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Structural analysis of an acidic polysaccharide secreted by *Xanthobacter* sp. (ATCC 53272)

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ABSTRACT

The structure of an acidic polysaccharide secreted by a *Xanthobacter* sp. has been investigated by glycosyl-residue and glycosyl-linkage composition analyses, and the characterization of oligoglycosyl fragments of the polysaccharide has been carried out by chemical analyses, ^{1}H-n.m.r. spectroscopy, fast-atom bombardment mass spectrometry, and electron-impact mass spectrometry. The polysaccharide, which contains *O*-acetyl groups (~5%) that have not been located, has the tetraglycosyl repeating unit **1** and belongs to a group of structurally related polysaccharides synthesized by both *Alcaligenes* and *Pseudomonas* species.

→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Man*p*-(1→
1

INTRODUCTION

Various polysaccharides secreted by bacteria have been found to have potential commercial applications as gelling or thickening agents[1]. Gellan gum, a linear polysaccharide composed of a partially *O*-acetylated tetraglycosyl repeating unit[2,3], was the first polysaccharide characterized from a closely related group of bacterial polysaccharides that possesses interesting rheological properties[4–8]. The repeating units of these polysaccharides differ from the tetraglycosyl repeating unit of gellan gum, either by the partial replacement of the 4-linked L-rhamnosyl residue with a 4-linked L-mannosyl residue[8,9] and or by the addition of mono- or di-glycosyl side chains[5–9]. We now describe the structure of a polysaccharide secreted by a *Xanthobacter* sp. that is the sixth member of this structurally and rheologically related group.

RESULTS AND DISCUSSION

Glycosyl-residue and glycosyl-linkage compositions. — The polysaccharide was shown by glycosyl composition analysis to contain mannose, glucose, and glucuronic

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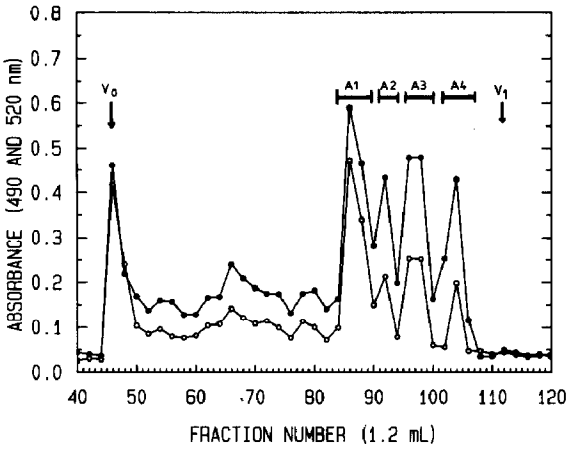

Fig. 1. Gel-permeation chromatography on Bio-Gel P-2 of the acidic oligosaccharides released by partial acidic hydrolysis of the polysaccharide produced by *Xanthobacter* sp. Fractions (1.2 mL) were collected, and portions (50 μL) were analyzed colorimetrically for hexose (o) and uronic acid (•). Fractions 84–90 (A1), 91–93 (A2), 94–9 (A3), and 101–106 (A4) were pooled, desalted and freeze dried.

acid in the molar ratios 1.0:1.9:0.9. On the basis of g.l.c. analysis of the per-*O*-(trimethylsilyl)ated (*R*)-(−)-2-butyl and (*S*)-(+)-2-butyl glycosides, respectively, the absolute configurations of glucose and glucuronic acid were shown to be D, while that of mannose was shown to be of the L configuration. Glycosyl-linkage composition analysis of the methylated and carboxyl-reduced polysaccharide showed that 4-linked mannosyl, 3- and 4-linked glucosyl, and 4-linked glucosyluronic acid residues were present in the ratios 1.0:0.9:1.0:0.9, respectively.

Partial acidic hydrolysis of the polysaccharide. — Hydrolysis of the polysaccharide for 2.5 h at 100° with 0.4M trifluoroacetic acid, followed by ion-exchange and gel-permeation chromatography on Bio-Gel P-2 (Fig. 1), yielded oligosaccharides that contained mannosyl, glucosyl, and glucosyluronic acid residues (Table I). The glycose residue at the reducing terminus of each oligosaccharide or mixture of oligosaccharides

TABLE I

Glycosyl composition of the acidic oligoglycosyl alditols derived from the polysaccharide secreted by *Xanthobacter* sp. by partial acidic hydrolysis and reduction

	Bio-Gel P-2 fraction			
	A1	*A2*	*A3*	*A4*
Glycosyl residue	*molar ratios*			
Mannose	1.0	1.0	1.0	1.0
Glucose	11.7	14.7	9.6	4.5
Glucuronic acid	6.9	10.8	7.2	13.7
Mannitol	5.2	0.6	12.4	0.7
Glucitol	0.7	7.6	1.0	9.5

was converted to its corresponding alditol, and the glycosyl-residue compositions were determined by g.l.c. of the trimethylsilyl derivatives (Table I).

The acidic oligosaccharides in the fractions (A1–A4, see Fig. 1) that were isolated by gel-permeation chromatography were reduced to their corresponding alditols, methylated, and separated by reversed-phase h.p.l.c. to give a series of methylated oligoglycosyl-alditol methyl esters. The major component (designated A1M, A2M, A3M, and A4M) isolated from each of the fractions by h.p.l.c. was characterized by ^{1}H-n.m.r., f.a.b.-m.s., g.l.c.–e.i.m.s., and glycosyl-linkage composition analyses.

The ^{1}H-n.m.r. spectrum of the methylated glycosyl alditol in fraction A4M contained a signal for an anomeric proton at δ 4.61 ($J_{1,2}$ 7.8 Hz) that was assigned to a β-linked glucosyluronic acid residue. F.a.b.-m.s. of the methylated glycosyl alditol in fraction A4M gave an ion at m/z 508, corresponding to $[M + Na]^+$ from a methylated monoglycosyl alditol methyl ester containing a hexuronosyl and a hexitol residue. G.l.c.–e.i.m.s. gave ions at m/z 169 (aA_3), 201 (aA_2), 233 (aA_1), 236 ($aldJ_2$), 296 ($aldJ_1$), and 395 (M − 90), which confirmed that the derivative in fraction A4M was an alkylated monoglycosyl alditol[10]. Glycosyl-linkage composition analysis of the carboxyl-reduced derivative showed that A4M was composed of terminal non-reducing

β-D-Glc*p*A-(1→4)-D-Glc*p*

2

β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-L-Man*p*

3

β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-D-Glc*p*

4

β-D-Glc-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-L-Man*p*

5

β-D-Glc-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-L-Man*p*-(→

6

β-D-Glc*p*-(1→4)-α-L-Man*p*-(1→3)-β-D-Glc*p*-(1→4)-xylitol

7

→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Man*p*-(1→3)-β-D-Glc*p*-(1→

8

Fig. 2. Structures of oligosaccharides derived from the polysaccharide produced by *Xanthobacter* sp. by partial acidic hydrolysis (fragments **2–5**) or by cleavage of the methylated polysaccharide by treatment with lead tetraacetate (fragment **1**). The structure **6** is the partially characterized tetraglycosyl repeating unit that was deduced from overlapping fragments (**2–5**) obtained after partial hydrolysis of the polysaccharide. The fully characterized tetraglycosyl repeating unit is structure **8**.

glucosyluronic acid and 4-linked glucitol residues in the ratio 1.0:1.2. The derivative in fraction A4M originated from **2** (Fig. 2).

The ^{1}H-n.m.r. spectrum of the methylated oligoglycosyl alditol in fraction A3M contained signals for anomeric protons at δ 4.31 ($J_{1,2}$ 7.8 Hz), and δ 4.50 ($J_{1,2}$ 7.8 Hz) that were assigned to β-glucosyl and β-glucosyluronic acid residues, respectively. F.a.b.-m.s. of the methylated oligoglycosyl alditol in fraction A3M gave an ion at *m/z* 712, corresponding to $[M + Na]^+$ from a methylated diglycosyl alditol methyl ester containing one hexosyl, one hexuronosyl, and one hexitol residue. G.l.c.–e.i.m.s. gave ions at *m/z* 201 (aA_2), 233 (aA_1), 236 ($aldJ_2$), 296 ($aldJ_1$), 405 (baA_1), and 436 (baA_1), confirming that the derivative in fraction A3M was a diglycosyl alditol. Glycosyl-linkage composition analysis of the carboxyl-reduced derivative showed that it was composed of terminal non-reducing glucosyluronic acid, 4-linked glucosyl, and 4-linked mannitol residues in the ratios 1.0:1.0:1.2. Thus, the derivative in fraction A3M originated from **3** (Fig. 2).

F.a.b.-m.s. of the methylated oligoglycosyl alditol in fraction A2M yielded an ion at *m/z* 712, corresponding to [M + Na] from a methylated diglycosyl alditol methyl ester containing one hexosyl, one hexuronosyl, and one hexitol residue. G.l.c.–e.i.m.s. gave ions at *m/z* 189 (aA_2), 219 (aA_1), and 236 ($aldJ_2$). Glycosyl-linkage composition analysis of the carboxyl-reduced derivative showed that it was composed of terminal non-reducing glucosyl, 4-linked glucosyluronic acid, and 4-linked glucitol residues in the ratios 1.0:0.9:0.9. These data, in combination with the results of the analysis of fractions A4M and A3M, respectively, show that the alkylated diglycosyl alditol in fraction A2M originated from **4** (Fig. 2).

The ^{1}H-n.m.r. spectrum of the methylated oligoglycosyl alditol in fraction A1M contained signals for anomeric protons at δ 4.26 ($J_{1,2}$ 7.3 Hz), δ 4.33 ($J_{1,2}$ 7.3 Hz) and δ 4.53 ($J_{1,2}$ 7.7 Hz) that were assigned to two β-glucosyl residues and one β-linked glucosyluronic acid residue, respectively. F.a.b.-m.s. of the methylated oligoglycosyl alditol in fraction 1AM gave an ion at *m/z* 916, corresponding to $[M + Na]^+$ from a methylated triglycosyl alditol methyl ester containing two hexosyl, one hexuronosyl, and one hexitol residue. G.l.c.–e.i.m.s. gave ions at *m/z* 187 (aA_2), 219 (aA_1), 236 ($aldJ_2$), 296 ($aldJ_1$), 437 (baA_1), and 440 ($cbaldJ_2$), which confirmed that the derivative in fraction A1M was an alkylated triglycosyl alditol. Glycosyl-linkage composition analysis of the carboxyl-reduced derivative showed that it was composed of terminal non-reducing glucosyl, 4-linked glucosyl, 4-linked glucosyluronic acid, and 4-linked mannitol residues in the ratios 1.0:1.0:0.9:0.9. Thus, the derivative in fraction A1M originated from **5** (Fig. 2).

The series of overlapping sequences (**2–5**, see Fig. 2) defined the partial sequence **6** of the polysaccharide secreted by *Xanthobacter* sp.

Degradation of the methylated polysaccharide with lead tetraacetate. — The anomeric configuration of the glycosidic linkage of the 4-linked L-mannosyl residue was elucidated, and the sequence of the *Xanthobacter* polysaccharide was confirmed as **6** (Fig. 2) by subjecting the methylated polysaccharide to decarboxylation and cleavage of the glycosidic bond of the uronosyl residue by treatment with lead tetraacetate in

pyridine–benzene[11,12]. The hydroxyl groups of the glycosyl and alditol residues that were exposed as a result of the lead tetraacetate procedure were methylated, and the resulting alkylated oligoglycosyl alditols were separated by reversed-phase h.p.l.c. The major fraction (PB1) isolated by h.p.l.c. was analyzed by ^{1}H-n.m.r., f.a.b.-m.s., g.l.c.–e.i.m.s., and glycosyl-residue linkage composition analyses.

The ^{1}H-n.m.r. spectrum of the methylated oligoglycosyl alditol in fraction PB1 contained signals for anomeric protons at δ 4.54 ($J_{1,2}$ 7.8 Hz), δ 4.61 ($J_{1,2}$ 7.7 Hz), and δ 5.42 ($J_{1,2}$ 1.6 Hz) that were assigned to two β-linked glucosyl residues and one α-linked mannosyl residue, respectively. F.a.b.-m.s. analysis of the methylated oligoglycosyl alditol in fraction PB1 gave an ion at m/z 859, corresponding to $[M + Na]^+$ from a methylated trihexosyl pentitol composed of three hexosyl residues, one pentitol residue, and two deuterium atoms. G.l.c.–e.i.m.s. gave ions at m/z 187 (aA_2), 193 ($aldJ_2$), 219 (aA_1), 397 ($caldJ_2$), 423 (baA_1), 601 ($bcaldJ_2$), 627 ($cbaA_1$), and 661 ($bcaldJ_1$), confirming that the derivative in fraction PB1 was an alkylated trihexosyl pentitol. Glycosyl-residue linkage analysis of the derivative in fraction PB1 showed that it was composed of terminal non-reducing glucosyl, 4-linked mannosyl, 3-linked glucosyl, and 4-linked xylitol residues in the ratios 1.0:1.1:1.1:0.6. The low recovery of the xylitol derivative, 2-*O*-acetyl-1,5-dideuterio-1,3,4,5-tetra-*O*-methylxylitol, probably resulted from evaporative loss of this volatile derivative during sample work-up. The data are consistent with the derivative having structure **7** in fraction PB1 (Fig. 2). As expected, the xylitol residue in **7** was derived by the lead tetraacetate oxidative decarboxylation and reductive cleavage[11,12] of the glucosyluronic acid residue in the methylated polysaccharide.

The results of methylation analyses, selective chemical fragmentation procedures, and m.s. and ^{1}H-n.m.r. analyses have established that the polysaccharide secreted by *Xanthobacter* sp. is composed of a tetraglycosyl repeating unit **8** (Fig. 1).

CONCLUSIONS

The polysaccharide secreted by this *Xanthobacter* sp. was selected for study because of its interesting rheological properties, including its ability to form gels[13], which were very different from those of other structurally related polysaccharides[13]. The primary structures of the polysaccharides secreted by *Xanthobacter* sp. and *Pseudomonas elodea* (gellan gum producer) are related, but chemically different, as the 4-linked L-rhamnosyl residue in the repeating unit of gellan gum[2,3] is replaced by a 4-linked L-mannosyl residue in the polysaccharide secreted by the *Xanthobacter* sp. Since the rheological properties of the two polysaccharides are different, the three-dimensional conformation of the polysaccharides is probably affected by the substitution of the 4-linked L-mannosyl for the 4-linked L-rhamnosyl residues.

EXPERIMENTAL

Cultivation of Xanthobacter *sp.* — *Xanthobacter* sp. (strain NW11, ATCC 53272) was grown for 5–7 days at 30° with shaking (200 r.p.m.) in media (100 mL per 500 mL

shake flask) that contained 1.75% glucose, 0.5% K_2HPO_4, 0.02% $MgSO_4$, and 1.4% corn-steep liquor. Approximately 1% of the fermentation broth was precipitated by the addition of 2-propanol to 70% v/v.

Purification of the polysaccharide. — The bacterial culture was diluted with an equal volume of water, adjusted to pH 7, and autoclaved. Polymeric material was precipitated by the addition of 2-propanol (to 70% v/v), the precipitate was redissolved in water, and reprecipitated by the addition of 2-propanol (to 70% v/v). The procedure for the precipitation of the polysaccharide was repeated four times. A solution of the final precipitate, in water, was subjected to ultrafiltration using a membrane (having a 200 000-daltons cut-off, then freeze-drying, to yield the purified polysaccharide (yield 200 mg per 100 mL of culture). The purified polysaccharide contained $<0.1\%$ protein, as determined by the Lowry procedure[14].

Glycosyl-residue composition analyses. — Neutral monosaccharides were generated by hydrolysis for 1 h at 121° with 2M trifluoroacetic acid (TFA). The neutral monosaccharides were analyzed as their alditol acetates by g.l.c.[15]. Neutral and acidic monosaccharide methyl glycosides (methyl esters) were generated by methanolysis and analyzed as their per-*O*-(trimethylsilyl)ated methyl glycosides by g.l.c.[15].

Determination of the absolute configuration of the glycosyl residues. — The polysaccharide (1 mg) was heated for 1 h at 120° with 2M TFA. The TFA was removed with a stream of air, and the residue was dried under vacuum. Portions (~ 500 μg) of the residue were reacted with either (*R*)-(−)-2-butanol or (*S*)-(+)-2-butanol (100 μL) containing 10 μL TFA for 16 h at 80° to yield the (*R*)-(−)-2-butyl or (*S*)-(+)-2-butyl glycosides, respectively. The chiral butyl glycosides were trimethylsilylated, analyzed by g.l.c.[16,17], and their retention times were compared to those obtained from the per-*O*-(trimethylsilyl)ated chiral butyl glycosides of glucose, mannose, and glucuronic acid, respectively.

Glycosyl-linkage composition analyses. — The polysaccharide (1 mg) was methylated as previously described[15] using potassium methylsulfinylmethanide. The methylated polysaccharide was recovered after dialysis against aqueous 50% ethanol. The glycosyl-linkage composition of a portion (~ 250 μg) of the methylated polysaccharide was analyzed[15] by formation of its alditol acetates and analysis by g.l.c. and g.l.c.–m.s. The remaining methylated polysaccharide (~ 750 μg) was carboxyl-reduced by treatment for 1 h at 20° with M lithium triethylborodeuteride in tetrahydrofuran (250 μL). Excess reductant was destroyed by addition of glacial acetic acid (50 μL), and boric acid was removed by codistillation with methanol containing 10% acetic acid (3 x 250 μL), and then methanol (3 x 500 μL). The methylated and carboxyl-reduced polysaccharide was hydrolyzed, reduced, and acetylated[15], and the resulting partially methylated alditol acetates were analyzed by g.l.c. and g.l.c.–m.s.[15].

Partial acid hydrolysis and chromatographic separation of the oligosaccharides and methylated oligoglycosyl alditols. — The polysaccharide (30 mg) was dissolved in water (30 mL) at 100°, and trifluoroacetic acid was added to a concentration of 0.4M. The solution was kept for 2.5 h at 100°. The cooled hydrolyzate was filtered, concentrated to dryness, and the residue was washed with 2-propanol (3 x 5 mL) to remove traces of the

acid. A solution of the residue in 10mM NH_4OH (5 mL) was fractionated on a column (1.5 x 15 cm) containing QAE-Sephadex ($HCOO^-$) by elution with water (200 mL) and then aqueous 10% formic acid (250 mL). The material that eluted with aqueous 10% formic acid was concentrated to dryness, and the residue, in 50mM NaOAc, pH 5.1 (2 mL), was chromatographed on a column (1.6 x 90 cm) containing Bio-Gel P-2 (−400 mesh) in 50mM NaOAc, pH 5.1, at 15 mL·h^{-1}. Fractions (1.2 mL) were collected, and portions (50 μL) were analyzed[14] colorimetrically for hexose and uronic acid. Appropriate fractions were pooled, desalted, and freeze-dried. The partially purified oligosaccharides were converted to their corresponding oligoglycosyl alditols by reduction with NaB^2H_4, and portions (~100 μg) were analyzed for their glycosyl-residue compositions by g.l.c. of their per-*O*-(trimethylsilyl)ated methyl glycosides[15]. The remaining material was methylated, and the methylated oligoglycosyl alditols were isolated using Sep-Pak C_{18} reversed-phase extraction cartridges[18]. The methylated oligoglycosyl alditols were purified by reversed-phase h.p.l.c. on a Supelco 50DS2 column (0.46 x 25 cm) by elution with aqueous 50% acetonitrile. The eluant was monitored by differential refractometry (Hewlett–Packard model HP1037A), and fractions were collected manually.

Treatment of the methylated polysaccharide with lead tetraacetate. — A suspension of the methylated polysaccharide (15 mg) in water (30 mL) was de-esterified at pH 12.0 for 3 h at 0° and then 3 h at 20°. The solution was desalted with Dowex-50 [H^+] resin and freeze-dried. A solution of the methylated and de-esterified polysaccharide in benzene (1.5 mL) containing pyridine (75 μL) was treated for 15 h at 90° with lead tetraacetate (50 mg)[11,12]. The cooled solution was passed through a glass fiber filter (GF/C), and the filtrate was washed with water (5 x 2 mL). The organic phase was concentrated by rotary evaporation and then treated for 4 h at 20° with tetrahydrofuran–water (1:1 v/v, 1 mL) containing NaB^2H_4 (15 mg). The excess reductant was destroyed by the addition of glacial acetic acid. The resulting solution was desalted using Dowex-50 [H^+], and boric acid was removed by codistillation with methanol containing 10% acetic acid (3 x 5 mL) and then methanol (3 x 5 mL). The products were methylated and purified by reversed-phase h.p.l.c. on a 0.46 x 25-cm Supelco 50DS2 column (with a C_{18} guard column) by elution with 40:60 water–acetonitrile. The eluant was monitored by differential refractometry, and fractions were collected manually.

Combined gas-liquid chromatography-electron impact mass spectrometry. — Methylated oligoglycosyl alditols were separated on a 15-m DB-1 column with on-column injection on a Hewlett–Packard model 5887 GC/MS. The g.l.c. was programmed to remain for 2 min at 80° and then rise from 80 to 205° at 25°·min^{-1}, and finally from 205 to 335° at 10°·min^{-1}.

Fast-atom bombardment mass spectroscopy (f.a.b.-m.s.). — F.a.b.-m.s. was performed on a VG Zab-SE mass spectrometer operating in the positive-ion mode. Samples (~5 μg) in acetone (1 μL) were loaded onto glycerol or diethanolamine (1 μL) on the probe tip.

^{1}H-N.m.r. spectroscopy. — ^{1}H-N.m.r. spectroscopy was carried out using either a Bruker AM250 or AM500 spectrometer operating at 250 MHz or 500 MHz, respectively. Spectra were obtained from samples in $(C^2H_3)_2CO$ (500 μL) and chemical shifts (δ) are reported in p.p.m. relative to internal tetramethylsilane (δ = 0.00).

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