichi Azuma [b,*], Ryuichiro Kurane [c]

[a] *Yokkaichi Laboratory, Hakuto Co., Ltd., 6-6-9, Betsumei, Yokkaichi, Mie 510, Japan*
[b] *Faculty of Agriculture, Kyoto University, Kitashirakawa Oiwake-cho, Kyoto 606-01, Japan*
[c] *National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, 1-1 Higashi Tsukuba, Ibaraki 305, Japan*

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Abstract

The structure of a neutral polysaccharide, isolated from the culture medium of *Alcaligenes latus* strain B-16, has been investigated by monosaccharide analysis, methylation analysis, one- (1D) and two-dimensional (2D) ^{1}H and ^{13}C NMR spectroscopy, and enzymatic digestion, and by MALDI-TOF-MS and LC–MS analyses of oligosaccharides isolated from a partial acid hydrolysate. The polysaccharide has the linear disaccharide repeating unit

→ 2)-α-D-Man*p*-(1 → 3)-α-L-Fuc*p*-(1 →

Keywords: *Alcaligenes latus*; Neutral polysaccharide; Mannofucopyranan

1. Introduction

Alcaligenes bacteria are Gram-negative rods in shape, with peritrichous flagella which have been known to produce a family of extracellular acidic heteropolysaccharides [1–4]. Previous studies on microbial flocculants and water-adsorbing polysaccharides resulted in the discovery of a new strain, *Alcaligenes latus* strain B-16 [5], which was able to produce a new super extracellular water-adsorbent polysaccharide [6].

* Corresponding author.

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During the course of the investigation into the production and chemical properties of this bioadsorbent, we found that it consisted of a mixture of at least two components, an acidic and a neutral polysaccharide [7]. We now report on the chemical structure of the neutral polysaccharide.

2. Results and discussion

Isolation, monosaccharide composition, and linkage analyses.—The crude neutral polysaccharide was isolated as an ethanol precipitate from the culture broth of *Alcaligenes latus* strain B-16, and purified to a protein- and acidic-polysaccharide-free material by treatment with cetavlon and passage through Dowex 50W-X8 (H^+ form) and Dowex 1-X4 (acetate form). About 500 mg of the purified neutral polysaccharide was obtained from 1 L of culture broth. The polysaccharide had $[\alpha]_D$ −7.8° (*c* 0.5, H_2O) and an average molecular mass of 64 kDa as determined by size-exclusion chromatography on a pullulan-calibrated column of YMC Diol S-5 (200A). The monosaccharide components were identified as mannose and fucose in an equal molar ratio. GLC analysis of the acetylated (+)-2-octyl glycosides [8] showed that mannose had the D-configuration and fucose the L-configuration. Conventional methylation analysis of the neutral polysaccharide revealed the presence of 2-linked D-mannopyranose (3,4,6-tri-*O*-methyl-D-mannose) and 3-linked L-fucopyranose (2,4-di-*O*-methyl-L-fucose) residues in a molar ratio of 1:1.25.

NMR spectroscopic analyses of the neutral polysaccharide.—The NMR spectra of the neutral polysaccharide revealed the presence of a disaccharide repeating unit incorporating one 6-deoxyhexose unit. Thus, the ^1H NMR spectrum (Fig. 1a) showed two anomeric ^1H signals at δ 4.79 (fucopyranose, $J_{1,2}$ 2.5 Hz) and 5.05 (mannopyranose, unresolved), and one methyl doublet at δ 1.07 (H-6 of fucopyranose, $J_{5,6}$ 6.5 Hz). A broad quartet at δ 4.210 was characteristic of H-5 of L-fucopyranose in α-configuration. The ^1H signal assignments obtained by H,H-COSY [9] and TOCSY [10] experiments are listed in Table 1. The occurrence of both L-fucopyranose and D-mannopyranose in α-configuration was supported by ^{13}C NMR analysis (Fig. 1b). The spectrum showed 12 discrete signals including two C-1 signals for pyranosyl residues at δ 98.96 (mannopyranose, $J_{C\text{-}1,H\text{-}1}$ 175 Hz) and δ 97.61 (fucopyranose, $J_{C\text{-}1,H\text{-}1}$ 169 Hz), a C-6 signal at δ 16.02 (fucopyranose), and a C-6 signal at δ 61.02 (mannopyranose unsubstituted at C-6). The ^{13}C signals were assigned using H,C-COSY [11] and gradient HSQC [12] (Fig. 2) experiments (Table 1). Examination of chemical shift differences for the carbon signals as compared to the values in the corresponding monomers [13] indicated that C-2 of D-mannopyranose and C-3 of L-fucopyranose were shifted 4.7 and 7.7 ppm downfield, respectively, confirming the linking positions of these sugars. The glycosyl-linkage analysis was confirmed by the use of NOESY [14] and gradient HMBC [15] experiments. The NOESY spectrum showed an interresidual cross-peak between H-1 of L-fucopyranose and H-2 of D-mannopyranose, while the cross-peak between H-1 of D-mannopyranose and H-3 of L-fucopyranose could not be seen because of overlap with signals due to the other ring protons (H-3 and H-4) of L-fucopyranose. However, the latter linkage could be determined from the gradient HMBC map (Fig. 3) which

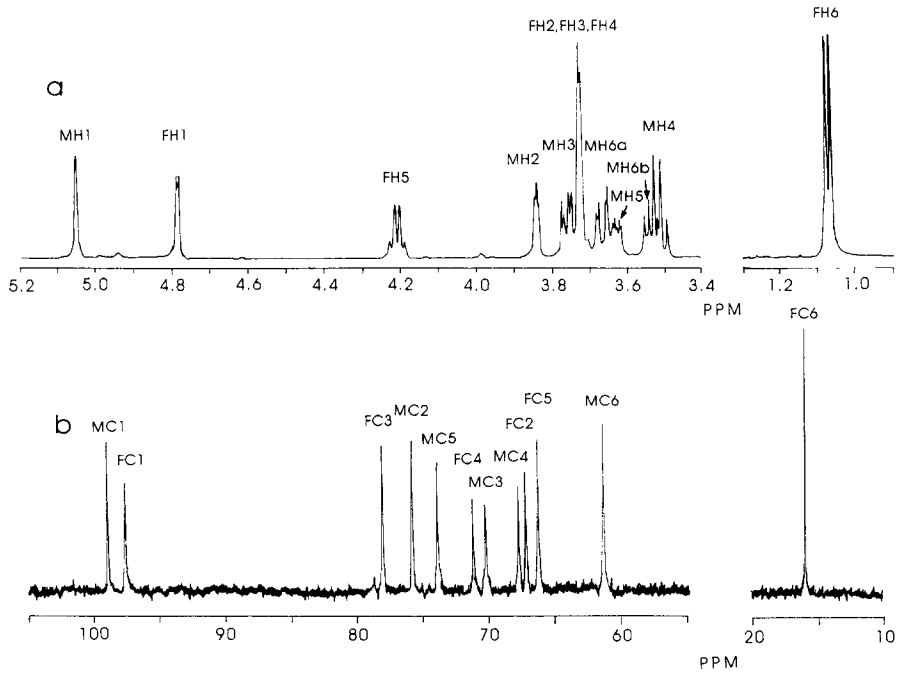

Fig. 1. 500-MHz 1D ^{1}H (a) and 125-MHz ^{13}C (b) NMR spectra of the neutral polysaccharide from *Alcaligenes latus* B-16, recorded in 17:3 Me_2SO-d_6–D_2O at 90 °C and 70 °C, respectively. Signals in MH (MC) and FH (FC) originated from protons (carbons) of D-Man*p* and L-Fuc*p*.

Table 1
NMR Data for the neutral polysaccharide in 7:3 Me_2SO-d_6–D_2O at 90 °C

Residue	^{1}H Chemical shifts (δ) [a]						
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
→ 2)-α-Man*p*-(1 →	5.051	3.843	3.762	3.511	3.624	3.667	3.536
	$J_{1,2}$ (n.r.)	$J_{2,3}$ (3.1)	$J_{3,4}$ (9.0)	$J_{4,5}$ (9.2)	$J_{5,6a}$, $J_{5,6b}$ (2.2, 5.4)	$J_{6a,6b}$ (11.2)	
→ 3)-α-Fuc*p*-(1 →	4.785	3.724	3.727	3.724	4.210	1.073	
	$J_{1,2}$ (2.5)	$J_{2,3}$ (n.d. [b])	$J_{3,4}$ (n.d.)	$J_{4,5}$ (~ 1)	$J_{5,6}$ (6.5)		

Residue	^{13}C Chemical shifts (δ) [a]					
	C-1	C-2	C-3	C-4	C-5	C-6
→ 2)-α-Man*p*-(1 →	98.96	75.80	70.20	67.72	73.88	61.02
	$J_{C\text{-}1,H\text{-}1}$ (175)					
→ 3)-α-Fuc*p*-(1 →	97.61	67.21	78.05	71.14	66.25	16.02
	$J_{C\text{-}1,H\text{-}1}$ (169)					

[a] Relative to Me_2SO-d_6 (δ_H 2.490 and δ_C 34.50), and coupling constants are given in parentheses (Hz); n.r., not resolved.

[b] n.d., The coupling constant was not determined because of overlapping of the signals.

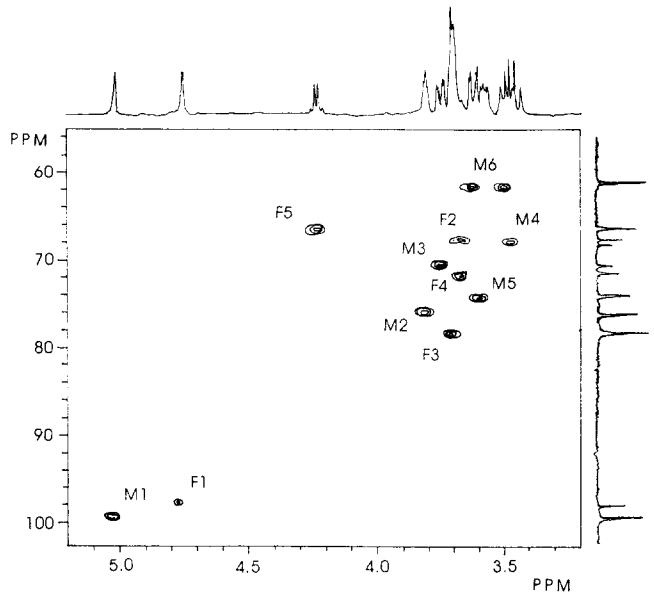

Fig. 2. 500-MHz gradient HSQC spectrum of the neutral polysaccharide from *Alcaligenes latus* B-16, recorded in 17:3 Me_2SO-d_6–D_2O at 50 °C. M and F denote the set of cross-peaks between protons and carbons of D-Man*p* and L-Fuc*p*.

showed not only interresidual cross-peaks between H-1 of L-fucopyranose and C-2 of D-mannopyranose together with C-3 and C-5 of L-fucopyranose, but also cross-peaks between H-1 of D-mannopyranose and C-3 of L-fucopyranose together with C-2, C-3, and C-5 of D-mannopyranose. These NMR spectroscopic results together with those from the methylation analysis indicated that the neutral polysaccharide is composed of equimolar amounts of 2-linked α-D-mannopyranose and 3-linked α-L-fucopyranose residues, and has the sequence →2)-α-D-Man*p*-(1→3)-α-L-Fuc*p*-(1→.

Partial acid hydrolysis analysis.—Further support for this structure was obtained via partial acid hydrolysis experiments. Partial acid hydrolysis of the neutral polysaccharide gave 30 oligosaccharides as revealed by HPAEC analysis (Fig. 4). MALDI-TOF-MS analysis of the same oligosaccharide mixture gave further information about their molecular masses. The MS spectrum showed the presence of a series of 20 homologous oligosaccharides as their sodium and potassium (minor) adducts whose molecular masses were all even numbers, i.e. degrees of polymerization (dp) of 2, 4,..., 40 (Fig. 5). A series of 12 oligosaccharides having dps up to 24 could also be detected by LC–MS using the analytical amino-bonded polymer-based column (Asahipak NH2P-50); electrospray ionization revealed identification of the separated oligosaccharides as ammonia adducts. For further analysis of the structure of the oligosaccharides, nine oligosaccharides (Nos. 1–9) were isolated by preparative HPLC on the same type amino-bonded column. MALDI-TOF-MS analysis confirmed their dps to be 2, 4,..., 18. Monosaccharide composition and reducing-end analyses indicated that all the isolated oligosaccharides were composed of equal moles of D-mannose and L-fucose

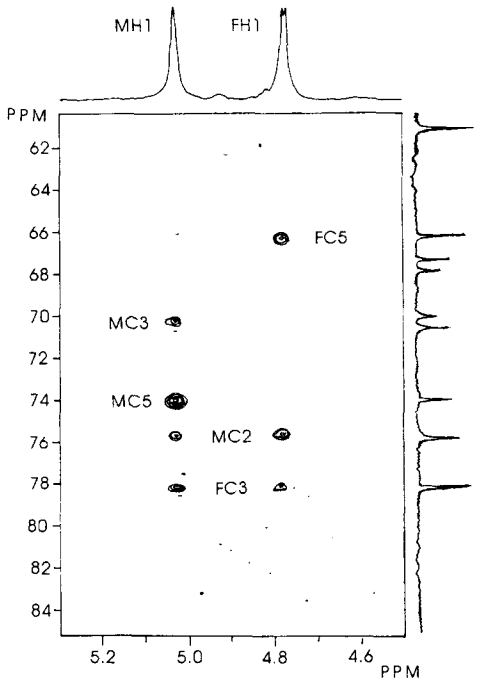

Fig. 3. 500-MHz gradient HMBC spectrum of the neutral polysaccharide from *Alcaligenes latus* B-16, recorded in 17:3 Me_2SO-d_6–D_2O at 50 °C. MH1 and FH1 denote anomeric protons of D-Man*p* and L-Fuc*p*. Cross-peaks in MC and FC originated from carbons of D-Man*p* and L-Fuc*p*.

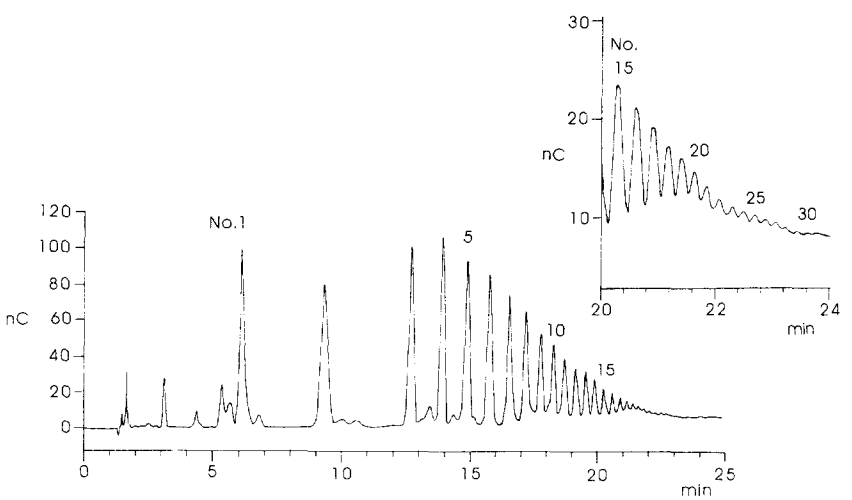

Fig. 4. HPAEC profile of a partial acid hydrolyzate of the neutral polysaccharide from *Alcaligenes latus* B-16.

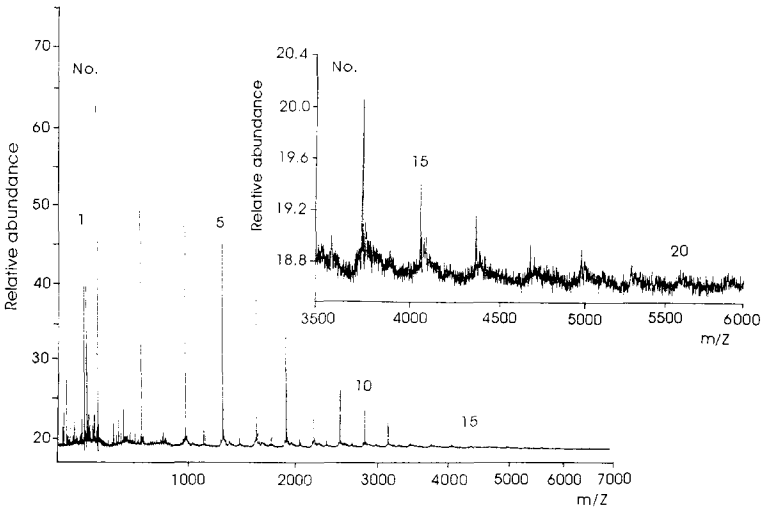

Fig. 5. MALDI-TOF mass spectrum of a partial acid hydrolyzate of the neutral polysaccharide from *Alcaligenes latus* B-16.

having L-fucose at their reducing end. In order to provide evidence of the non-reducing-end monosaccharide, α-fucosidase, and α- and β-mannosidase treatments were performed. The oligosaccharides were inert to the α-fucosidase and β-mannosidase treatments, while α-mannosidase released one mole of mannose from each oligosaccharide, indicating that all isolated oligosaccharides had an α-linked non-reducing-end D-mannopyranose residue. The results of the analysis of the anomeric region of the ^{1}H NMR spectra of the isolated oligosaccharides were consistent with the chemical and enzymatic glycosidic-linkage analyses (Table 2). Five anomeric ^{1}H signal regions at δ 5.03 ($J_{1,2}$ 3.4–3.9 Hz), 4.40 ($J_{1,2}$ 7.8 Hz), 4.83 ($J_{1,2}$ 3.3–3.9 Hz), 5.06 ($J_{1,2}$ 1.3–2.0 Hz), and 4.96 ($J_{1,2}$ 1.3–1.7 Hz) corresponding to anomeric protons of the α and β reducing-end L-fucopyranose, internal L-fucopyranose, internal D-mannopyranose, and non-reducing-end D-mannopyranose, respectively, were present in the oligosaccharides having dp ≥ 4 (Nos. 2–9), while only three signals due to non-reducing-end D-mannopyranose (H-1α; δ 4.95, $J_{1,2}$ 1.3 Hz) and reducing-end L-fucopyranose (H-1α; δ 5.03, $J_{1,2}$ 3.1 Hz and H-1β; δ 4.40, $J_{1,2}$ 7.7 Hz) were detected in the dimer, α-D-Man*p*-(1 → 3)-L-Fuc*p* (No. 1).

In conclusion, all results permit structure **1** to be assigned to the repeating unit of the neutral exopolysaccharide:

→ 2)-α-D-Man*p*-(1 → 3)-α-L-Fuc*p*-(1 →

1

Table 2
Analytical properties of the isolated oligosaccharides

No.	dp			Molar ratio	Molecular mass			^{1}H Chemical shifts (δ) [e]			
	Obsd [a]	Obsd [b]	Obsd [c]	D-Man*p*/L-Fuc*p*	LC–MS *m/z*	MALDI-TOF-MS *m/z*	Calcd [d]	D-Man*p*		L-Fuc*p*	
								Non-reducing end	Internal	Internal	Reducing end
1	2.0	2.0	2.0	1.1	326.0	326.8	326.3	4.952(1.0) [f]	–	–	α 5.030 (0.4) [3.1] [g]
								[1.3]			β 4.402 (0.6) [7.7]
2	4.0	4.2	3.7	0.9	634.2	634.2	634.6	4.955(1.0)	5.057(0.9)	4.831(0.8)	α 5.030 (0.4) [3.4]
								[1.3]	[2.0]	[3.6]	β 4.404 (0.6) [7.8]
3	6.0	6.2	6.4	0.9	942.5	642.6	942.0	4.957(1.0)	5.058(2.3)	4.831(2.1)	α 5.020 (0.4) [3.6]
								[1.7]	[2.0]	[3.3]	β 4.402 (0.6) [7.8]
4	8.0	7.9	7.6	1.0	1250.8	1251.5	1251.3	4.956(1.0)	5.059(2.9)	4.831(2.7)	α 5.020 (0.4) [3.5]
								[1.4]	[1.2]	[3.7]	β 4.402 (0.6) [7.8]
5	10.0	10.1	9.7	0.8	1559.0	1559.9	1559.6	4.956(1.0)	5.059(3.8)	4.830(3.9)	α 5.020 (0.4) [3.5]
								[1.3]	[1.2]	[3.7]	β 4.402 (0.6) [7.8]
6	12.0	12.2	11.6	0.9	1867.0	1868.8	1867.9	4.957(1.0)	5.059(4.7)	4.831(4.9)	α 5.031 (0.4) [3.6]
								[1.4]	[1.4]	[3.9]	β 4.404 (0.6) [7.8]
7	14.0	14.3	13.4	0.9	2175.3	2176.9	2176.2	4.956(1.0)	5.059(5.7)	4.830(5.7)	α 5.030 (0.4) [3.6]
								[1.7]	[1.6]	[3.9]	β 4.403 (0.6) [7.8]
8	16.0	16.3	15.5	1.0	2483.5	2485.9	2484.6	4.957(1.0)	5.058(6.7)	4.830(6.8)	α 5.020 (0.4) [3.9]
								[1.5]	[1.4]	[3.8]	β 4.402 (0.6) [7.8]
9	18.0	18.0	17.5	0.9	2792.0	2794.1	2792.9	4.957(1.0)	5.059(7.8)	4.831(7.7)	α 5.020 (0.4) [3.6]
								[1.5]	[1.3]	[3.8]	β 4.402 (0.6) [7.8]

[a] Values calculated from the molecular mass determination by LC–MS and MALDI-TOF-MS.
[b] Values determined by the reducing-end analysis [(moles of D-Man*p* + L-Fuc*p* + fucitol)/(moles of fucitol)].
[c] Values estimated by the ^{1}H NMR spectra in D_2O at 90 °C.
[d] Calculated by the chemical structure of each oligosaccharide.
[e] Relative to internal acetone (δ_H 2.225).
[f] Molar ratios calculated from the signal area, non-reducing-end D-Man*p* being taken as 1.0, in brackets.
[g] Coupling constants in Hz, in square brackets.

3. Experimental

General methods.—The polarimetric measurement was made with a JASCO DIP-1000 instrument at 25 °C. The average molecular mass measurement was carried out by HPLC on a column of Diol S-5 (200A) (YMC, 8.0 × 500 mm) using 25 mM sodium phosphate buffer (pH 6.8) as an eluant at 25 °C and 0.6 mL/min. The eluant was monitored by a differential refractometer (TOSOH RI-8). A mixture of pullulans having known molecular masses (Shodex P-82, Showa Denko) was used as a calibration standard.

Preparation of the neutral polysaccharide.—*Alcaligenes latus* B-16 was grown for 5 days at 30 °C with rotary shaking (180 rpm) in media (100 mL/500 mL shake flask) that contained 2.0% glucose, 0.15% KH_2PO_4, 0.45% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, 0.1% urea or $NaNO_3$, and 0.05% yeast extract (pH 7.0). Neutral polysaccharide was isolated from the culture broth by a modified method described previously [7]. Briefly, the culture broth was precipitated by 2 vol of ethanol. The precipitate was redissolved in hot water (60–70 °C) and precipitated again by addition of 5 vol of ethanol. This solubilization and precipitation procedure was repeated until the coloured materials that originated from the culture medium were completely eliminated. The obtained material was suspended in distilled water (4 g/5 L). The water-insoluble cells and acidic polymers were removed by filtration through a membrane filter (Millipore, pore size 0.22 μm) and treatment with 2% cetavlon (cetyltrimethylammonium bromide) to precipitate acidic materials. The neutral water-soluble polysaccharide was recovered by ethanol precipitation and further purified by passage through columns of Dowex 50W-X8 (H^+ form) and Dowex 1-X4 (acetate form).

Monosaccharide composition analysis.—Neutral monosaccharides, released by hydrolysis with 1 M H_2SO_4 for 6 h at 100 °C or 2 M trifluoroacetic acid (TFA) for 2 h at 121 °C, were analyzed as their alditol acetates by GLC on a column (15 m × 0.25 mm) of DB-225 (J&W Scientific) at 200 °C. The absolute configuration of the neutral monosaccharides was determined by GLC [8] of their acetylated (+)-2-octyl glycosides on the same column with a temperature gradient program from 140 to 220 °C at 2.0 °C/min.

Methylation analysis.—The polysaccharide (1 mg) dissolved in 1 mL dry Me_2SO was methylated twice with sodium methylsulfinylmethanide and methyl iodide, and the methylated polysaccharide extracted into chloroform was hydrolyzed and reduced [16]. The resulting partially methylated alditol acetates were analyzed by GLC (Shimadzu GC-9A) and GLC–MS (Shimadzu QP-1000) on a column (25 m × 0.2 mm) of CBP-20 (Shimadzu) using a temperature program from 150 to 210 °C at 2.0 °C/min after maintaining for 2 min at 150 °C [17].

Partial acid hydrolysis and chromatographic separation of oligosaccharides.—Partial hydrolysis of the neutral polysaccharide was carried out in 0.25 M TFA for 30 min at 80 °C. The cooled hydrolyzate was neutralized with DEAE-Sephadex A-50 (carbonate form) and then passed through a small column of this resin. The eluant and column washings were combined and freeze-dried. The production of oligosaccharides was monitored by HPLC on Asahipak NH2P-50 (Showa Denko, 4.6 × 300 mm) eluted with 3:2 acetonitrile–water at 30 °C and 0.6 mL/min. Nine oligosaccharides were isolated by

preparative HPLC on Asahipak NH2P-90 2F (Showa Denko, 21.5 × 300 mm) eluted with the same solvent at 4.0 mL/min. The eluant was monitored with a differential refractometer (Shodex SE-50). The distribution of the oligosaccharides was also analyzed by HPAEC (Dionex DX-500 equipped with an ED40 electrochemical detector in an integrated amperometry mode) on a column of CarboPac PA1 (4 × 250 mm) with a gradient elution from 100 mM NaOH to 100 mM NaOAc in 100 mM NaOH in 30 min at 1.0 mL/min.

Structural analysis of the isolated oligosaccharides.—The molecular mass distribution of the oligosaccharide mixture and the molecular masses of the isolated oligosaccharides were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and combined liquid chromatography–mass spectrometry (LC–MS). MALDI-TOF-MS was performed by a Vision 2000 instrument (ThermoQuest, analyzer length 1.7 m) operated in the positive-ion mode at 5 kV using 2,5-dihydroxybenzoic acid as a matrix. LC–MS was performed on a TSQ7000 triple stage quadrupole LC–MS instrument (ThermoQuest) fitted with a Finnigan electrospray (ESI) interface. The mass spectrometer was operated in the positive-ion mode at 4.5 kV and the capillary temperature was 215 °C. LC was carried out on a HP-1050 instrument (Hewlet–Packard) using a column of Asahipak NH2P-50 and 3:2 acetonitrile–water as eluant at 0.6 mL/min.

Reducing-end analysis of the isolated oligosaccharides was carried out by reduction, acid hydrolysis, and HPLC separation of the released alditol and monosaccharides. Each oligosaccharide was reduced overnight with sodium borohydride in distilled water in a plastic vial, and hydrolyzed with 2 M TFA for 4 h at 100 °C in a glass ampule. After the removal of TFA by treatment with DEAE-Sephadex A-50 (carbonate form), reducing monosaccharides and alditols were separated by HPLC on HPX-87P (Bio-Rad, 7.8 × 300 mm) using distilled water as an eluant at 80 °C. The dp value of each oligosaccharide was calculated by subtracting moles of reducing monosaccharides plus alditol by moles of alditol.

Non-reducing-end analysis of the isolated oligosaccharides was performed enzymatically. Each oligosaccharide (250 μg) was treated with 0.1 U α-fucosidase (*Fusarium oxysporum*, Seikagaku Kogyo), α-mannosidase (Jack bean, Seikagaku Kogyo) or β-mannosidase (*Achatina fulica*, Seikagaku Kogyo) in 50 μL 10 mM NaOAc buffer, pH 4.5, for 3 h at 37 °C in a plastic micro tube. The solutions were heated for 5 min at 100 °C to inactivate the enzymes and then passed through a small column of Dowex 50-X8 (H^+ form) and Dowex 1-X8 (acetate form). The eluant and washings with distilled water were then combined and freeze-dried. Released sugar composition of the isolated oligosaccharides having dp ≥ 4 was analyzed by HPLC on Asahipak NH2P-50 as described above. The digest obtained from the dimer was analyzed by HPAEC (Dionex DX-500) on CarboPac PA1 (4 × 250 mm) using 80 mM NaOH as an eluant at 1.0 mL/min.

NMR spectroscopy.—500-MHz 1D ^1H and ^{13}C NMR, H,H-COSY, C,H-COSY, and NOESY spectra of the native exopolysaccharide in 17:3 Me_2SO-d_6–D_2O were recorded using a Bruker ARX-500 spectrometer with standard Bruker programs at 70–90 °C. 1D ^1H NMR spectra of the isolated oligosaccharides were recorded in D_2O. 500-MHz TOCSY, gradient HSQC, and gradient HMBC spectra of the native exopolysaccharides

were recorded using a Varian Unity 500 spectrometer with standard Varian programs at 50 °C. Chemical shifts are reported in ppm, using Me_2SO-d_6 (δ_H 2.45 and δ_C 39.5) or acetone (δ_H 2.225) as internal references.

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