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Structure of a colitose-containing O-specific polysaccharide of the marine bacterium *Pseudoalteromonas tetraodonis* IAM 14160^{T}☆

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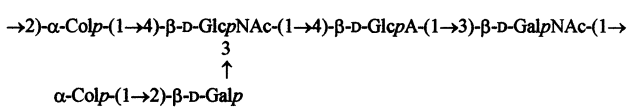
Abstract

O-specific polysaccharide was isolated by mild acid degradation of the lipopolysaccharide of *Pseudoalteromonas tetraodonis* type strain IAM 14160^{T} and studied by sugar and methylation analyses along with ^{1}H and ^{13}C NMR spectroscopy, including 2D COSY, TOCSY, ^{1}H,^{13}C HMQC and HMBC experiments. The polysaccharide was found to consist of hexasaccharide repeating units containing one residue each of D-Gal, D-GlcA, D-GalNAc and D-GlcNAc and two residues of 3,6-dideoxy-L-*xylo*-hexose (colitose, Col) and having the following structure:



In common with the polysaccharides of some other bacteria, the polysaccharide studied contains a tetrasaccharide fragment α-Col*p*-(1→2)-β-D-Gal*p*-(1→3)-[α-Col*p*-(1→4)]-β-D-Glc*p*NAc, which is a colitose ('3-deoxy-L-fucose') analogue of the Lewis^{b} blood group antigenic determinant.

Keywords: Polysaccharide; Colitose; Methylation

1. Introduction

Gram-negative bacteria of the genera *Alteromonas* and *Pseudoalteromonas* are aerobic marine heterotrophic prokaryotes widely distributed in the marine environment.[1] These bacteria produce a wide range of biologically active compounds, such as antibiotics, enzymes, antitoxins, antitumour and antiviral agents.[2] In 1990 a new marine, tetrodotoxin-producing bacterium *Alteromonas tetraodonis* was described.[3] Later, based on DNA–DNA hybridisation data, *A. tetraodonis* was recognised as a junior subjective synonym of *A. haloplanktis*,[4] and in accordance with results

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of phylogenetic biochemical analyses classified as *Pseudoalteromonas haloplanktis* subsp. *tetraodonis*.[5] However, further taxonomic investigations afforded new phenotypic and chemotaxonomic data, genomic fingerprint patterns, DNA–DNA hybridisation data, and data of phylogenetic analysis of 16S rRNA, which led to a conclusion that the species rank of this bacterium should be retrieved and it should be classified as *Pseudoalteromonas tetraodonis* comb. nov. with the type strain IAM 14160^T.[6]

Structures of the polysaccharides of a number of *Alteromonas* and *Pseudoalteromonas* strains have been established (Refs. 7 and 8 and references cited therein). Distinctive features of the extracellular and O-specific polysaccharides of the two genera are their acidic character and the presence of unusual sugars and non-sugar substituents with the absence of any marked structural similarity of the repeating units. Typical components include various *N*-acyl derivatives of 6-deoxyamino sugars.

In this paper we report on the elucidation of the structure of the O-specific polysaccharide of the type strain of *P. tetraodonis*, IAM 14160^T, containing a new component of *Pseudoalteromonas* polysaccharides, 3,6-dideoxy-L-*xylo*-hexose (colitose).

2. Results and discussion

The O-specific polysaccharide was obtained by mild acid degradation of the lipopolysaccharide isolated from *P. tetraodonis* IAM 14160^T by the phenol–water procedure.[9] Sugar analysis of the polysaccharide by GLC of the alditol acetates derived after acid hydrolysis with CF_3CO_2H (120 °C, 2 h) revealed the presence of Gal, GalN, and GlcN. When milder hydrolysis conditions were applied (100 °C, 1 h), a 3,6-dideoxy-*xylo*-hexose was identified in addition to the above sugars. GLC analysis of the acetylated glycosides with chiral alcohols showed that Gal, GalN, and GlcN have the D configuration, whereas 3,6-dideoxy-*xylo*-hexose has the L configuration and is thus colitose (Col). The latter analysis also showed the presence of D-glucuronic acid (D-GlcA).

Methylation analysis of the polysaccharide resulted in identification of 2,4-di-*O*-methylcolitose, 4-*O*-methyl-3,6-colitose, 3,4,6-tri-*O*-methylgalactose, 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methyl)acetamidogalactose and 2-deoxy-6-*O*-methyl-2-(*N*-methyl)acetamidoglucose. When the methylated polysaccharide was carboxyl-reduced prior to hydrolysis, 2,3-di-*O*-methylglucose was identified in addition to the sugars mentioned above, and so was evidently derived from GlcA. Therefore, the polysaccharide is branched and contains terminal Col, 2-substituted Col, 2-substituted Gal, 4-substituted GlcA, 3-substituted GalN, and 3,4-disubstituted GlcN.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) showed the presence of a hexasaccharide repeating unit. The anomeric region contained signals for six carbons at δ 96.6–105.0. Additionally present were signals for

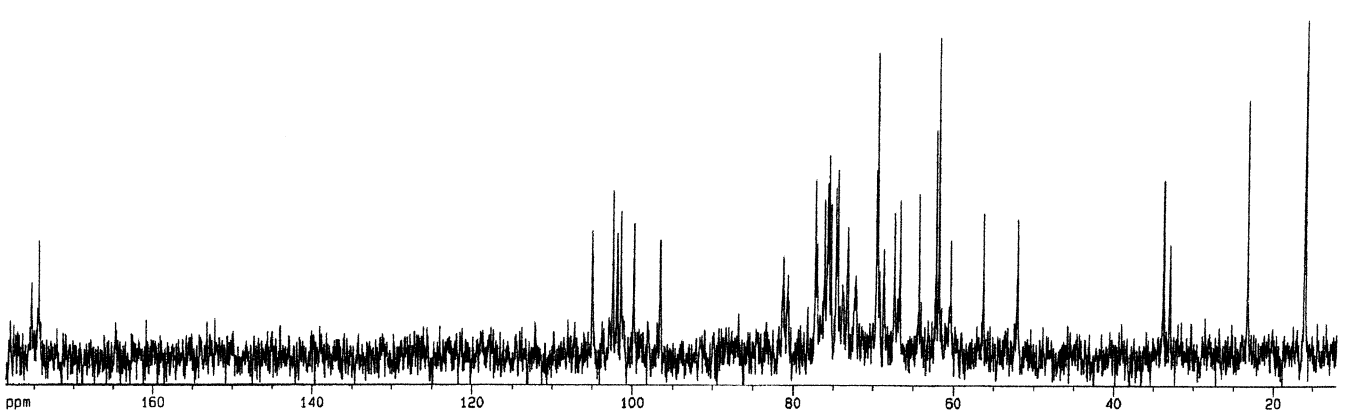


Fig. 1. 125 MHz ^{13}C NMR spectrum of the polysaccharide at 60 °C.

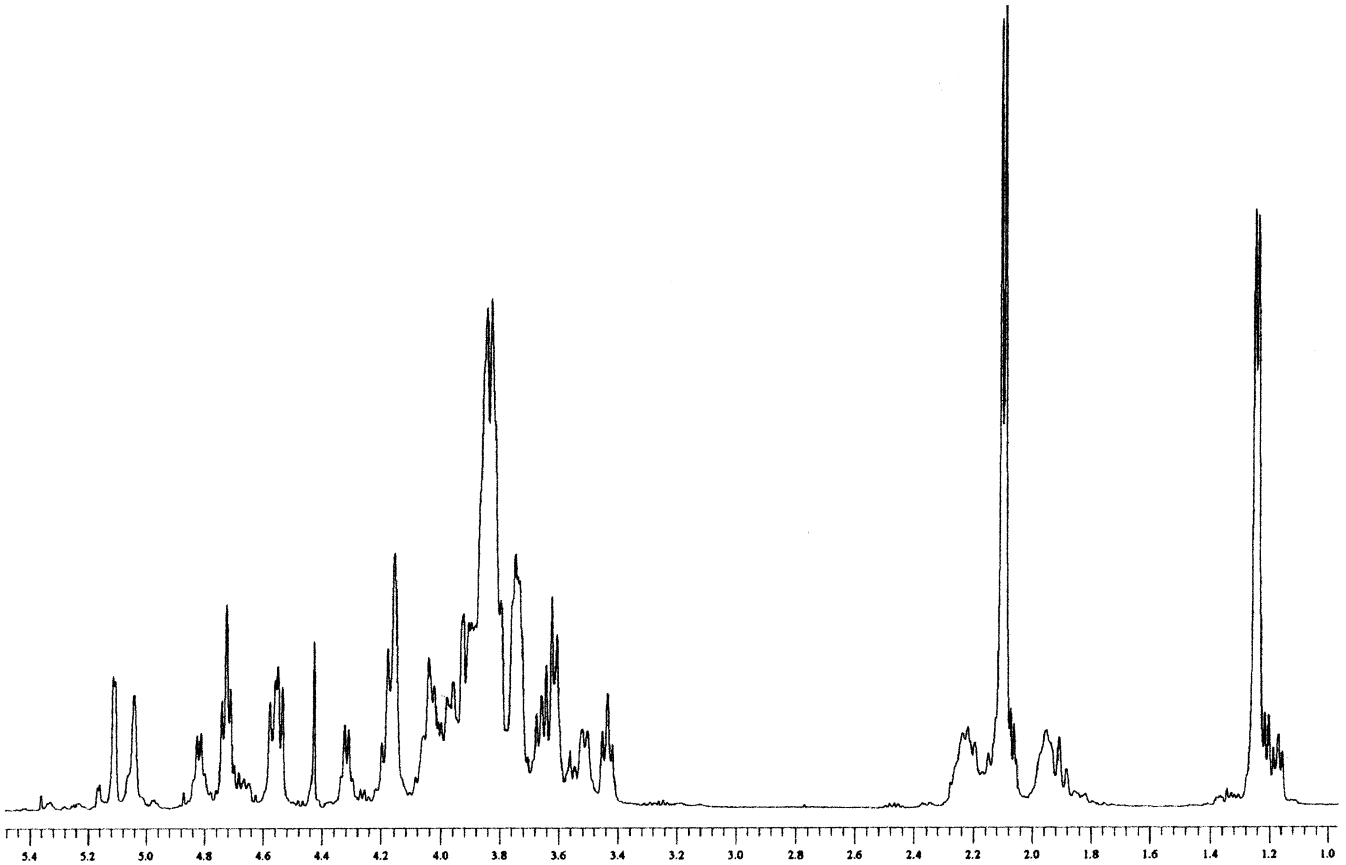


Fig. 2. 500 MHz ^{1}H NMR spectrum of the polysaccharide at 60 °C.

two carbons bearing nitrogen at δ 52.1 and 56.3 (C-2 of GalN and GlcN), three hydroxymethyl groups in the region δ 60.4–62.2 (C-6 of Gal, GalN, and GlcN), two C–CH_2–C groups at δ 33.0 and 33.8 (C-3 of colitose), two CH_3–C groups at δ 16.1 (2C, C-6 of colitose), and two *N*-acetyl groups at δ 23.3 (2C, Me) and 174.4–175.5 (CO). The absence from the ^{13}C NMR spectrum of any signals for ring carbons at a lower field than δ 82 demonstrated the pyranose form of all sugar residues.[10]

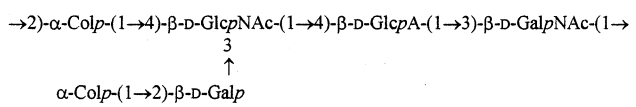
The low-field region of the ^{1}H NMR spectrum of the polysaccharide (Fig. 2) contained signals for six anomeric protons at δ 4.55, 4.57, 4.72, 4.74, 5.04, and 5.11, as well as signals for H-5 of two colitose residues at δ 4.31 and 4.81. The high-field region contained typical signals for two sets of C–CH_2–C signals of colitose in the regions of δ 1.96 and 2.24, and two CH_3–C groups at δ 1.25 (H-6 of colitose), as well as a signal for two *N*-acetyl groups at δ 2.10 (3 H) and 2.11 (3 H).

The ^{1}H and ^{13}C NMR spectra of the polysaccharide were assigned using 2D COSY, TOCSY, NOESY, and H-detected ^{1}H,^{13}C HMQC experiments (Tables 1 and 2). Spin-systems for two sugar residues having the *gluco* configuration (GlcA and GlcNAc) and two residues with the *galacto* configuration (Gal and GalNAc) were identified on the basis of characteristic $J_{H,H}$ coupling constant values. In addition, there were spin-systems for two 3,6-dideoxy hexose residues present with an axial proton at C-2 and equatorial proton at C-4, which was in agreement with the *xylo* configuration of colitose. Assignments of H-3ax and H-3eq of the two colitose residues (Table 1) were made on the basis of their patterns of coupling constants. Relatively large $J_{1,2}$ coupling constant values of 7.2–8.7 Hz showed that Gal, GlcA, GlcNAc, and GalNAc are β-linked, whereas both colitose residues are α-linked ($J_{1,2} < 3$ Hz).

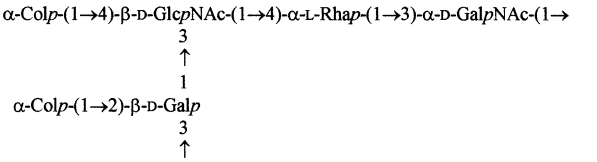
Large low-field displacements, by 5–10 ppm, were observed for C-2 of galactose and

one of the colitose residues, C-3 of GalNAc, C-4 of GlcNAc and GlcA, as compared with their positions in the corresponding free monosaccharides.[11–13] These displacements confirmed the modes of substitution of the monosaccharides. For the GlcNAc residue at the branching point, the shifts for the C-3 and C-4 were not so significant owing to interaction between two sugar substituents attached at vicinal hydroxy groups.[14] The second colitose residue showed no significant displacements for the C-2–C-6 signals, which confirmed its terminal position in the repeating unit.

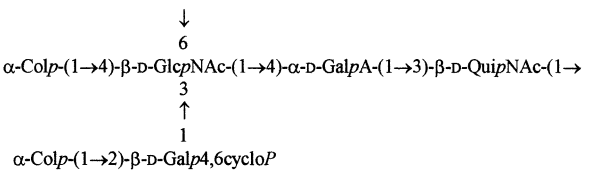
Sequence and linkage analyses of the polysaccharide were performed using NOESY and $^{1}H,^{13}C$ HMBC experiments (Table 3). They confirmed the substitution pattern and revealed the sugar sequence in the polysaccharide repeating unit. Therefore, the O-specific polysaccharide studied has the structure shown below.



P. tetraodonis IAM 14160^{T}



Aeromonas trota [15]



Vibrio cholerae O139 [16]

Interestingly, in common with the O-specific polysaccharide of *Aeromonas trota*[15] and the capsular polysaccharide of *Vibrio cholerae* O139,[16] the polysaccharide studied has a hexasaccharide repeating unit that includes a tetrasaccharide fragment α-Col*p*-(1→2)-β-D-Gal*p*-(1→3)-[α-Col*p*-(1→4)]-β-D-Glc*p*-NAc. Although in all three polysaccharides this fragment is substituted differently in the interior repeating units and two other sugar components of the repeating units are not the same, the polysaccharides may have great sim-

Table 1
^{1}H NMR data (δ, ppm) for the polysaccharide. The chemical shift for NAc is δ 2.10 (6 H)

Sugar residue	H-1	H-2	H-3	H-3eq	H-4	H-5	H-6	H-6b
→2)-α-Col-(1→	5.04	4.16	2.20 [a]	2.24	3.92	4.81	1.25	
→3,4)-β-Glc*p*NAc-(1	4.57	3.85	4.19		3.84	3.53	3.81 [b]	3.97
→4)-β-Glc*p*A-(1→	4.55	3.44	3.63		3.82	3.76		
→3)-β-Gal*p*NAc-(1→	4.74	4.04	3.90		4.16	3.73	3.83 [b]	3.83
α-Col-(1→	5.11	4.03	1.91 [a]	1.96	3.86	4.31	1.25	
→2)-β-Gal*p*-(1→	4.72	3.67	3.84		3.94	3.60	3.74 [b]	3.85

[a] H-3ax.
[b] H-6a.

Table 2
^{13}C NMR data (δ, ppm) for the polysaccharide. The chemical shifts for NAc are δ 23.3 (Me) and 174.4 [a] and 175.5 [a] (CO)

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
→2)-α-Col-(1→	96.6	73.9	32.3	69.5	67.4	16.1
→3,4)-β-Glc*p*NAc-(1→	101.9	56.3	75.3	72.3	76.1	60.4
→4)-β-Glc*p*A-(1→	105.0	73.3	74.5	80.7	77.1	174.8 [a]
→3)-β-Gal*p*NAc-(1→	102.4	52.1	81.2	68.8	75.6	61.9
α-Col-(1→	99.9	64.3	33.8	69.5	66.7	16.1
→2)-β-Gal*p*-(1→	101.4	77.3	74.7	69.6	75.7	62.2

[a] Assignment could be interchanged.

Table 3
Homonuclear (NOESY) and heteronuclear (^{1}H,^{13}C HMBC) inter-residue connectivities for the anomeric atoms in the polysaccharide

Sugar residue	δ_{H-1}	δ_{C-1}	δ_{H}	δ_{C}	Connectivity to
NOESY data					
→2)-α-Col*p*-(1→	5.04		3.84		GlcNAc H-4
→3,4)-β-Glc*p*NAc-1→	4.57		3.82		GlcA H-4
→4)-β-Glc*p*A-(1→	4.55		3.90		GalNAc H-3
→3)-β-Gal*p*NAc-1→	4.74		4.16		Col H-2
α-Col*p*-(1→	5.11		3.67		Gal H-2
→2)-β-Gal*p*-(1→	4.72		4.19		GlcNAc H-3
HMBC data					
→2)-α-Col*p*-(1→	5.04			72.3	GlcNAc C-4
		96.6	3.84		GlcNAc H-4
→3,4)-β-Glc*p*NAc-1→	4.57			80.7	GlcA C-4
		101.9	3.82		GlcA C-4
→4)-β-Glc*p*A-(1→	4.55			81.2	GalNAc C-3
		105.0	3.90		GalNAc H-3
→3)-β-Gal*p*NAc-1→	4.74			73.9	Col C-2
		102.4	4.16		Col H-2
α-Col*p*-(1→	5.11			77.3	Gal C-2
		99.9	3.67		Gal H-2
→2)-β-Gal*p*-(1→	4.72			75.3	GlcNAc C-3
		101.4	4.19		GlcNAc H-3

ilarity at the non-reducing end, as shown above. The putative common terminal tetrasaccharide fragment represents a colitose ('3-deoxy-L-fucose') analogue of the Lewis^{b} blood group antigenic determinant α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-[L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc, and its presence in bacterial polysaccharides may play a role in mimicry by microorganisms of the animal host structures.

3. Experimental

Bacterial growth, isolation and degradation of the lipopolysaccharide.—The bacterial strain was kindly provided by Dr U. Simidu and Dr T. Kita-Tsukamoto. The bacteria were grown in Youschimizu-Kimura medium,[17] and the lipopolysaccharide was isolated from wet bacterial cells by phenol–water extraction[9] followed precipitation of nucleic acids with trichloroacetic acid at pH 2. The lipopolysaccharide was degraded with aq 2% HOAc at 100 °C until precipitation of lipid (6% of the lipopolysaccharide weight), and a high-molecular mass O-specific polysaccharide (40% of the lipopolysaccharide weight) was obtained by GPC of the supernatant on Sephadex G-50 (S).

Sugar analysis.—The O-specific polysaccharide was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h for hexose and amino sugars) or 1 M CF_3CO_2H (100 °C, 1 h for colitose), monosaccharides were reduced with 0.25 M $NaBH_4$ in aq 1 M ammonia (20 °C, 2 h), acetylated with a 1:1 (v/v) mixture of pyridine and Ac_2O (120 °C for hexose and amino sugars or 100 °C for colitose, 40 min) and analysed by GLC. The absolute configurations of the monosaccharides were determined by GLC of acetylated (*S*)-2-butyl glycosides (for GlcN and GalN) or (*R*)-2-octyl glycosides (for Gal and Col) according to the published method[18,19] modified as described.[20] GLC was performed using a Hewlett–Packard 5890 instrument equipped with a DB-5 fused-silica capillary column (25 m × 0.25 mm) and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C/min. The authentic sample of colitose was derived from the capsular polysaccharide of *V. cholerae* O139.[16]

Methylation analysis.—Methylation of the polysaccharide was performed with CH_3I in Me_2SO in the presence of sodium methylsulfi-

nylmethanide.[21] A portion of the methylated polysaccharide was reduced with $LiBH_4$ in aq 70% 2-propanol (20 °C, 16 h). Partially methylated monosaccharides were derived by hydrolysis (2 M CF_3CO_2H, 120 °C, 2 h), converted into the alditol acetates, and analysed by GLC–MS on a Hewlett–Packard 5890 chromatograph equipped with a NERMAG R10-10L mass spectrometer, using the same chromatographic conditions as in GLC.

NMR spectroscopy.—Samples were deuterium-exchanged by freeze-drying three times from D_2O and then examined as solutions in 99.97% D_2O, using internal acetone as a reference (δ_H 2.225, δ_C 31.45). NMR spectra were recorded on a Bruker DRX-500 spectrometer at 60 °C and processed using standard Bruker software (XWINNMR 1.2). The parameters used for 2D experiments were essentially the same as described previously.[22,23] A mixing time of 300 ms were used in 2D TOCSY and NOESY experiments.

Acknowledgements

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