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Structure of the extracellular polysaccharide from slime-forming *Lactococcus lactis* subsp. *cremoris* SBT 0495

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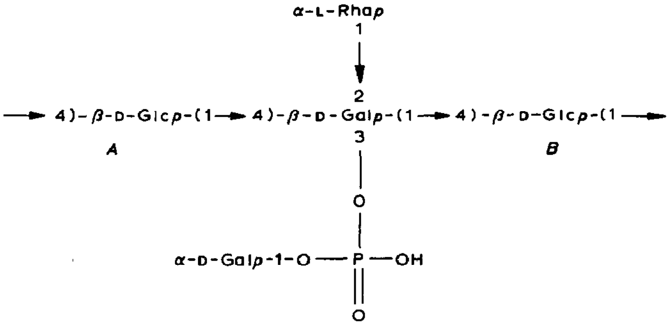
ABSTRACT

The extracellular polysaccharide obtained from slime-forming *Lactococcus lactis* subsp. *cremoris* SBT 0495 is composed of D-glucose, D-galactose, L-rhamnose, and phosphate. Methylation analysis of the native and dephosphorylated polysaccharides provided information on the linkage of the sugar residues and the location of the phosphate group. N.m.r. spectroscopy confirmed the structure of the polysaccharide, which is assigned the following repeating-unit:



$$\begin{array}{ccc} & \alpha\text{-L-Rha}p & \\ & 1 & \\ & \downarrow & \\ & 2 & \\ \rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow & 4)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow & 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow \\ A & 3 & B \\ & | & \\ & \text{O} & \\ & | & \\ & \alpha\text{-D-Gal}p\text{-1-O}-\text{P}-\text{OH} & \\ & \| & \\ & \text{O} & \end{array}$$

INTRODUCTION

Nordic fermented-milk products "viili", "långfil", and "taette" are well known to have a characteristic "ropy" consistency that resembles egg white. They are manufactured using starter cultures containing slime-forming strains of *Lactococcus lactis* subsp. *cremoris* and/or *Lactococcus lactis* subsp. *lactis*[1]. We have reported[2] scanning-electron-microscopic and texture studies on fermented-milk gels produced by slime-forming *L. lactis* subsp. *cremoris* SBT 0495 and its non-slime-forming variant, and these

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indicated that slime materials played important roles in the characteristic ropy consistency and decreased susceptibility to syneresis.

There have been several reports on the nature of the slime material. Sundman[3], and Nilsson and Nilsson[4] reported that it consists of a protein-like substance. Macura and Townsley[5] obtained slime material containing 47% of protein and 28% of carbohydrate, and they stated that it is most likely a glycoprotein. However, we showed that the slime material produced by slime-forming *L. lactis* subsp. *cremoris* SBT 0495 isolated from Finnish "viili" is a mixture of a phosphate-containing polysaccharide and proteins[6]. As separation of polysaccharide from protein by ion-exchange chromatography was unsuccessful, we have purified the polysaccharide by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by ion-exchange chromatography[6].

There are numerous reports concerning structural studies of extracellular polysaccharides secreted by microorganisms[7] but none devoted to lactococci (dairy lactic streptococci[8]). We now report the structure of the polysaccharide purified from the culture supernatant of *L. lactis* subsp. *cremoris* SBT 0495.

RESULTS

Dephosphorylation of the polysaccharide. — The extracellular polysaccharide of *L. lactis* subsp. *cremoris* SBT 0495 contained[6] rhamnose, glucose, galactose, and phosphorus in the molar ratio of 1.3:1.8:2.1:1.0. The D configuration was assigned to glucose and galactose, and the L configuration to rhamnose, by the method of Gerwig *et al.*[9,10]

Treatment of the polysaccharide with 48% hydrogen fluoride (HF) solution and subsequent gel-filtration chromatography on Toyopearl HW-55S yielded three fractions (Fig. 1). Fraction A was a polymeric product which gave a single peak by h.p.l.c. on Asahipak GS-710 and had an apparent molecular weight of 2.5×10^5. This fraction contained L-rhamnose, D-glucose, and D-galactose in the molar proportions 1.0:2.2:1.3 but not phosphorus. Fraction B contained only inorganic phosphate, and no sugar. Fraction C contained only D-galactose and no phosphorus. Phosphate was not released from the native polysaccharide by treatment with alkaline phosphatase.

These results indicated that treatment with 48% HF solution selectively cleaves the phosphate diester linkage without hydrolysis of glycosidic linkages. It is assumed that D-galactose is linked to polysaccharide backbone *via* phosphate diester linkages.

Structure of the dephosphorylated polysaccharide. — Methylation analysis of the dephosphorylated polysaccharide yielded 2,3,4-tri-*O*-methyl-1,5-di-*O*-acetyl-L-rhamnitol, 3,6-di-*O*-methyl-1,2,4,5-tetra-*O*-acetyl-D-galactitol, and 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetyl-D-glucitol in the molar ratio of 1:1:2 (Table I), indicating that the dephosphorylated polysaccharide consisted of tetrasaccharide repeating-unit containing one terminal L-rhamnopyranosyl residue, two D-glucopyranosyl residues linked through O-4, and one D-galactopyranosyl residue linked through O-2 and O-4.

The ^{13}C-n.m.r. spectrum of the dephosphorylated polysaccharide (Fig. 2) gave signals for four anomeric carbons at δ 104.43, 103.17, 101.70, and 101.28, suggesting a

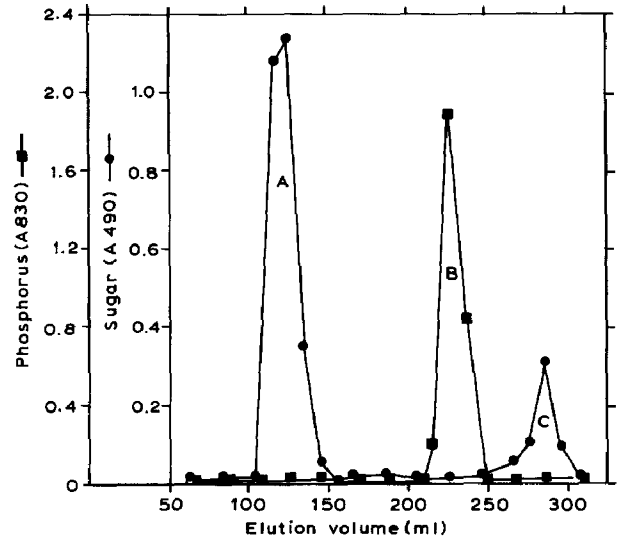


Fig. 1. Gel-filtration chromatography of the HF-treated polysaccharide of *L. lactis* subsp. *cremoris* SBT 0495 on Toyopearl HW-55S. [The polysaccharide was treated with 48% HF solution and 10 mg of hydrolyzate was applied to a column (2.2 mm × 1000 mm) packed with Toyopearl HW-55S and eluted with 0.05M $(NH_4)_2CO_3$. Fractions of 10 mL were collected and analyzed for sugar (●) and phosphorus (■).]

tetrasaccharide repeating-unit. The ^{1}H-n.m.r. spectrum of the dephosphorylated polysaccharide also indicated the presence of four anomeric protons, at δ 5.158 (1 H, not resolved), 4.653 (1 H, $J_{1,2}$ 6.2 Hz), and 4.501 (2 H, $J_{1,2}$ 7.0 Hz). The chemical shifts and coupling constants of the latter two signals suggested the presence of three β-linked sugar residues, which was also confirmed by the ^{13}C-n.m.r. spectrum. Overlapping of two anomeric protons in the last signal was confirmed from a ^{13}C–^{1}H COSY experiment; this signal (δ_H 4.501) gave two cross peaks at δ_c 104.43 and 101.28. Most of the ^{1}H-n.m.r. signals could be assigned by ^{1}H–^{1}H and ^{13}C–^{1}H COSY experiments. The signal at δ 1.256 (3 H, $J_{5,6}$ 5.5 Hz) was assigned to the methyl group of an L-rhamnopyranosyl residue. Starting from this signal, all of the L-rhamnose protons were assigned from cross peaks

TABLE I

Methylation analysis of native (A) and dephosphorylated (B) polysaccharide of *L. lactis* subsp. *cremoris* SBT 0495

Methylated sugars	*Molar ratio*	
	A	*B*
2,3,4,6-Tetra-*O*-methyl-1,5-di-*O*-acetyl-D-(1-^{2}H)galactitol	0.3–0.5[a]	n.d.[b]
2,3,6-Tri-*O*-methyl-1,4,5-tri-*O*-acetyl-D-(1-^{2}H)glucitol	2	2
2,3,4-Tri-*O*-methyl-1,5-di-*O*-acetyl-L-(1-^{2}H)rhamnitol	1	1
3,6-Di-*O*-methyl-1,2,4,5-tetra-*O*-acetyl-D-(1-^{2}H)galactitol	1[c]	1

[a] Molar ratio varied between different analyses. [b] Not detected. [c] Labeled with deuterium at O-3.

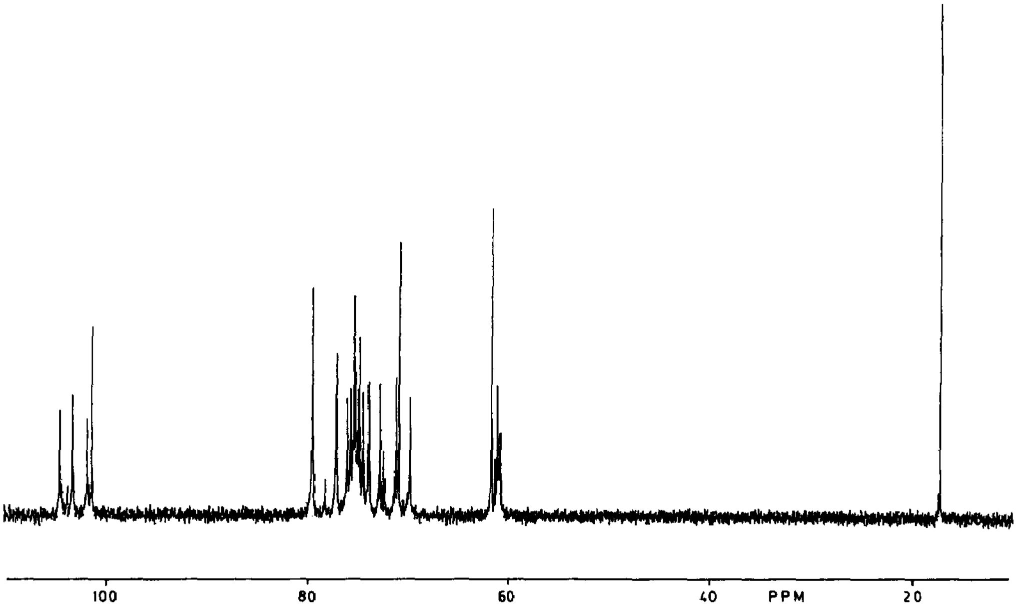


Fig. 2. ^{13}C-N.m.r. spectrum of the dephosphorylated polysaccharide of *L. lactis* subsp. *cremoris* SBT 0495. [The spectrum was recorded at 60° in D$_2$O with 1,4-dioxane (δ_c 67.40) as external standard.]

between contiguous protons in the ^{1}H–^{1}H COSY spectrum; the signal at δ 5.158 was assigned to H-1 of the α-L-rhamnopyranosyl residue. From ^{13}C–^{1}H COSY experiments the signal at δ 101.70 were assigned as the anomeric carbon of an α-L-rhamnose residue. Concerning the remaining three anomeric protons, the overlapped signal at δ 4.501 showed two cross peaks of H-2 protons at both δ 3.698 and 3.352, and another at δ 4.653 was coupled to H-2 at δ 3.393 from the ^{1}H–^{1}H COSY experiment. As the branched β-D-galactopyranosyl residue was bound through O-2 and O-4, the lowest-field H-2 proton at δ 3.698 was assigned to the β-D-galactopyranosyl residue. This result means that one of the overlapped anomeric protons at δ 4.501 was from a β-D-galactopyranosyl residue. Furthermore, since the anomeric-proton chemical shifts of β-D-glucopyranosyl residues bound to different sugars did not have the same value, the other overlapped signal at δ 4.501 was the anomeric proton of a β-D-glucopyranosyl residue. The remaining signal at δ 4.653 was assigned to another β-D-glucopyranosyl residue. Signals at δ 79.39 and δ 77.02 were assigned respectively as C-4 and C-2 of a β-D-galactopyranosyl residue. From cross peaks in ^{13}C–^{1}H ^{1}H–^{1}H COSY experiments, signals at δ 4.501 and δ 101.28 were assigned as H-1 and C-1 of a β-D-galactopyranosyl residue, respectively. A signal at δ 77.12 was assigned as C-4 of the β-D-glucopyranosyl residue labeled as *A* in **1**. Assignments of signals at δ 4.501 and δ 104.43 respectively as H-1 and C-1 of β-D-glucopyranosyl residue *A* in **1** were made from the ^{13}C–^{1}H and ^{1}H–^{1}H COSY spectra. The NOESY results, shown later, confirmed that the anomeric proton of the β-D-glucopyranosyl residue that bound a β-D-galactopyranosyl residue was the signal at δ 4.501. Signals at δ 4.653 and δ 103.17 were assigned as H-1 and C-1 of

TABLE II

^{1}H- And ^{13}C-n.m.r. chemical shifts[a] of dephosphorylated polysaccharide of *L. lactis* subsp. *cremoris* SBT 0495

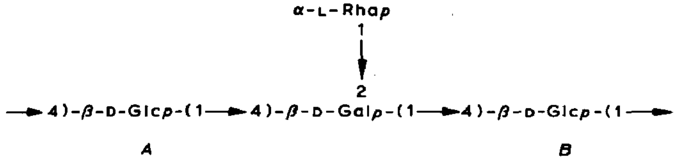
Sugar residue	*H/C*					
	1	*2*	*3*	*4*	*5*	*6*
α-L-Rha*p*-(1-	5.158	4.127	3.784	3.447	3.867	1.256
	101.70	70.88	71.18	72.75	69.80	17.29
-4)-β-D-Glc*p*-(1-*A*	4.501	3.352	3.593	3.698	3.885	n.a.[b]
	104.43	73.87	74.96	77.12	75.33	61.72
-4)-β-D-Gal*p*-(1-2	4.501	3.698	3.885	4.127	n.a.[b]	n.a.[b]
	101.28	77.02	74.78	79.39	75.20	61.08
-4)-β-D-Glc*p*-(1-*B*	4.653	3.393	3.625	3.546	3.784	n.a.[b]
	103.17	74.43	75.64	79.39	76.00	61.72

[a] ^{1}H And ^{13}C-n.m.r. spectra were obtained at 60°. Chemical shifts were relative to the signal of 1,4-dioxane (δ_c 67.40, and δ_H 3.700). [b] Not assigned.

β-D-glucopyranosyl residue *B in* **1**. All of the carbon signals and most of the proton signals were assigned, as shown in Table II.

The sequence of sugar residues in the dephosphoryrated polysaccharide was determined by NOESY spectra. The H-1 signals of the α-L-rhamnopyranosyl residue (δ 5.158) and β-D-glucopyranosyl residue *A* (δ 4.501) showed respectively n.O.e. contacted with H-2 (δ 3.698) of the β-D-galactopyranosyl residue and H-4 of the β-D-galactopyranosyl residue (δ 4.127). The results indicated that the β-D-galactopyranosyl residue was linked through O-2 to a terminal α-L-rhamnopyranosyl residue and O-4 to a β-D-glucopyranosyl residue, in agreement with the results of Jones[11]. The possibility of a β-D-Glc-(1→2)-β-D-Gal sequence in the polysaccharide was ruled out because the chemical shift of C-2 of the β-D-galactopyranosyl residue has been reported[12] at δ 81.04. The n.O.e. results confirmed the assignment of two β-D-glucopyranosyl residues (*A* and *B* in structure **1**) as shown in Table II.

On the basis of the foregoing results, the repeating-unit of the dephosphorylated polysaccharide is formulated as:



$$\begin{array}{c} \alpha\text{-L-Rha}p \\ 1 \\ \downarrow \\ 2 \\ \rightarrow 4)\text{-}\underset{A}{\beta\text{-D-Glc}p}\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\underset{B}{\beta\text{-D-Glc}p}\text{-}(1\rightarrow \end{array}$$

1

Location of the phosphate group. — The linkage position of the phosphate group to the repeating-unit of the polysaccharide was determined by the methods of Lugowski and Jennings[13]. The fully methylated polysaccharide was treated with 48% HF solution to remove the phosphate group, and then re-methylated with trideuteriomethyl iodide in order to label the hydroxyl group originally involved in the phosphate linkage. G.l.c.–m.s. analysis of the hydrolyzate showed that the trideuteriomethyl group was fully incorporated into O-3 of 3,6-di-*O*-methyl-1,2,4,5-tetra-*O*-acetyl-D-galactitol (Table I), indicating that the phosphate group was attached to O-3 of the branched *β*-D-galactopyranosyl residue in **1**. The presence of 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl-D-galactitol in the native polysaccharide and absence of it in the dephosphorylated one (Table I) indicated that a terminal D-galactopyranosyl residue was linked through O-1 to phosphate. Although the molar ratio of 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl-D-galactitol to the other residues varied between the analyses, this phenomenon was pointed out by Jansson, *et al.*[14] They indicated that phosphorus-containing polysaccharides could not be satisfactorily methylated because of partial dephosphorylation during methylation and incomplete hydrolysis of phosphate esters.

The ^{1}H-n.m.r. spectrum of the native polysaccharide is shown in Fig. 3. One additional signal at δ 5.702 (not resolved), absent in the dephosphorylated polysaccharide, was observed the furthest downfield in the anomeric region. It was assigned as a terminal α-D-galactopyranosyl residue in accordance with literature reports[15,16].

From the combined evidence, it is concluded that the polysaccharide is composed of pentasaccharide repeating-units having the following structure:

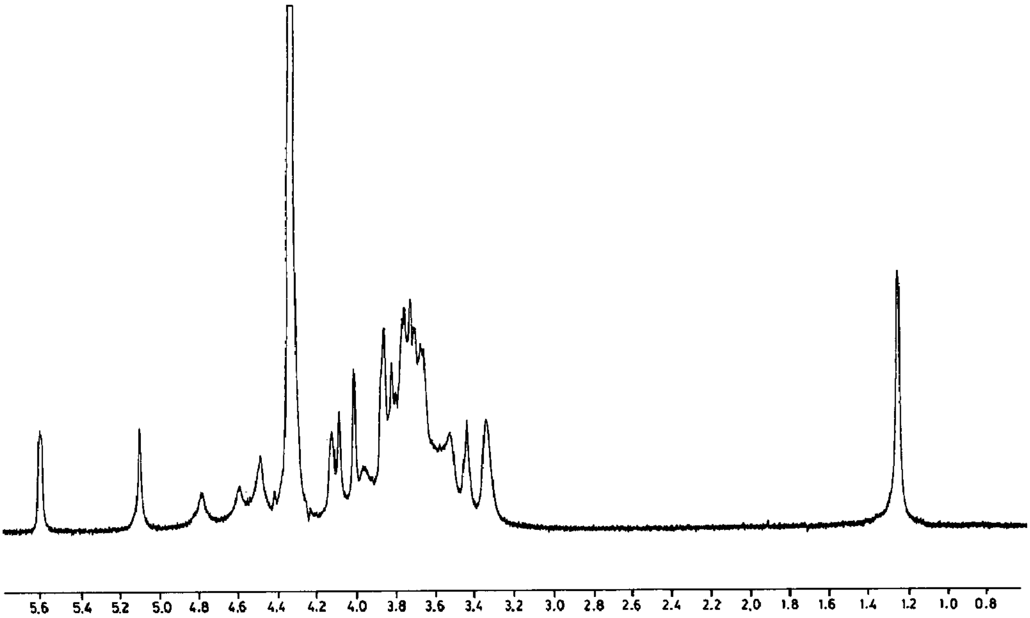
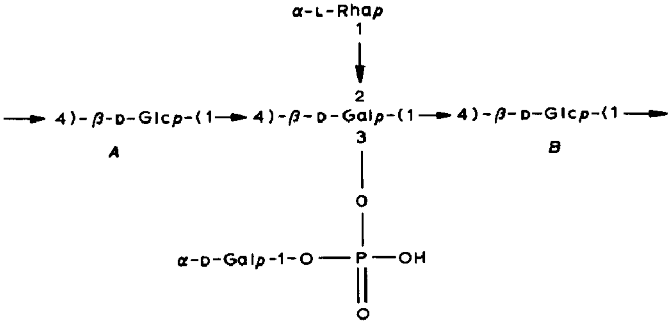


Fig. 3. ^{1}H-N.m.r. spectrum of the native polysaccharide of *L. lactis* subsp. *cremoris* SBT 0495. [The spectrum was measured at 60° in D_2O with 1,4-dioxane (δ_H 3.700) as external standard.]



DISCUSSION

Some strains of dairy lactic acid bacteria are known to produce extracellular polysaccharides[17]. However, the structures of only two of these extracellular polysaccharides have been elucidated: the one produced by *Streptococcus salivarius* subsp. *thermophilus* CNCMI 733, 734, and 735 was shown to have a branched tetrasaccharide repeating-unit consisting of D-galactose, D-glucose, and 2-acetamido-2-deoxy-D-galactose[18]. The capsular polysaccharide produced by *Lactobacillus kefiranofaciens* K_1 was shown to have branched hexa- and hepta-saccharide repeating-unit consisting of D-glucose and D-galactose[16].

In this article, we have elucidated the chemical structure of the extracellular polysaccharide from *L. lactis* subsp. *cremoris* SBT 0495. This polysaccharide is composed of branched pentasaccharide repeating-unit consisting of L-rhamnose, D-galactose, D-glucose, and phosphate. A scanning-electron-microscopic study[2] suggested that the characteristic "ropy" property of milk fermented by *L. lactis* subsp. *cremoris* SBT 0495 is caused by strong interaction between acid-coagulated milk protein and the polysaccharide. Lipoteichoic acid, which has also negatively charged phosphate groups, is considered to interact strongly with proteins[19–21]. It may be easily suspected that the negative charge of the phosphate group in the polysaccharide causes formation of a complex with protein, and results in the "ropy" character of the milk gel.

It has been reported that extracellular polysaccharides from *S. salivarius* subsp. *thermophilus*, CNRZ 1100, 1068, and 1066 (ref. 22) and those from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 416 and 737 (ref. 23) were difficult to separate from proteins by DEAE ion-exchange chromatography, suggesting that these polysaccharides also interact with the milk proteins. Those polysaccharides were shown to be composed of D-glucose, D-galactose, and L-rhamnose (absent in *S. salivarius* subsp. *thermophilus* strains), and contained neither uronic acids nor amino sugars. However, it is unclear whether phosphate groups are included in the aforementioned polysaccharides or not.

EXPERIMENTAL

Bacterial growth and isolation of the extracellular polysaccharide. — *Lactococcus lactis* subsp. *cremoris* SBT 0495 was isolated from Finnish fermented milk "viili" and maintained as described previously[6]. The bacterium was grown in whey permeate medium, and the extracellular polysaccharide was isolated from culture supernatant and purified by preparative SDS-PAGE[6].

General methods. — Colorimetric determination of sugar and phosphorus was performed as described previously[6]. Monosaccharide composition was determined by g.l.c.[6]. H.p.l.c. analysis of the polysaccharide was performed on an Asahipak GS-710 column using 0.1M NaCl as solvent[6]. Absolute configurations of sugars were determined by g.l.c. analysis of their trimethylsilylated (−)-2-butyl glycosides according to the method of Gerwing *et al.*[9,10]

N.m.r. spectroscopy. — N.m.r. spectra were recorded for solutions in D_2O at 60° with a Bruker-AM600 spectrometer. Chemical shifts are given in p.p.m., using external 1,4-dioxane (δ_c 67.40, δ_H 3.700) as reference. ^{13}C–^{1}H and ^{1}H–^{1}H COSY, and NOESY experiments were performed according to Bruker standard pulse-sequences. For the correlation spectrometry, a 90° mixing pulse was used. The NOESY experiment was performed using a mixing time of 200 ms.

Dephosphorylation of the polysaccharide. — The dried sample of polysaccharide (10 mg) was treated[24] with 48% HF solution (0.5 mL) for 48 h at 0°. The excess of HF was removed under vacuum over KOH at 4° in a plastic desiccator. The residue was dissolved in 0.05M NH_4HCO_3 and fractionated on a TSK gel Toyopearl HW-55S (Tosoh Corporation, Tokyo, Japan) column (22 × 1000 mm) using 0.05M NH_4HCO_3 as an eluant.

Methylation analysis. — Methylation of polysaccharides was performed according to the method of Kvernheim[25]. The methylated polysaccharide was hydrolyzed and the partially methylated monosaccharides were converted into their alditol acetate derivatives by the method of Harris *et al.*[26]. G.l.c.–m.s. of the partially methylated alditol acetates was achieved as described previously[27].

In order to determine the location of phosphate group in the polysaccharide, the method of Lugowski and Jennings[11] was adopted. The methylated polysaccharide was purified by silica-gel chromatography (Wako gel S-1, Wako Pure Chemicals, Osaka, Japan). The permethylated polysaccharide was treated with 48% HF solution for 2 days at 0° to remove phosphate groups. The resultant, partially methylated polysaccharide, was remethylated with CD_3I. The permethylated polysaccharide was then hydrolyzed and the component monosaccharides were identified as alditol acetate derivatives.

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