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The structure of the antigenic polysaccharide produced by *Eubactrium saburreum* T15

Naomi Sato,[a,*] Futoshi Nakazawa,[a] Teiichiro Ito,[a] Tsutomu Hoshino,[b] Etsuro Hoshino[a]

[a]*Department of Oral Health Science, Division of Oral Ecology in Health and Infection, Course for Oral Life Science, Niigata University Graduate School of Medical and Dental Sciences Gakkocho-dori 2, Niigata 951-8514, Japan*
[b]*Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, Ikarashi, Niigata 950-2181, Japan*

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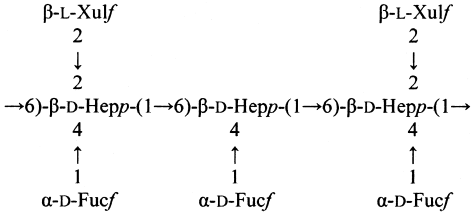
Abstract

The antigenic polysaccharide was obtained from the cell wall of *Eubacterium saburreum* strain T15 by trypsin digestion followed by gel permeation and ion-exchange chromatography. Its structure was determined using acid hydrolysis, methylation analysis, and 1D and 2D NMR spectroscopy. It contained L-*threo*-pent-2-ulose (Xul), D-fucose (Fuc), and D-*glycero*-D-*galacto*-heptose (Hep) in 2:3:3 ratio. Methylation analysis indicated an octasaccharide repeating-unit containing five branches. The ^{1}H and ^{13}C signals in NMR spectra of the sugar residues were assigned by COSY, HOHAHA, and HMQC 2D experiments, and the sequence of sugar residues in the repeating unit was determined by NOESY and HMBC experiments. The polysaccharide also contains two *O*-acetyl groups in the repeating unit, located on the Hep residue. The repeating structure can be written as:



This is a novel structure in bacterial cell-wall polysaccharides from Gram-positive bacteria.

Keywords: *Eubacterium saburreum*; Antigenic polysaccharide; L-*threo*-Pent-2-ulose (L-xylulose); D-Fucofuranose; D-*glycero*-D-*galacto*-Heptopyranose; 2D NMR spectroscopy data

1. Introduction

The bacterium *Eubacterium saburreum* is a Gram-positive, anaerobic and filamentous rod which is frequently isolated from human dental plaques,[1,2] periodontal pockets,[3] and infectious dental pulp.[4] It is known that bacteria produces antigenic polysaccharide with unique sugar compositions in the cell walls and that they contain a linear chain composed of Hep as their core structure.[6–14] According to their sugar components, *E. saburreum* has been classified previously into three chemotypes.[5] *E. saburreum* strains L44,[6] T17,[7] T18[8] and T27,[9] all of which produce homopolysaccharides composed of Hep, are representatives of chemotype I. *E. saburreum* strains L49,[10] O2,[11] S29,[12] T21[13] and T110[14] are representatives of chemotype II since their heteropolysaccharides are composed of Hep and 6-deoxy-D-*altro*-heptose. *E. saburreum* strain T19,[5] belongs to chemotype III, and produces another heteropolysaccharide that is composed of Fuc and Hep.

* Corresponding author. Tel.: +81-25-2272839; fax: +81-25-2270806
E-mail address: sa-naomi@dent.niigata-u.ac.jp (N. Sato).

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E. saburreum T15 was isolated from a human supragingival plaque in our laboratory and is a novel strain.[1] The antigenic polysaccharide of *E. saburreum* T15 is composed of unusual sugar components, namely Fuc, Hep, and a ketopentose.[15] The ketopentose had been identified as Xul, a specific component in the antigenic polysaccharide of *E. saburreum* T15. The antigenic polysaccharide of *E. saburreum* T15 did not react against any antisera of *previously isolated strains* of *E. saburreum* in immunodiffusion tests, suggesting that this polysaccharide may be different from any antigenic polysaccharides reported previously. We describe here the novel structure of this antigenic polysaccharide.

2. Results and discussion

2.1. Sugar composition of the antigenic polysaccharide

Gel permeation chromatography shows that the molecular weight (M_W 56,000) of the purified antigenic polysaccharide of *E. saburreum* T15 is similar to that of *E. saburreum* T19.[5] To elucidate the structure of this antigenic polysaccharide, mild acid hydrolysis was performed with 10 mM hydrochloric acid at 25 °C for 48 h, resulting in a degraded polysaccharide (modified polysaccharide) and a ketopentose. After borodeuteride reduction and peracetylation, the ketopentose was identified as Xul[15] by GC, GC–MS, and ^{1}H NMR and as L-Xul[16] from its specific optical rotation ($[\alpha]_D^{20}$ + 24.3°, *c* 1.0, water).[15] The modified antigenic polysaccharide yielded two sugars upon stronger acid hydrolysis. The GC and GC–MS analysis of their alditol acetate indicated that they were a 6-deoxyhexose and an aldoheptose. Their ^{1}H NMR spectra and optical rotations showed them to be D-Fuc ($[\alpha]_D^{20}$ + 73.0°, *c* 1.0, water) and D-Hep ($[\alpha]_D^{20}$ + 58.0°, *c* 1.0, water).

The anomeric configurations of the sugar components were determined by comparison of optical rotation of the polysaccharide with those of its partially hydrolyzed products, and indicated that L-Xul is β-linked, and D-Fuc and D-Hep are α- and β-linked, respectively.

The polysaccharide contained Xul, Fuc, and Hep in the approximate molar ratio of 2:3:3, estimated from the peak areas of their alditol acetate derivatives.[15]

2.2. Analysis of the antigenic polysaccharide

Methylation analysis of the polysaccharide revealed the presence of 1,3,4-tri-*O*-methyl-Xul, 2,3,5-tri-*O*-methyl-Fuc, 2,3,7-*O*-tri-methyl-Hep, and 3,7-*O*-di-methyl-Hep in the ratios 1.7:3.0:1.0:2.0, respectively (Table 1, column A). This proportion is different from that (2.0:3.0:1.0:2.0) expected from sugar components, probably because of the volatility and lability of 1,3,4-tri-*O*-methyl-Xul under conditions of acid hydrolysis. These results suggest that Xul and Fuc are terminal furanosyl groups and that Hep is a pyranosyl branching residue linked at *O*-4,6 and *O*-2,4,6. The molar ratios of sugar residues indicated that the repeating unit is an octasaccharide containing two L-*threo*-pent-2-ulo-furanosyl (Xul*f*) groups, three D-fucofuranosyl (Fuc*f*) groups and three D-*glycero*-D-*galacto*-heptopyranosyl (Hep*p*) residues.

High resolution 1D ^{1}H NMR data for the polysaccharide in D_2O at 600 MHz showed two signals in the anomeric region at δ 5.19 and 4.65 (Table 2) which agreed with chemical shifts of anomeric protons of terminal α-fucofuranosyl group (δ 5.17)[5] and β-(1→6)-linked D-Hep*p* (δ 4.62)[17] in the antigenic polysaccharides of *E. saburreum* T19 and O2, respectively. A sharp three-proton signal at δ 1.22 (3 H, d, $J_{5,6}$ 6.3 Hz) was assigned to H-6 (CH_3) of Fuc*f*. Another sharp signal at

Table 1
Alditol acetates obtained by methylation analysis of the antigenic polysaccharide from *E. saburreum* strain T15 and of the modified polysaccharide by mild hydrolysis

O-Methylalditol acetates of Xul, Fuc and Hep	T_R [a]	Polysaccharides		Decrease or increase after mild acid hydrolysis (B–A)
		PS [c] (A)	MP [d] (B)	
1,3,4-*O*-Me-Xul	0.39 [b]	1.7	0	−1.7
2,3,5-*O*-Me-Fuc	0.41	3.0	3.0	0
2,3,7-*O*-Me-Hep	1.00	1.0	3.0	+2.0
3,7-*O*-Me-Hep	1.78	2.0	0	−2.0

[a] Retention times of partial methyl alditol acetates relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an OV 225 column.
[b] The methyl acetates of xylitol and arbinitol from Xul are not separated on this column.
[c] Antigenic polysaccharide.
[d] Modified polysaccharide.

Table 2
^{1}H NMR data (δ, ppm) for the antigenic polysaccharide, the O-deacetylated polysaccharide and the modified polysaccharides from *E. saburreum* T15

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6	H-7
Antigenic polysaccharide **							
β-L-Xul*f*-(2→	3.64 *		4.05	4.40	4.28 *		
	3.75 *				3.55 *		
α-D-Fuc*f*-(1→	5.19	4.01	4.15	3.63	3.84	1.22	
→6)-β-D-Hep*p*-(1→	4.65	3.50	3.65	4.11	4.20	3.77	4.5–4.6 *
		3.83					
O-Deacetylated polysaccharide							
β-L-Xul*f*-(2→ [a]	3.52 *		4.05	4.37	4.27 *		
	3.67 *				3.56 *		
α-D-Fuc*f*-(1→ [b]	5.22	3.96	4.13	3.63	3.83	1.20	
	5.33		4.21	4.21			
→6)-β-D-Hep*p*-(1→ [c]	4.72	3.50	3.64	4.11	4.19	3.72	3.78 *
		3.87				4.11	3.80 *
							4.08 *
							4.02 *
Modified polysaccharides ***							
α-D-Fuc*f*-(1→	5.14	4.02	4.14	3.63	3.85	1.20	
→6)-β-D-Hep*p*-(1→	4.65	3.63	3.73	4.14	4.15	3.46	3.72 *
							4.02 *

* Non-equivalent CH_2 protons.
** Chemical shift for *O*-acetyl group is δ 2.11 (3 H).
*** Chemical shift for *O*-acetyl group is δ 2.07 (3 H).
[a] β-L-*threo*-Pent-2-ulo-furanose-(2→.
[b] α-D-Fucofuranose-(1→.
[c] →6)-β-D-*glycero*-D-*galacto*-Heptopyranose-(1→.

δ 2.11 (3 H) was assigned to CH_3 of the *O*-acetyl group. The *proton-decoupled* ^{13}C NMR spectrum (Fig. 1, Table 3) showed four signals for anomeric carbons at δ 105.0, 101.8, 98.9, and 97.0. Three signals, at δ 19.1, 20.7 and 173.9, were attributed to *C*H$_3$ of Fuc*f* *C*H$_3$ and *C*=O of the *O*-acetyl group, respectively. The ^{1}H NMR analysis suggested that the above polysaccharide bore approximately two *O*-acetyl groups per repeating unit, according to the ratio of integral intensities between the CH_3 signal of the *O*-acetyl group and the CH_3 signal of the Fuc*f* group.

A DEPT 135 spectrum indicated that the characteristic signals of one –C–*C*H$_2$–O– group at δ 70.4 and two *C*H$_2$OH (unsubstituted) signals at δ 61.6 and 58.7 were belonged to C-5 and C-1 of β-Xul*f* and C-7 of β-Hep*p*, respectively. The ^{1}H and ^{13}C resonances for sugar residues (Tables 2 and 3) were assigned using combinations of DEPT and 2D NMR (COSY, HOHAHA, and HMQC) experiments, according to the established chemical shift values.[16–20] A signal at δ 105.0 in the polysaccharide (Table 3), proved to be that of a non-protonated carbon by an HMQC experiment, and was consistent with the chemical shift (δ 105.8) of C-2 of methyl β-D-*threo*-pent-2-ulofuranoside,[18] its enantiomer. Therefore, Xul proved to be a terminal β-L-Xul*f* group. A signal at δ 101.8 was assigned to the anomeric carbon of the terminal α-D-Fuc*f* group because the chemical shift agreed with those (δ 103.0 and 102.8) of anomeric carbons of methyl α-D-fucofuranoside[20] and of the terminal α-D-Fuc*f* group from the O-specific polysaccharide of *Pseudomonas syringe*.[20] Two signals, at δ 98.9 and 97.0, belonged to the anomeric carbon of Hep*p* by comparison with those of methyl β-D-*glycero*-D-*galacto*-heptopyranoside.[17] Furthermore, the substituted Hep*p* was characterized by low-field displacements of carbons C-2, C-4, and C-6 (δ 78.4, 76.8 and 71.7) when compared with their corresponding positions in methyl β-D-*glycero*-D-*galacto*-heptopyranoside (Table 3). This indicated the presence of 2,4,6-*O*-tri-substituted Hep*p*. Another chemical shift (δ 71.7) of C-2 of Hep*p* agreed with that of C-2 of methyl β-D-*glycero*-D-*galacto*-heptopyranoside and showed the presence of 4,6-*O*-di-substituted Hep*p*. Thus, ^{13}C NMR data proved the presence of terminal Xul*f*, terminal Fuc*f*, 4,6-*O*-di-substituted Hep*p* and 2,4,6-*O*-tri-substituted Hep*p* in the polysaccharide. These results further confirmed that the substitution pattern was defined by methylation analysis.

2.3. Analysis of the O-deacetylated polysaccharide

To locate the *O*-acetyl group, the O-deacetylated polysaccharide was prepared from the antigenic polysaccharide by dilute alkaline treatment. Comparison of ^{1}H NMR spectra of the original polysaccharide and its O-deacetylated derivative showed a downfield shift from δ 3.78–4.08 to 4.5–4.6 of the H-7 signal of Hep*p*, reflecting the presence of an *O*-acetyl group at O-7 of Hep*p* (Table 2). This was confirmed by the effect of O-acetylation on the C-7 signal of Hep*p* which was shifted from δ 55.8 in the ^{13}C NMR spectrum of the O-deacetylated polysaccharide to δ 58.7 in that of the original polysaccharide (Table 3).

2.4. Analysis of the modified polysaccharide

To confirm the glycosidic linkage position of the terminal Xul*f* group, the polysaccharide was subjected to mild acid hydrolysis. The modified polysaccharide obtained was composed of Fuc and Hep in approximately equimolecular amounts and was subjected to methylation analysis (Table 1, column B). The major changes observed after mild acid hydrolysis are decreases in the amounts of 1,3,4-tri-*O*-methyl-Xul and 3,7-di-*O*-methyl-Hep, an increase in the amount of 2,3,7-tri-*O*-methyl-Hep, and no recognized change in the amount of 2,3,5-tri-*O*-methyl-Fuc (Table 1, column A–B). These results indicates that the terminal Xul*f* group was linked to O-2 of 2,4,6-tri-*O*-substituted Hep*p* and that the terminal Fuc*f* group was not liberated from the modified polysaccharide and was linked to O-4 or O-6 of 4,6-di-*O*-substituted Hep*p*.

The ^{1}H NMR data of the modified polysaccharide showed that two signals, at δ 5.14 and 4.65, could be assigned to Fuc*f* and Hep*p*, and a doublet signal at δ 1.20 ($J = 6.3$ Hz) to H-6 of Fuc*f* (Table 2). The signal at δ 4.65, doublet ($J_{1,2} = 7$ Hz),[17] indicated that the Hep residues are β-linked. The ^{13}C NMR data showed that two signals, at δ 102.4 and 99.9, could be assigned to anomeric carbons of Fuc*f* and Hep*p* (Table 3), respectively. A signal at δ 58.8 was ascribed to C-7 (CH_2OH) of Hep*p* by a DEPT 135 experiment. The proton and carbon assignments for two sugar residues in the modified polysaccharide were performed using an HMQC experiment (Tables 2 and 3). The C-2 (δ 78.4) signal of Hep*p* in the antigenic polysaccharide was shifted 6.4 ppm upfield as compared with that of C-2 (δ

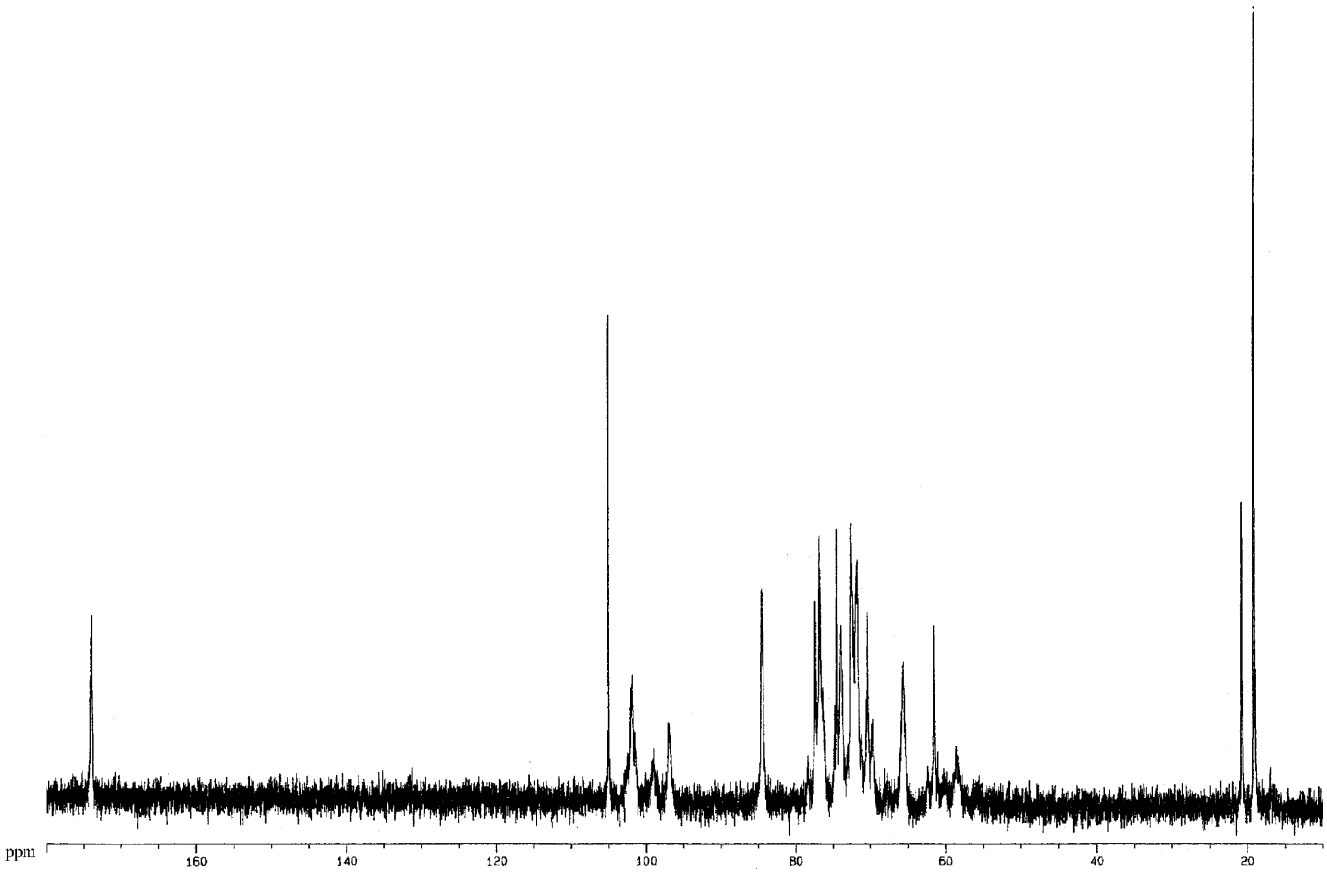


Fig. 1. 150.9 MHz ^{13}C NMR spectrum of the antigenic polysaccharide from *E. saburreum* T15 in D_2O at 23 °C.

Table 3
^{13}C NMR data (δ, ppm) for the antigenic polysaccharide, the O-deacetylated polysaccharide and the modified polysaccharides from *E. saburreum* T15

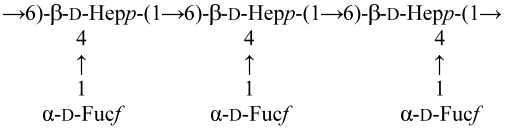
Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	C-7
Antigenic polysaccharide *							
β-L-Xul*f*-(2→	61.6	105.0	77.4	74.5	70.4		
α-D-Fuc*f*-(1→	101.8	76.8	74.0	84.5	65.6	19.1	
→6)-β-D-Hep*p*-(1→	97.0	71.7	72.6	76.8	74.0	71.7	58.7
	98.9	78.4					
O-Deacetylated polysaccharide							
β-L-Xul*f*-(2→	62.3	105.0	78.5	74.8	70.5		
α-D-Fuc*f*-(1→	101.3	76.9	73.8	84.4	65.7	19.0	
	101.7					19.1	
→6)-β-D-Hep*p*-(1→	96.7	71.9	72.6	76.9	73.8	71.9	55.8
	99.3	76.9					58.5
Modified polysaccharide *							
α-D-Fuc*f*-(1→	102.4	76.7	73.8	84.8	65.9	19.9	
→6)-β-D-Hep*p*-(1→	99.9	72.0	72.3	77.3	74.5	71.7	58.8
Methyl glycosides							
β-D-Xul*f*-(2→Me [a]	60.7	105.8	79.1	76.1	71.2		
α-D-Fuc*f*-(1→Me [b]	103.0	77.8	76.1	86.4	70.6	18.6	
β-D-Hep*p*-(1→Me [c]	104.9	71.9	74.2	69.9	75.6	68.8	64.0

* The chemical shifts for *O*-acetyl group are δ 20.7 (CH_3CO) and 173.9 (CH_3CO).
[a] Methyl β-D-*threo*-pent-2-ulo-furanoside.
[b] Methyl α-D-fucofuranoside.
[c] Methyl β-D-*glycero*-D-*galacto*-heptopyranoside.

72.0) of Hep*p* in the modified polysaccharide. This indicated that O-2 of 4,6-di-*O*-substituted Hep*p* in the polysaccharide was substituted by a terminal Xul*f* group. Low-field displacements of the signals at δ 77.3 for C-4 and δ 71.7 for C-6 of Hep*p* were observed and corresponded with their position in methyl β-D-*glycero*-D-*galacto*-heptopyranoside.[18] These data confirmed that the repeating unit of the modified polysaccharide is composed of equimolecular amounts of 4,6-di-*O*-substituted Hep*p* and the terminal Fuc*f* group.

2.5. Analysis of the polymeric product

To determine the position of attachment of the terminal Fuc*f* group, the modified polysaccharide was subjected to a second mild hydrolysis (50 mM HCl at 80 °C for 210 min). The polymeric product obtained was composed of Hep and yielded 2,3,4,7-tetra-*O*-methyl-Hep upon methylation analysis (data not shown). This result strongly suggested that the polymeric product was composed of 6-*O*-substituted Hep*p* residues. These results show that the main chain of the modified polysaccharide is composed of 4,6-di-*O*-substituted Hep*p* substituted with terminal α-D-fucofuranose groups at O-4 (structure **1**).



1

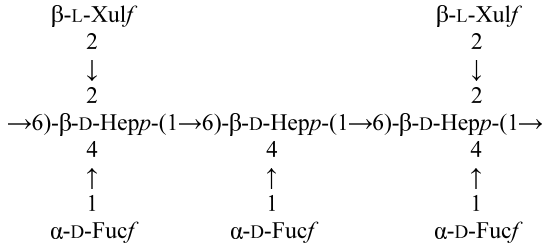
The positions of glycosylation and sequences of the sugar residues in the antigenic polysaccharide, the O-deacetylated polysaccharide, and the modified polysaccharide were examined by two-dimensional NOESY experiments (Table 4). These revealed the following correlations between the transglycosidic protons, Xul*f* H-1/Hep*p* H-2 (δ 3.64/3.83, antigenic polysaccharide; δ 3.67/3.87, O-deacetylated polysaccharide), Fuc*f* H-1/Hep*p* H-4 (δ 5.19/4.11, antigenic polysaccharide; δ 5.22/4.11, O-deacetylated polysaccharide; δ 5.14/4.14, modified polysaccharide), Hep*p* H-1/Hep*p* H-6 (δ 4.72/3.72, O-deacetylated polysaccharide; δ 4.65/3.46, modified polysaccharide). The NOE correlation between H-1 of Xul*f* and H-2 of Hep*p* suggested that these protons were close to each other and C-2 of Xul*f* was linked to C-2 of Hep*p* by ketoglycosidic linkage. The other NOE correlations suggested glycosyl linkages between H-1 of Fuc*f* and H-4 of Hep*p*, and H-1 of Hep*p* and H-6 of Hep*p*, respectively. In addition to the

interresidue correlation peaks, there were intraresidue cross-peaks Hep*p* H-1/H-3 (δ 4.72/3.64) and H-1/H-5 (δ 4.72/4.19) for Hep*p* and Fuc*f* H-1/H-2 (δ 5.22/3.96) for Fuc*f* in the NOESY spectrum of the O-deacetylated polysaccharide (data not shown). These results indicated that Fuc*f* and Hep*p* were α- and β-linked, respectively, in agreement with the anomeric configuration of glycosidic linkages established by specific optical rotation.

To determine the sequence of sugar residues, an HMBC experiment was carried out. The data revealed the following interresidue correlations between the transglycosidic protons and carbons separated by three bonds (Table 4); Fuc*f* H-1/Hep*p* C-4 (δ 5.22/76.90, O-deacetylated polysaccharide; δ 5.14/77.30, modified polysaccharide), Fuc*f* C-1/Hep*p* H-4 (102.4/4.14, modified polysaccharide), Hep*p* H-1/Hep*p* C-6 (δ 4.65/71.70, modified polysaccharide), Hep*p* C-1/Hep*p* H-6 (δ 99.9/3.46, modified polysaccharide), respectively.

Interresidue NOE and HMBC correlations were observed for the following glycosidic linkages; α-Fuc*f*-(1→4)-β-Hep*p*-(1→ and →6)-β-Hep*p*-(1→6)-β-Hep*p*-(1→. Although a correlation peak for Xul*f*-β-(2→2)-Hep*p*-β-(1→ was not observed, the ketoglycosidic linkage was been proved by methylation analysis and a NOESY experiment.

On the basis of the aforementioned results, it can be concluded that the antigenic polysaccharide of *E. saburreum* T15 is composed of an octasaccharide repeating unit with five branches having following structure:



It has been reported previously that the antigenic polysaccharide of *E. saburreum* T19 classified in chemotype III is composed of α-D-Fuc*f* and β-D-Hep*p*.[5] β-L-Xul*f* has been reported as one of the sugar components in the lipopolysaccharide from *Yersinia enterocolitica*.[16] Immunodiffusion tests have also demonstrated previously that the antigenic polysaccharide of *E. saburreum* T15 does not react with anti-T19 serum and that L-Xul*f* is an immunodeterminant sugar in the antigenic polysaccharide of *E. saburreum* T15.[15] According to the novel chemical structure and the immunological specificity, it is now suggested that *E. saburreum* T15 should be classified in a new chemotype, IV.

3. Experimental

3.1. Bacterial strain and growth condition

E. saburreum strain T15 was isolated from dental plaque of the human oral cavity. The same culture conditions were used as previously described.[11]

Table 4
Interglycosidic correlations from the anomeric atoms observed in NOESY and HMBC spectra of the antigenic polysaccharide, the O-deacetylated polysaccharide and the modified polysaccharide from *E. saburreum* T15

Sugar residue	H-1(δ) or C-1(δ)	NOE to proton (δ)	HMBC to C or H(δ)	Correlation	Polysaccharides
β-L-Xul*f*-(2→	3.64	3.83		Xul H-1 to Hep H-2	PS [a]
	3.67	3.87		Xul H-1 to Hep H-2	DP [b]
α-D-Fuc*f*-(1→	5.19	4.11		Fuc H-1 to Hep H-4	PS
	5.22	4.11		Fuc H-1 to Hep H-4	DP
	5.22		76.9	Fuc H-1 to Hep C-4	DP
	5.14	4.14		Fuc H-1 to Hep H-4	MP [c]
	5.14		77.3	Fuc H-1 to Hep C-4	MP
	102.4		4.14	Fuc C-1 to Hep H-4	MP
β-D-Hep*p*-(1→	4.72	3.72		Hep H-1 to Hep H-6	DP
	4.65	3.46		Hep H-1 to Hep H-6	MP
	4.65		71.7	Hep H-1 to Hep C-6	MP
	99.9		3.46	Hep C-1 to Hep H-6	MP

[a] Antigenic polysaccharide.
[b] O-Deacetylated polysaccharide.
[c] Modified polysaccharide.

3.2. Extraction and purification of the antigenic polysaccharide

The antigenic polysaccharide was extracted from the bacterial cells by digestion with trypsin.[21] The digest was dialyzed against distilled water and purified by gel permeation chromatography on a Sepharose 6B column (1.4 × 145 cm), by ion-exchange chromatography on a DEAE cellulose column (2.4 × 30 cm), and on a hydroxyapatite column (2.4 × 10 cm). All of the eluates from the columns were monitored by the phenol–H_2SO_4 method.[22] The antigenic activity of the polysaccharide was examined by a capillary precipitation test[23] with anti-*E. saburreum* T15 rabbit serum that had been prepared by intravenous injection of formaldehyde-killed whole cells of strain T15. One gram of the bacterial cells gave 12 mg (dry weight) of the purified antigenic polysaccharide. The molecular weight of the antigenic polysaccharide was determined on a TSK HW-55 (superfine) high resolution column (1.4 × 300 cm) eluting with 20 mM phosphate buffer, pH 7.2, containing 0.02% NaN_3. The molecular-weight range and retention characteristics of the column were determined by using dextran standards (T10, T40, and T70).

3.3. Preparation of O-deacetylated polysaccharide, modified polysaccharide, and polymeric product

The antigenic polysaccharide was treated with 10 mM NaOH at 25 °C for 48 h and the O-deacetylated polysaccharide obtained by dialysis and lyophilization. The antigenic polysaccharide was mild acid hydrolyzed with 10 mM HCl at 25 °C for 48 h. After neutralization, the hydrolyzate was applied to a column (1.4 × 300 cm) of TSK HW-55 gel and gave two peaks. The first peak, in the high molecular weight region, was termed the modified polysaccharide, and the second peak, in the monosaccharide region, was concentrated and purified by preparative paper chromatography on Whatman No. 1 paper using 6:4:3 (v/v) 1-butanol–pyridine–water and then identified as *threo*-pent-2-ulose.[15] After a second mild acid hydrolysis with 50 mM HCl at 80 °C for 210 min followed by dialysis, the modified polysaccharide gave the polymeric product.

3.4. Sugar components

The antigenic polysaccharides (2 mg) were hydrolyzed with 1 M HCl (1 mL) for 5 h at 100 °C. Monosaccharides were reduced with $NaBD_4$ and per-*O*-acetylated.[24] The derivatives were analyzed in a Hitachi 163 gas chromatograph equipped with a flame-ionization detector at 150 °C using a capillary glass column (0.25 mm × 50 m) coated with OV-225. Optical rotations were determined in distilled water using a photoelectric polarimeter (Horiba SEPA-200).

3.5. Determination of α, β anomeric configuration

Anomeric configurations of the sugar components in the antigenic polysaccharide were determined as follows. The specific rotation of the antigenic polysaccharide, $[\alpha]_D^{20}$ + 39.0° (*c* 0.7, water), decreased on mild acid hydrolysis and from which ~95% of L-*threo*-pent-2-ulose had been released. The remaining modified polysaccharide had $[\alpha]_D^{20}$ + 20.0° (*c* 1.0, water). The difference of optical rotation between the former and the latter was + 19°. The specific rotations of methyl α-L-*threo*-pent-2-ulo-furanoside, $[\alpha]_D$ − 74° (water), and methyl β-L-*threo*-pent-2-ulo-furanoside, $[\alpha]_D$ + 86° (water), were estimated from the values for their enantiomers.[25] These results indicated that L-Xul in the antigenic polysaccharide is β-linked. The second mild acid hydrolysis (50 mM HCl at 80 °C for 210 min) removed ~90% of D-fucofuranose, $[\alpha]_D^{20}$ + 73.0° (*c* 1.0, water), from the modified polysaccharide. The remaining polymeric product, composed of a D-*glycero*-D-*galacto*-heptopyranose polymer, showed a negative value, $[\alpha]_D^{20}$ − 20.1° (*c* 1.1, water). These results indicated that D-Hep is β- and D-Fuc is α-linked, respectively.

3.6. Methylation analysis

Methylation was performed with sodium methylsulfinyl-methanide in Me_2SO according to Hakomori[26] as previously described. Complete hydrolysis of the fully methylated antigenic polysaccharide was achieved by 90% formic acid for 2 h and then in 0.25 M H_2SO_4 for 16 h at 100 °C. In order to identify the partial methylated L-*threo*-pent-2-ulose derivative, a portion of the methylated antigenic polysaccharide (10 mg) was hydrolyzed with a mixture of 0.05 M oxalic acid (0.64 mL) and MeOH (2 mL) at 80 °C in a sealed tube.[27] The hydrolyzate was concentrated to dryness by rotary evaporation in vacuo below 40 °C. Distilled water (2 mL) was added to the residue which was then hydrolyzed at 100 °C for 3 h. The partially methylated sugars obtained were converted into their alditol acetates by reduction with borodeuteride (10 mg) and heating with pyridine and Ac_2O (1:1) at 100 °C for 2 h. GC–MS was performed at 150 °C with a Hitachi M-80 B instrument and a capillary column (0.25 mm × 50 m) coated with OV-225.

3.7. NMR spectroscopy

Samples (15 mg) were dissolved in 0.4 mL D_2O (99.99% D) and transferred into a 5-mm $^{13}C/^{1}H$ NMR tube. Sodium 4,4-dimethyl-silapentane-1-sufonate (DSS) at δ_H 0.0 ppm for ^{1}H spectra and 1,2-dioxane at δ_C 67.4 ppm for ^{13}C spectra were added as the external standard for chemical shift calibration. NMR experiments

were performed for the *O*-acetylated polysaccharide and modified polysaccharide at 65 °C, but at 25 °C for the antigenic polysaccharide in order to protect L-*threo*-pent-2-ulose from degradation by elevated temperatures. Measurements were made on a Bruker DMX-600 spectrometer operating at 600.1 MHz for ^{1}H and 150.9 MHz for ^{13}C, using standard Bruker software. DEPT spectra were recorded with a 135° read pulse for polarization transfer with decoupling during acquisition. For sequence information, two-dimensional nuclear Overhauser effect spectra (NOESY) were recorded with mixing times of 200 ms and H-detected $^{1}H,^{13}C$ heteronuclear multiple bond connectivity (HMBC) spectra were measured with 65 ms delay for the evolution of long-range connectivities. H-detected $^{1}H,^{13}C$ heteronuclear multiple quantum coherence (HMQC) spectra were recorded without ^{13}C decoupling for chemical shifts. Chemical shifts of the anomeric and methyl protons were determined from a one-dimensional spectrum but all other ^{1}H and all ^{13}C chemical shifts were obtained from HMQC spectrum.

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