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Structure of a galactan from cell walls of *Bifidobacterium catenulatum* YIT4016

Masato Nagaoka *, Shusuke Hashimoto, Hideyuki Shibata, Itsuko Kimura, Kazumasa Kimura, Haruji Sawada, Teruo Yokokura

Yakult Central Institute for Microbiological Research, Kunitachi-shi, Tokyo 186, Japan

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Abstract

A structural study was carried out on a galactose-rich polysaccharide fraction isolated from cell walls of *Bifidobacterium catenulatum* YIT4016 after *N*-acetylmuramidase digestion. The polysaccharide contained galactose and glucosamine in a molar ratio of 16.9:1.0. Data obtained by ^{13}C NMR spectroscopy showed that the backbone chain of this polysaccharide is composed of galactofuranose residues, while the branches consist of galactopyranosyl residues. Furthermore, the data obtained from $NaIO_4$ oxidation, partial methanolysis and methylation analysis indicated that this polysaccharide consists of a trisaccharide repeating unit having the following structure:

$$[\rightarrow 6)\text{-}\beta\text{-}D\text{-}Galf\text{-}(1\rightarrow 5)\text{-}\beta\text{-}D\text{-}Galf(1\rightarrow]_n$$
$$\begin{array}{c} 6 \\ \uparrow \\ 1 \\ \alpha\text{-}D\text{-}Galp \end{array}$$

Keywords: Bifidobacteria; Galactan; Cell wall

* Corresponding author.

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1. Introduction

Bifidobacteria are gram-positive enterobacteria related physiologically and immunologically to host homeostasis [1,2]. Recently, we found that some cell wall polysaccharides of these bacteria were effective in healing gastric ulcers [3]. Bacterial surface components, such as cell walls and their polysaccharides, are important for this biological activity [2]. In previous studies, these bacterial polysaccharides were classified into two types, 6-deoxyhexose-containing polysaccharides, and neutral hexose-containing ones such as galactan and glucogalactan [4–6]. Galactan-type polysaccharides have been observed in *B. pseudolongum* and *B. catenulatum*. It is of interest that these polysaccharides contain galactofuranose residues [4], but their polysaccharide structures have not yet been determined. The present paper reports the results of a structural study on a galactan from cell walls of *B. catenulatum* YIT4016.

2. Materials and methods

Cultivation of bacteria and preparation of cell walls.—*B. catenulatum* YIT4016 (ATCC 27539) was grown under anaerobic conditions and cell walls were prepared by a method similar to that described previously [5].

Isolation and fractionation of cell wall polysaccharides.—The method used for the preparation of cell wall polysaccharides has already been reported [5]. In brief, a suspension of cell walls (500 mg) was exhaustively digested with *N*-acetylmuramidase (10 mg) in 5 mM Tris-malate buffer (pH 5.8) containing 2 mM $MgCl_2$ at 37 °C for 20 h. The resultant solution was digested with trypsin (10 mg) and then dialyzed against distilled water. The non-diffusible material was chromatographed on a column of Sephacryl S-200 HR (2 × 90 cm, 50 mM $(NH_4)_2CO_3$). Fractions were collected and assayed for hexose. The molecular weights of the resulting polysaccharides were determined by comparison of their elution positions with those of standard dextrans, T-10 and T-40. The fractions obtained were designated as Fr.1 (21.7 mg), Fr.2 (45.5 mg) and Fr.3 (60.4 mg).

Periodate oxidation.—Periodate oxidation was performed on Fr.1, Fr.2 and Fr.3. Each fraction (2 mg) was oxidized with 0.1 M $NaIO_4$ in 0.5 mL of 0.1 M sodium acetate buffer (pH 5.0) in the dark for 20 h at 4 °C. After the addition of excess ethylene glycol, the products were reduced with $NaBH_4$ in 0.1 M sodium borate buffer (pH 9.5) for 16 h at 4 °C. The resulting non-diffusible fractions were hydrolyzed at 100 °C for 4 h in 2 M trifluoroacetic acid. The hydrolyzates were analyzed by GLC.

Partial methanolysis of Fr.2.—Partial methanolysis of Fr.2 (5 mg) was carried out by treatment with 0.05 M MeOH–HCl for 3.5 h at 80 °C. The product was then dried in vacuo and dissolved in a small volume of water. The solution was subjected to descending paper chromatography on Toyo No 50 filter paper in 1-BuOH/Pyr/H_2O (6:4:3). Oligosaccharides were detected with silver nitrate reagent [7].

Methylation analysis.—Fr.2 was permethylated by the method of Ciucanu and Kerek [8]. The permethylated product was subjected to hydrolysis, reduction and acetylation as

described in a previous paper [5]. The partially methylated alditol acetates of the methylated sugars were analyzed by GLC–MS using a Hitachi M80b instrument [7].

Analytical methods.—Unless otherwise indicated, the analytical methods were the same as described previously [5,7]. The total hexose content was determined by the method of Dubois et al. [9]. Glycerol, hexose and hexosamine were analyzed by GLC after acid hydrolysis, *N*-acetylation and trimethylsilylation [5]. The absolute configurations of component sugars were established by conversion to the corresponding trimethylsilylated *R*-(−)-2-butylgalactosides, followed by GLC [10].

Other materials and methods.—Unless otherwise indicated, the materials and methods were the same as described in previous papers [5,7]. ^{1}H NMR and ^{13}C NMR spectra were recorded (with acetone as the internal standard, δ_H 2.22 and δ_C 30.5) on D_2O solutions at 50 °C with a Jeol FX400 spectrometer. NOE connectivities were measured in the 1D mode by difference spectroscopy (DIFNOE) [11]. Phase sensitive double quantum filter correlated spectroscopy (PHDQF) was performed by the method of Kuman [12]. DEPT was measured by the method of Doddrell et al. [13]. HSQC, HSQC-TOCSY and HMBC spectra were recorded as described in previous reports [14–16].

3. Results and discussion

A cell wall digest prepared by *N*-acetylmuramidase treatment was chromatographed on a column of Sephacryl S-200. The material was separated into three fractions (Fr.1–Fr.3) on the basis of differences in molecular mass. From the elution positions of these fractions on gel filtration, the molecular masses were estimated to be about 50,000, 36,000 and 28,000 for Fr.1, Fr.2 and Fr.3, respectively. All of the fractions were composed of galactose in the D-configuration, and a small amount of glucosamine residues. These glucosamine residues might be derived from peptidoglycan components. Other peptidoglycan components, such as muramic acid and amino acids, were not investigated in this study.

Methylation analysis of Fr.2 was carried out. 2,3,4,6-Tetra-*O*-methylgalactitol, 2,3,5-tri-*O*-methylgalactitol and 2,3-di-*O*-methylgalactitol were detected in a peak area ratio of 1.00:1.01:1.03. Similar results were obtained for Fr.1 and Fr.3. Thus, these galactan fractions contain trisaccharides as the repeating unit. Two galactose residues of this repeating unit were furanosyl rings and one was a pyranosyl ring. These results were confirmed by ^{1}H and ^{13}C NMR data. The spectrum of Fr.2 is presented in Fig. 1; spectra of Fr.1 and 3 were the same as that of Fr.2. Therefore, these materials might have the same structure, but different molecular masses. Three main anomeric signals were observed in ^{1}H and ^{13}C NMR spectra. The two lowest field signals (δ 108.25 and δ 107.27) corresponded to β-galactofuranoside residues, since the anomeric signals of α- and β-galactopyranoside, and α-galactofuranoside do not exceed δ 105. A lower field signal than δ 105 is only observed for the β-configuration of galactofuranoside [17,18]. The signal at δ 98.94 corresponded to α-galactopyranoside residues [18]. These assignments were supported by ^{1}H NMR data. The coupling constants ($^{3}J_{H1,H2}$) of the anomeric protons at δ 5.09 and 5.28 were smaller than 2 Hz. These signals indicate the

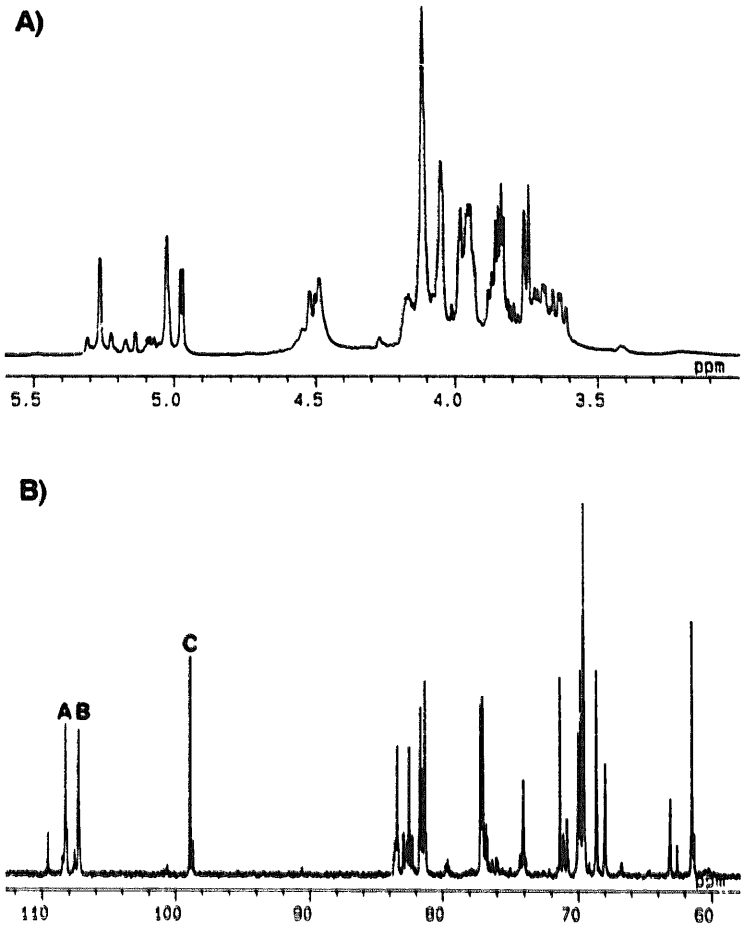


Fig. 1. ^1H (A) and ^{13}C NMR (B) spectra of the galactan (Fr.2), obtained in D_2O at 50 °C. The anomeric carbons are labelled A–C.

presence of two β-galactofuranosyl residues [17,18]. The anomeric signal at δ 4.90 with a coupling constant ($^3J_{H1,H2}$) of 3.3 Hz was assigned to an α-galactopyranosyl linkage. Therefore, this polysaccharide is a galactan consisting of two galactofuranosyl residues and one galactopyranosyl residue. These three residues were designated as Units A, B and C, based on the relative positions of their anomeric carbon signals (low to high field).

Partial acid degradation of Fr.2 was carried out by mild acid treatment (0.01 M HCl, 100 °C, 15 min). The rate of hydrolysis was very low under these conditions and prolonged hydrolysis gave the monosaccharide. Therefore, mild methanolysis was used to obtain the oligosaccharide. The product was separated into two fractions by paper chromatography. The latter were designated as *X* and *Y*. Fraction *X* migrated to the monosaccharide position (R_{Glc} = 0.85), while fraction *Y* migrated to the disaccharide position (R_{Glc} = 0.52). Methylation analysis of fraction *X* by GC–MS revealed the presence of 2,3,4,6-tetra-*O*-methylgalactitol and 2,3,5,6-tetra-*O*-methylgalactitol in a peak area ratio of about 1.00:1.07. This fraction is a mixture of methyl galactopyranosides and methyl galactofuranosides. In the same manner, fraction *Y* gave 2,3,4,6-tetra-

O-methylgalactitol, 2,3,4-tri-*O*-methylgalactitol and 2,3,5-tri-*O*-methylgalactitol in a peak area ratio of about 1.65:0.96:1.00. Considering its migration position on paper chromatography and the above methylation data, fraction *Y* is a mixture of Gal*p*-(1 → 6)-Gal*f*-(1 → OCH_3) and Gal*p*-(1 → 6)-Gal*p*-(1 → OCH_3). Although the reaction mechanism was not elucidated, half of the furanose ring is probably converted into a methylpyranoside through methanolysis, since a 6-*O*-substituted galactopyranoside was not found in the polysaccharide component. Based on the results of this experiment and methylation analysis, a galactopyranose residue is linked to the C-6 position of the galactofuranose residue as a side chain.

To determine the structures of these galactans, the correlation of proton–proton and proton–carbon signals of each unit was analyzed by PHDQF and HSQC. The assignment of each cross-peak was based on the information obtained by DIFNOE, DEPT and HSQC-TOCSY. For example, the C-6 signals of this polysaccharide were identified by the DEPT method. Negative peaks were observed at δ 61.5, δ 68.0 and δ 69.6, which reflect C-6 of each unit. From the results of methylation analysis and partial methanolysis, the signal at δ 61.5 was assigned to C-6 of galactopyranose residues and the other signals to C-6 of galactofuranoside residues, since these signals (δ 68.0 and δ 69.6) are shifted downfield due to the *O*-substitution effect (α-effect). Some information on the assignment of signals to Units A–C were obtained by DIFNOE. Using these results, assignments of the ring carbon and proton signals of each unit were made using HSQC-TOCSY spectra. In brief, the correlation between C-1 and H-1 of each unit was determined from HSQC spectra, and correlation between C-6 and H-6 of each unit was determined by the HSQC, PHDQF and DEPT. The correlations of ring carbons and protons at positions 1 to 3 and 5 to 6 were determined by HSQC-TOCSY. The correlations for positions 4 to 5 were also determined by this method, except in the case of unit C. Though the connectivity of these signals of unit C could not be observed in this experiment, the H-4 signals of each unit were determined by the PHDQF experiment. This assignment led to the assignment of C-4 of unit C in a HSQC experiment.

Table 1
^1H and ^{13}C NMR chemical shifts and coupling constants [a] of Fr.2

Unit	Chemical shift (δ)					
	H-1	H-2	H-3	H-4	H-5	H-6
A	5.09	4.12	4.12	4.17	4.12	3.70
[→ 5,6)-Gal*f*-(β-]	(< 2.0 [b])					3.95
B	5.28	4.12	4.04	4.05	3.95	3.64
[→ 6)-Gal*f*-(β-]	(1.83)					3.85
C	4.90	3.83	3.96	3.98	3.95	3.74
[Gal*p*(α →]	(3.30)					
Unit	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**	**C-6**
A	108.3	81.4	77.1	82.4	74.1	68.0
B	107.3	81.7	77.2	83.6	70.0	69.6
C	98.9	68.7	69.8	69.6	71.4	61.5

[a] Value in parentheses, Hz.
[b] The signal was not well resolved.

These assignments are listed in Table 1. The ^{1}H chemical shifts of units A and B are in good agreement with those reported by Parra et al. [16]. The ^{1}H chemical shift data show that unit A is substituted at positions C-5 and C-6, and unit B at position C-6. This conclusion is consistent with ^{13}C NMR spectral results. The signals of C-5 and C-6 of unit A, and C-6 of unit B are downfield relative to those of methyl β-galactofuranoside [16]. This effect indicates that these positions are glycosylated. The galactopyranose residue is linked to a galactofuranose residue as a side chain. The structure is supported by the results of methylation analysis and periodate oxidation. As judged from the results of periodate oxidation of this polysaccharide, all galactose residues are oxidized, and glycerol and threitol were detected in a molar ratio of about 1.0:2.1. As expected from the substituted positions of units A and B, two moles of threitol were derived from units A and B, and one mole of glycerol was derived from unit C on periodate oxidation.

Considering these results, three possible structures for this polysaccharide are postulated: →6)-Gal*f*-(1→5)-[Gal*p*-(1→6)]-Gal*f*-(1-, →5)[Gal*p*-(1→6)-Gal*f*-(1→6)]-Gal*f*-(β1-, or →6)-[Gal*p*-(1→6)-Gal*f*-(1→5)]-Gal*f*-(β1-. To determine the primary structure of this polysaccharide, HMBC spectra were recorded. Such spectra show long-range connectivities between carbon atoms and their coupled protons through two or three bonds. With these spectra, coupling through a glycosidic linkage can be detected with certainty. The above glycosidic connectivities were determined by the observation of cross-peaks between H-1 of unit A and C-6 of unit B, H-1 of unit B and

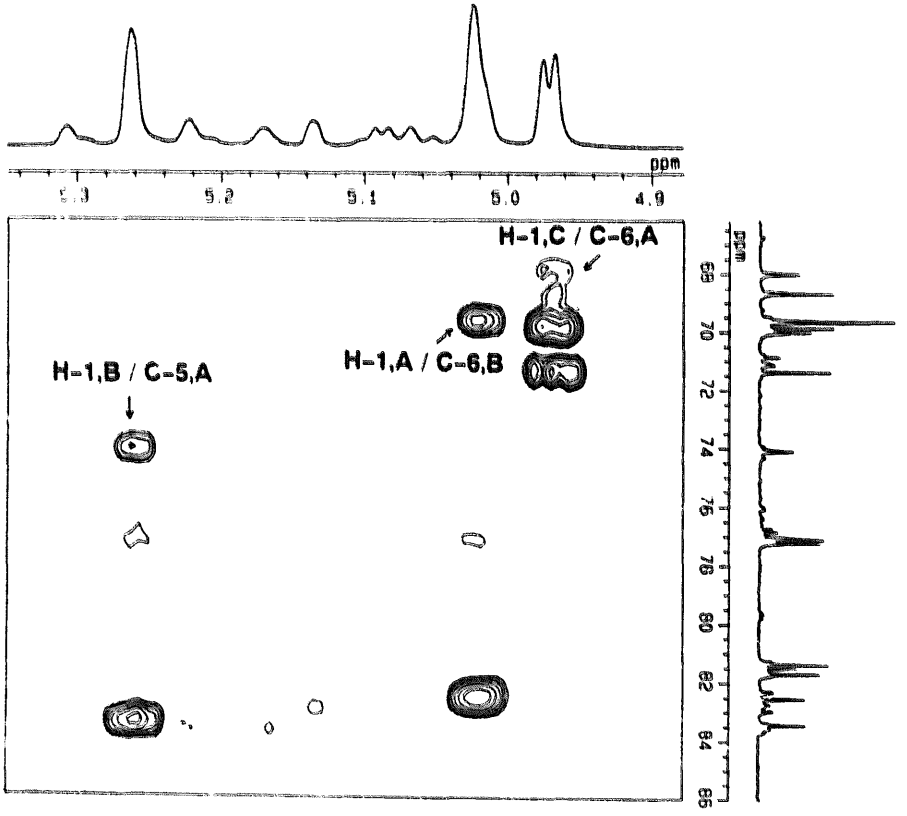
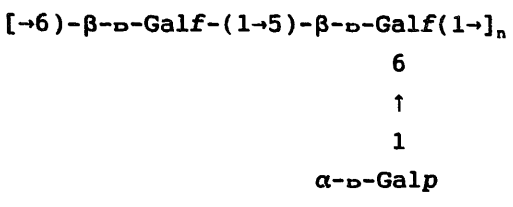


Fig. 2. HMBC spectra of Fr.2, showing ^{1}H–^{13}C long-range connectivities. The anomeric protons, carbons (units A–C), and significant cross-peaks are labelled.

C-5 of unit A, and H-1 of unit C and C-6 of unit A. Therefore, unit C is linked to C-6 of unit A, unit B is linked to C-5 of unit A, and unit A is linked to C-6 of unit B (Fig. 2), and the backbone chain is composed of galactofuranose residues substituted at C-5 and C-6 alternately. These results suggest that the repeating unit of Fr.2 is most probably



From the results of methylation analysis, periodate oxidation and ^{13}C NMR spectroscopy, the polysaccharides in Fr.1 and Fr.3 might have the same structure as that in Fr.2. The differences in molecular mass of these fractions might depend on the length of the repeating unit or the number of polysaccharide chains attached to the glycopeptide component [19]. In this study, we did not investigate the linkage region between the polysaccharide and peptidoglycan moieties of this galactan. The structure of this region and the polysaccharide's function are currently under investigation in our laboratory.

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