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Oligosaccharide β-glucans with unusual linkages from *Sarcina ventriculi*

Jeongrim Lee, Rawle I. Hollingsworth *

Department of Chemistry, Michigan State University, East Lansing, MI 48824, USA

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Abstract

The structure of a family of unusual glucans from *Sarcina ventriculi* has been characterized by NMR spectroscopy, methylation analysis, and mass spectrometry. One is a trisaccharide containing a β-(1 → 3) and a β-(1 → 4)-linkage. The other is a hexasaccharide that is simply a 1,4-linked dimer of the trisaccharide unit. This is the first report of β-glucan biosynthesis in a Gram-positive organism. Their occurrence in these organisms supports an even more general link between their synthesis and the adaptability of bacteria.

Keywords: β-Glucans; *Sarcina ventriculi*; Oligosaccharides; Linkage analysis; NMR spectroscopy; Adaptation

1. Introduction

β-(1 → 2)-linked glucans are regular glucose oligomers that have been found thus far only in Gram-negative bacteria. The levels produced are affected by the osmotic balance, and their synthesis is known to occur in the periplasm. The absence of these glucans in Gram-positive bacteria, which have no periplasm, supports this idea. β-(1 → 2)-Glucans have been found in members of the family *Rhizobiaceae*, notably *Rhizobium* [1,2], and *Agrobacterium* [3,4] that can adapt to surviving intracellularly in plants. They have also been found in a few other Gram-negative genera including *Xanthamonas* (plant pathogens) [5], *Brucella* sp. (mammalian pathogens) [6], *Alcaligenes* [7], and *Acetobacter xylinium* [8]. In *Rhizobium* and *Agrobacterium* they are typically between 14 and 25 glucosyl residues in length and are usually cyclic in structure [9]. There are instances where they are acyclic (linear) ranging from 6 to 42 glucose units in *Acetobacter* [8] or 6 to 19 units in some strains of *Rhizobium* [10]. Linear glucans ranging from 8 to 20 glucosyl units have been isolated from *Xanthamonas* species [5]. These β-(1 → 2)-glucans are related to those with mixed linkages found in *E. coli* and known generally as membrane-derived oligosaccharides [11]. They are thought to function in maintaining the osmolality of the periplasm, thus protecting the organism from osmotic stress [12–15]. As such, they have never before been found in Gram-positive organisms. *Rhizobium*, *Xanthamonas* and *Agrobacterium* are plant symbionts or pathogens and, since they must survive intracellularly inside the host cell, should be capable of readily adapting to differences in osmotic pressures. A role for the regulated synthesis of a molecule that might

* Corresponding author. Tel.: +1-517-3530613; fax: +1-517-3539334; e-mail rih@argus.cem.msu.edu.

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facilitate this is expected. In fact, bacterial mutants that are incapable of synthesizing these glucans are impaired in their ability to infect and live intracellularly in host plants. *Brucella* is a mammalian pathogen and the same is true of these species.

Sarcina ventriculi is an anaerobic, Gram-positive organism that is adaptable to a very wide range of environmental conditions including pH values ranging from 3 to 10 [16] and in the presence of a wide variety of organic solvents [17]. During normal growth, the pH of the medium becomes quite acidic and can drop to under 4 [16]. Recently, a family of fatty acylated β-(1 → 2)-linked glucose disaccharides were isolated from such cultures [18]. This indicated that *S. ventriculi* was one Gram-positive organism in which the synthesis of β-glucans might be demonstrated. This would expand the link that had already been established between adaptability and β-glucan biosynthesis [12–15]. Here we describe the isolation and characterization of two such glucans, a trisaccharide and a hexasaccharide from this anaerobic, Gram-positive organism.

2. Results and discussion

Thin-layer chromatographic analysis of a polar fraction of a propanol–water extract of *S. ventriculi* cells indicated the presence of carbohydrates in several components. Most of these corresponded to glycosides but some were much more polar and behaved like free oligosaccharides. The fastest moving of these components had an R_f value and a ^{1}H-NMR spectrum and mass spectrum identical to the previously characterized β-1-*O*-acylsophorose. The fast atom bombardment mass spectra of two slower moving components indicated that they were a trisaccharide and a hexasaccharide, respectively. Gas chromatography of alditol acetate derivatives indicated that glucose was the only component in both molecules. The negative ion FAB mass spectrum of the trisaccharide (Fig. 1) contained a signal for the pseudomolecular ion, $[M-H]^-$ at *m/z* 503. The negative ion FAB mass spectrum of the hexasaccharide (Fig. 2) contained the $[M-H]^-$ ion at *m/z* 989. The series ions at *m/z* 827, 665, and 503 corresponded to the sequential loss of glucose units from the pseudomolecular ion. A TLC analysis of the polar fraction from *S. ventriculi* and oligosaccharides of a partially hydrolyzed sample of *Agrobacterium tumefaciens* β-(1 → 2)-glucan as a standard was also performed. This also supported the chain lengths indicated by the mass spectra and the purity of the samples.

A more quantitative determination of the relative amounts of these three oligosaccharides present, and sufficient quantity to allow a rigorous structural characterization, were obtained by gel filtration chromatography of the eluant after adsorbing out the glycolipids and other more non-polar compounds in the *S. ventriculi* extract on a C_{18} column and eluting with water. Colorimetric analysis of the fractions indicated the presence of two major carbohydrate-containing peaks and a few minor ones.

A definitive study of the linkages in the oligosaccharides was performed by methylation analysis. Permethylation of the $NaBD_4$-reduced trisaccharide and conversion into alditol acetates followed by GC–MS analysis gave a profile containing three major

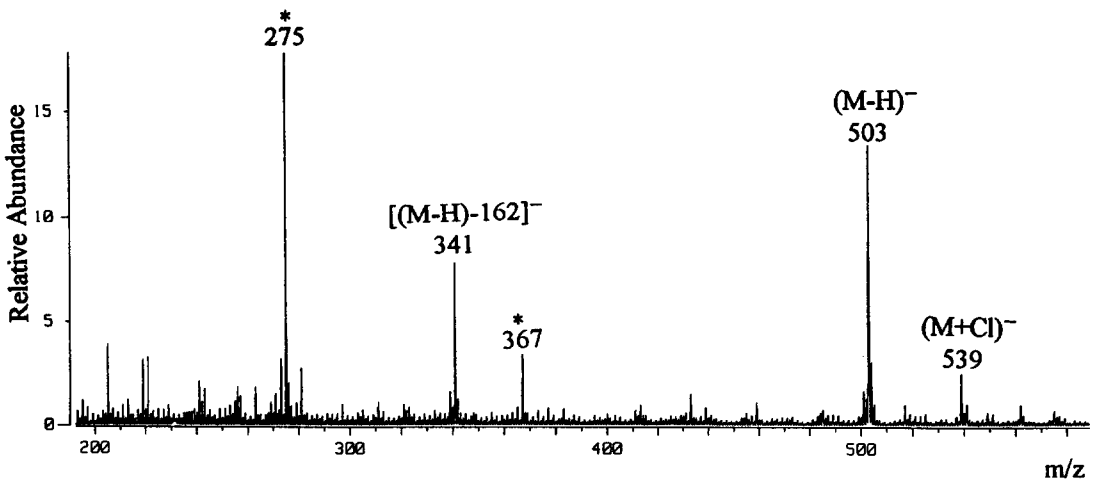


Fig. 1. Negative ion FAB mass spectrum of the trisaccharide. Glycerol matrix peaks are designated by an asterisk.

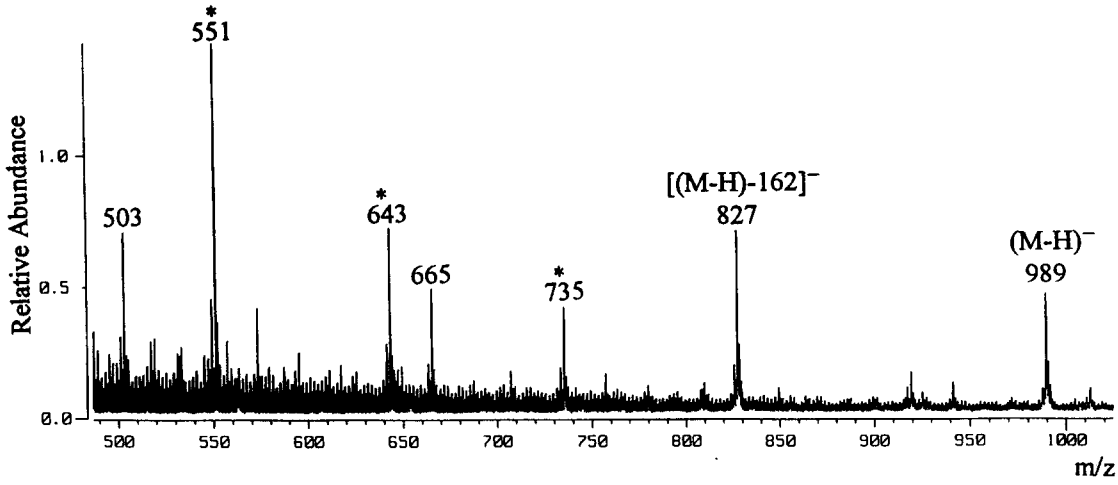


Fig. 2. Negative ion FAB mass spectrum of the hexasaccharide.

peaks. The first peak was identified as 1-deuterio-1,2,4,5,6-penta-*O*-methylglucitol acetate, arising from the 3-substituted reducing end. The later-eluting peaks had mass spectra consistent with 2,3,4,6-tetra- and 2,3,6-tri-*O*-methylglucitol derivatives. These results indicated that the trisaccharide was linear and contained a 1,3 and 1,4-linkage. The hexasaccharide yielded 1-deuterio-1,2,4,5,6-penta-, 2,3,4,6-tetra-, 2,4,6-tri-, and 2,3,6-tri-*O*-methyl-glucose in the ratio of 1:1:1:3. These results indicated that the hexasaccharide was probably a 1,4-linked dimer of the trisaccharide unit.

The configurations of the glycosidic linkages were established by 2D NMR spectroscopy experiments. In the ^{1}H-NMR spectrum of the trisaccharide (Fig. 3) four anomeric signals were observed at 4.36, 4.52,

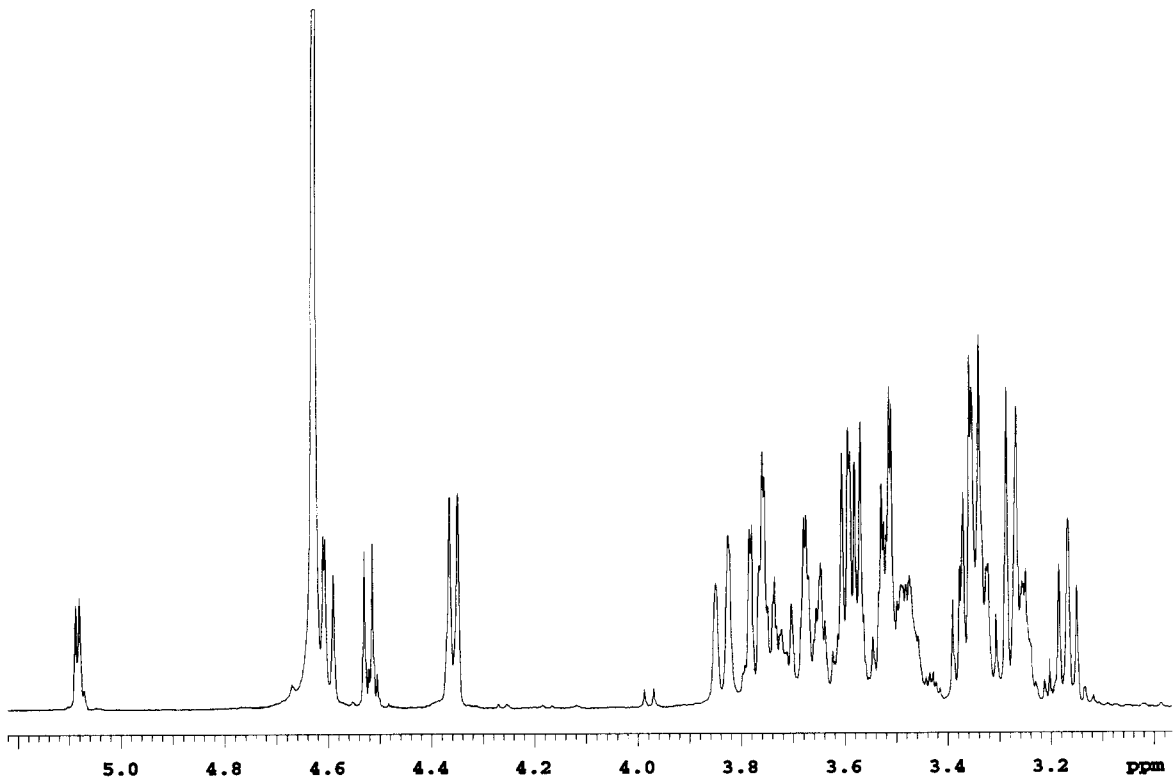


Fig. 3. ^{1}H-NMR spectrum of the trisaccharide.

Table 1
^{1}H- and ^{13}C-NMR chemical shifts[a] of the hexasaccharide

		Residues					
		A-α	A-β	B, E	C	D	F
1	H	5.09	4.525	4.601, 4.63	4.385	4.393	4.360
	C	92.9	96.5	103.4	103.2	103.2	103.4
2	H	3.59	3.29	3.26, 3.25	3.21	3.38	3.17
	C	71.9	74.7	74.1	73.8	73.8	74.0
3	H	3.79	3.60	3.52	3.51	3.63	3.36
	C	83.1	85.4	75.0	75.0	84.7	76.3
4	H	3.39	3.36	3.53	3.52	3.38	3.27
	C	69.0	69.0	79.2	79.4	68.8	70.3
5	H	3.73	3.37	3.48	3.50	3.37	3.34
	C	72.1	76.4	75.7	75.7	76.4	76.8
6	H	3.60/3.77	3.60/3.77	3.68/3.84	3.68/3.84	3.60/3.77	3.60/3.77
	C	61.4	61.4	60.8	60.8	61.4	61.4

[a]In ppm, D_2O solvent.

4.60, and 5.09 ppm. Two signals at 5.09 ppm ($J = 3.8$ Hz) and 4.52 ppm ($J = 8.0$ Hz) were assigned to the α- and β-anomeric proton signals, respectively, of the reducing end. The signals at 4.36 and 4.60 ppm were assigned to the two β-glucopyranosyl residues. The following structure was deduced for the trisaccharide: β-glucopyranosyl-(1 → 4)-β-glucopyranosyl-(1 → 3)-glucopyranose (**1**).

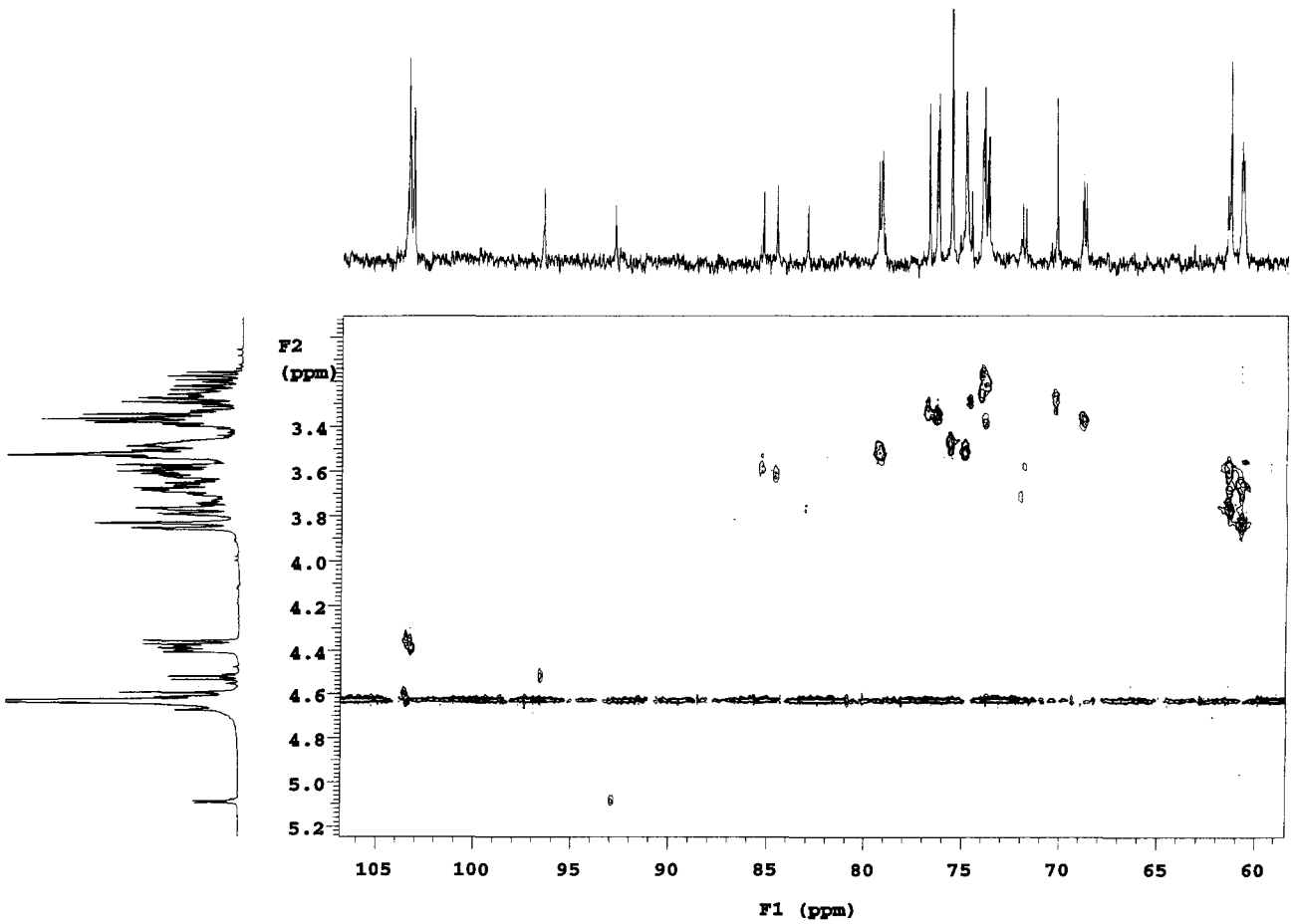
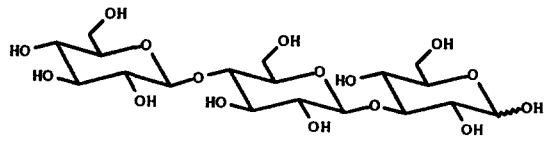


Fig. 4. ^{1}H–^{13}C-HMQC spectrum of the hexasaccharide.



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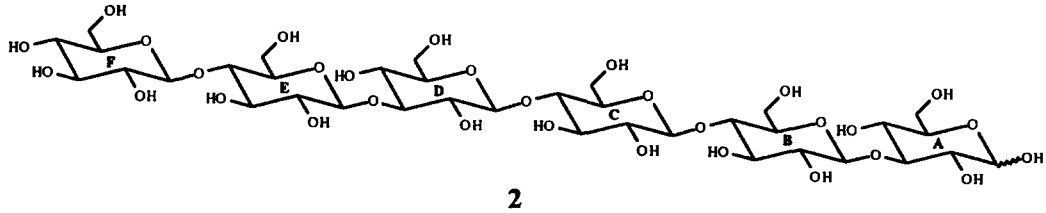

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The proposed structure of the hexasaccharide is shown as **2**. The complete assignments for ^{1}H- and ^{13}C-NMR signals of the hexasaccharide are given in Table 1. The ^{1}H-NMR spectrum of the hexasaccharide showed anomeric proton resonances in the region of 4.3–5.1 ppm. In the ^{1}H–^{13}C heteronuclear multiple quantum coherence (HMQC) spectrum (Fig. 4) a doublet at 5.09 ppm ($J = 4$ Hz) was correlated with a ^{13}C signal at 92.9 ppm and was assigned to the α-anomeric proton of the reducing end. A doublet at 4.52 ppm ($J = 8$ Hz) was assigned to the β-anomeric proton of the reducing end. The anomeric proton signal at 4.60 ppm ($J = 8$ Hz) corresponded to the 1,3-linked β-anomeric proton of the second glucose residue (residue B) seen before in the trisaccharide spectrum. The doublet at 4.36 ppm was assigned to the non-reducing terminal group (residue F) using the same reasoning. These two anomeric signals displayed the same chemical shifts as those of similar residues in the trisaccharide. The HMQC and double quantum filtered J-correlated spectroscopy (DQF–COSY) spectra (Fig. 5) strongly indicated the presence of two anomeric proton signals at ca. 4.6 ppm where one of the signals partially overlapped with the solvent signal. The signal at 4.63 ppm was assigned to the anomeric proton engaged in the 1,3-linkage. The second 3-substituted glucose residue could now either be residue C or D. This was determined by mass spectrometry. FAB-collision activated dissociation–tandem mass spectrometry (FAB–CAD–MS/MS) has been used to determine the linkage position and sequence of underivatized disaccharides and oligosaccharides [19]. In the last cited study the fragmentation pattern of 1,3- vs. 1,4-linkages was clearly delineated. This was confirmed by comparing the linked scan B/E mass spectra of the trisaccharide (βGlc1 → 4βGlc1 → 3Glc) obtained from this study and cellotriose (βGlc1 → 4βGlc1 → 4Glc) (data not shown). The linked scan B/E mass spectrum of cellotriose showed peaks at m/z 383, 425, and 443 which are characteristic ions of the 1,4-linkage to the reducing sugar. For the trisaccharide, only an intense ion at m/z 411 (loss of 92 amu) was present. The fragmentation pattern due to cleavage between the non-reducing and the middle glucose residues for both cases showed the same characteristic ions for the 1,4-linkage at m/z 161, 179, 221, 263, and 281. The linked scan B/E mass spectrum (Fig. 6A) of the hexasaccharide ion at m/z 989 showed the characteristic loss of 92 amu, indicating that the reducing sugar was linked by a 1,3-linkage. The ion at m/z 827 is attributable to the pentasaccharide formed by elimination of the reducing sugar (residues B–F). The linked scan B/E spectrum of the ion at m/z 827 showed the ions at m/z 707, 749, and 767 and not a simple loss of 92 amu, indicating a 1,4-linkage between the residues B and C (Fig. 6B). The presence of peaks at m/z 545, 587, and 605 confirmed the 1,4-linkage between residues C and D. Thus, the second 3-substituted glucose was assigned to residue D.

The assignments for the other proton and ^{13}C signals were readily made and were consistent with the methylation analysis and the CAD–MS/MS spectra. The assignments were also consistent with those of model oligosaccharides such as laminaribiose and cellotriose. The other signals at ca. 4.38–4.41 ppm ($J = 8$ Hz) in the NMR spectrum of the hexasaccharide corresponded to the two anomeric proton signals of the other internal residues engaged in the 1,4-linkages (residues C and D). The cross peaks between ca. 79.2–85.4 ppm in the proton–carbon HMQC spectrum were assigned to the carbons involved in linkages. These signals are typically 10 ppm downfield of the other carbon signals. The signals for protons at carbon atoms involved in the

linkages were readily identifiable through correlation with the corresponding ^{13}C signals in the HMQC spectrum. The α-anomeric proton of residue A showed three cross peaks at 3.39, 3.59 and 3.79 ppm in the HOHAHA spectrum (data not shown). The α-anomeric proton at 5.09 ppm was coupled to the H-2 proton of residue A at 3.59 ppm in the DQF–COSY spectrum. The H-2 signal of residue A showed a cross peak with H-3 at 3.79 ppm. This latter proton signal was correlated with a ^{13}C signal at 83.1 ppm, indicating that C-3 was a linkage site. The HOHAHA-traces for the β-anomeric proton of residue A (4.525 ppm) showed connectivities with three signals at 3.29, 3.36 and 3.60 ppm. In the DQF–COSY spectrum, the β-anomeric proton of residue A was coupled to the H-2 proton signal at 3.29 ppm. The H-2 proton signal was also correlated with a signal at 3.60 ppm (H-3). The latter signal at 3.60 ppm showed a cross peak in the HMQC spectrum with a ^{13}C chemical shift of 85.4 ppm. The assignments of ^{13}C chemical shifts of residue A were in good agreement with those of the reducing end of laminaribiose [20]. The H-1 signal of residue B was coupled to the H-2 signal at 3.26 ppm. The H-1 signal of residue E showed a cross peak with a signal at 3.25 ppm (H-2). The H-2 signals of residue B and E were coupled to the H-3 signal at 3.52 ppm. The remaining ^{1}H and ^{13}C signals of residue B and E showed the same chemical shifts. The H-1 signal of residue C was correlated with the H-2 signal at 3.21 ppm, which was, in turn, coupled to the H-3 signal at 3.51 ppm. These three H-3 signals of residues B, C, and E were correlated with H-4 signals which, in the HMQC spectrum, had cross peaks with ^{13}C chemical shifts between 79.4 and 79.2 ppm, indicating that residues B, C and E were the sites of 1,4-linkage. The H-1

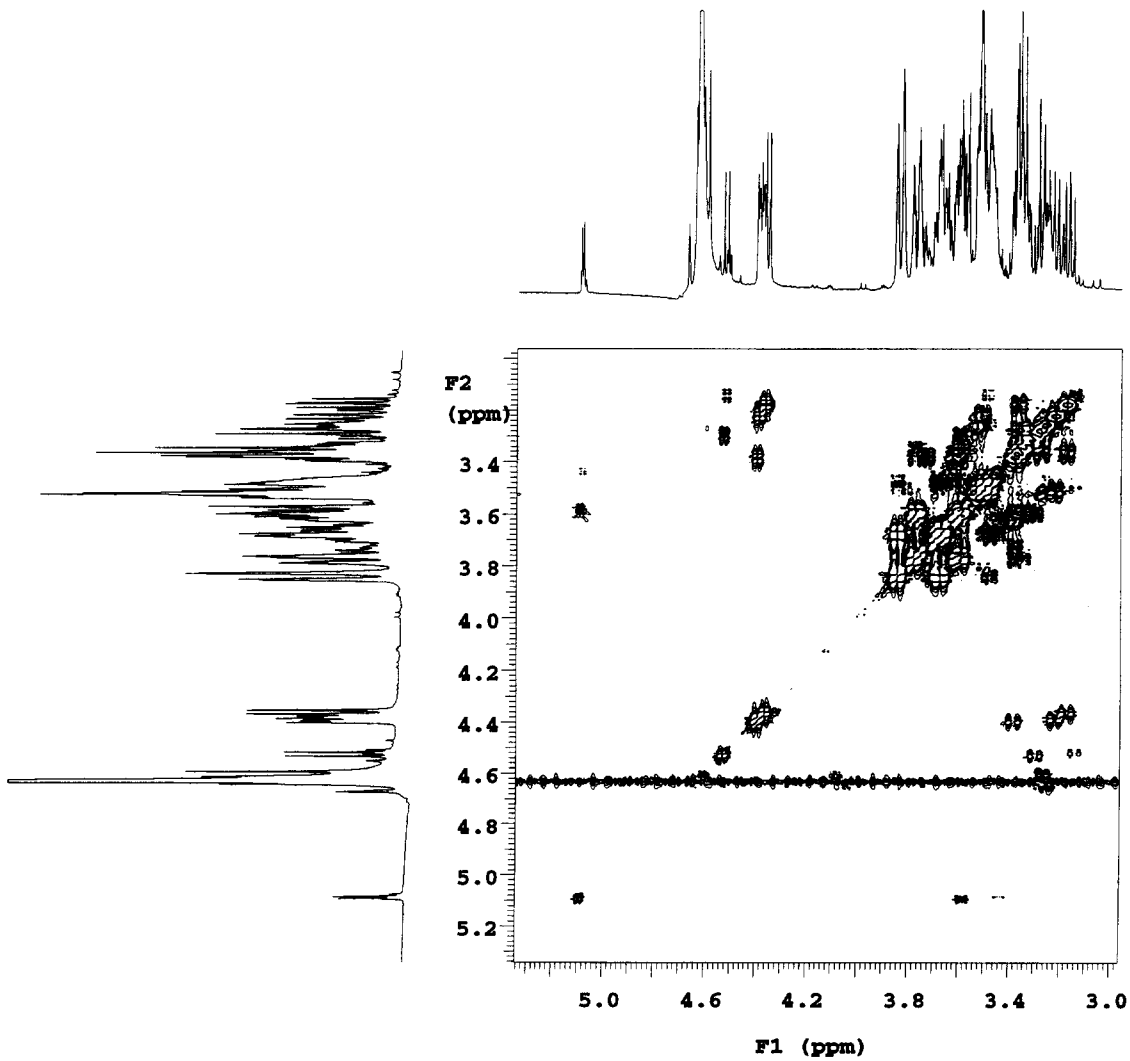

Fig. 5. DQF–COSY spectrum of the hexasaccharide.

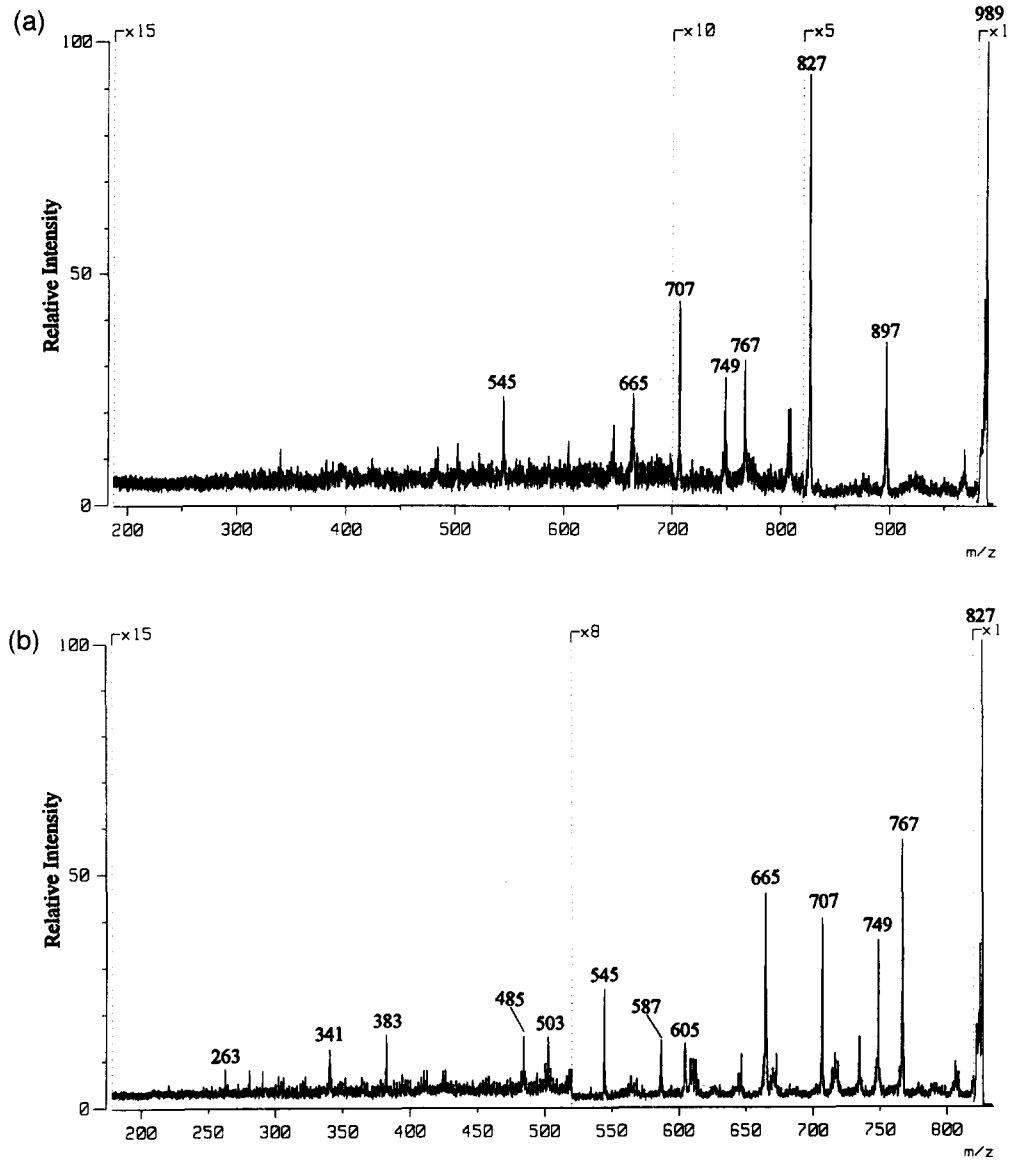

Fig. 6. Negative ion B/E linked scans FAB mass spectra of the hexasaccharide: (a) pseudomolecular ion at *m/z* 989 (b) ion at *m/z* 827.

signal of the 1,3-linked residue D was coupled to the H-2 signal at 3.38 ppm. The H-2 signal was coupled to the H-3 signal at 3.63 ppm. In the HMQC spectrum, the H-3 signal was correlated with the ^{13}C signal at 84.7 ppm, confirming the 1,3-linkage. The assignments of ^{13}C chemical shifts for residue C and F were consistent with those of the internal residue and non-reducing group of cellotriose, respectively [21].

3. Experimental

Isolation and purification of oligosaccharides.—*S. ventriculi* was cultured as previously described [16]. Cells were extracted by treating a suspension in a buffer consisting of 25 mM EDTA and 50 mM Tris–HCl (1:1) with lysozyme (50,000 units) and protease (7 units) at 37 °C for 5 h. The slurry was then extracted with *n*-propanol. The propanol–water solution was concentrated to dryness and chromatographed on a C_{18} column (30 × 2.5 cm) using water, water–methanol (1:1), methanol, methanol–chloroform (1:1) and chloroform as eluents. The water fractions were pooled and chromatographed on a Bio-Gel P4 column (200 × 2) cm using water as eluant. Fractions (6 mL) were collected and assayed for carbohydrate by the phenol–sulfuric acid method [22]. Fractions 47–60 were pooled and further puri-

fied on a silica gel column using a solvent system composed of 2-propanol–ammonia–water in the ratio 6:4:1. Fractions were assayed by the orcinol–sulfuric acid method [23].

Isolation and purification of β-(1 → 2)-glucan.—*A. tumefaciens* strain C58 was cultured as previously reported [24]. Cells were extracted twice with a mixture of water, methanol and chloroform (3:1:5). The aqueous layer was recovered, concentrated to a syrup and chromatographed on a Sepharose 4B column (75 cm × 6 cm) in 1% aqueous acetic acid. Column fractions were monitored for carbohydrate using the phenol–sulfuric acid assay [22]. The last eluting peak containing the glucan was then subjected to chromatography on a Bio-Gel P2 column (100 cm × 2 cm) in water. This fraction was lyophilized and then subjected to limited hydrolysis in 0.2 M trifluoroacetic acid at 100 °C for use as a standard in thin-layer chromatography. This was carried out on silica plates using a solvent system composed of 1-butanol–ethanol–water in the ratio of 5:3:2.

Methylation analysis.—To differentiate between a 3- and 4-substituted reducing end, the samples of the trisaccharide and hexasaccharide (each ca. 100 μg) were reduced with $NaBD_4$ (ca. 100 μg) in water (100 μL) for 2 h before methylation. The solutions were treated with HOAc (50 μL) to decompose excess reducing agent and then concentrated to dryness. Methanol (0.5 mL) was added and the solutions were evaporated to dryness. Four more 0.5 mL aliquots of MeOH were added and the solutions again concentrated to dryness after each addition to remove methyl borate. The mixtures were treated with 500 μL of a 1.5 M solution of sodium methylsulfinyl anion in dry Me_2SO [25]. The mixtures were stirred for 24 h at room temperature and then treated 100 μL of iodomethane. The resulting suspensions were stirred for 1 h and then diluted to 3 mL with water and passed through C_{18} Sep-Pak cartridges. The cartridges were eluted sequentially with 3 mL each water, 1:1 MeOH–water, and finally MeOH. The fractions of MeOH–water and MeOH were collected separately and subjected to acid hydrolysis with 2 *M* trifluoroacetic acid (1.5 h, 120 °C). The hyrolyzates were reduced with $NaBH_4$ as described earlier and then acetylated with dry pyridine (200 μL) and Ac_2O (100 μL) for 24 h at room temperature and then dried under N_2. The acetylated products were partitioned between water and $CHCl_3$. The chloroform fraction was removed and dried under N_2 at room temperature. The partially methylated, acetylated alditols were analyzed by GC–MS.

Mass spectrometry.—The fractions corresponding to a single peak by silica column chromatography were pooled and analyzed by fast atom bombardment (FAB) mass spectrometry. Negative ion FAB mass spectra were recorded using a JEOL HX-110 double-focusing mass spectrometer with glycerol as a matrix. Collisionally activated dissociation tandem mass spectrometry (CAD–MS/MS) was conducted by scanning the electric sector and magnetic sector in a fixed ratio (B/E linked scan) [26]. Helium was used as the collision gas in a cell located in the first field-free region. The helium pressure was adjusted to reduce the abundance of the precursor ion by 30%. GC/MS analyses of the partially methylated alditol acetates were performed on a Hewlett–Packard 5995C GC/MS, equipped with a Supelco DB-225 fused-silica capillary column using a temperature program of 170 °C (3 min) −230 °C at 2 °C/min. Helium was used as the carrier gas.

NMR spectroscopy.—All NMR spectra were measured in D_2O at 500 MHz for ^1H or 125 MHz for ^{13}C with a Varian VXR 500 spectrometer. For the HMQC experiments, a spectral width of 7114 Hz was employed for the ^{13}C dimension. A total of 32 transients were required at 1024 points each. A total of 512 data sets were acquired. The DQF–COSY spectrum was obtained using a total of 512 data sets (16 transients at 2048 data points each).

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