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Evidence that the acidic polysaccharide secreted by *Agrobacterium radiobacter* (ATCC 53271) has a seventeen glycosyl-residue repeating unit

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ABSTRACT

The extracellular anionic polysaccharide produced by the bacterium *Agrobacterium radiobacter* (ATCC 53271) contains D-galactose, D-glucose, and pyruvic acid in the molar ratio 2:15:2. Analysis of the methylated polysaccharide indicated the presence of terminal, non-reducing glucosyl, 3-, 4-, 6-, 2,4-, and 4,6-linked glucosyl residues, 3-linked 4,6-*O*-[(*S*)-1-carboxyethylidene]glucosyl residues, and 3-linked galactosyl residues. Partial acid hydrolysis of the methylated polysaccharide, followed by reduction with NaB^2H_4 and then *O*-ethylation, gave a mixture of alkylated oligoglycosyl alditols that were separated by reversed-phase h.p.l.c. and analyzed by ^{1}H-n.m.r. spectroscopy, g.l.c.–m.s., and glycosyl-linkage composition analysis. Smith degradation of the polysaccharide gave three diglycosyl alditols that were separated by semi-preparative, high-pH anion-exchange chromatography, and were analyzed by ^{1}H-n.m.r. spectroscopy, g.l.c.–m.s., and glycosyl-linkage composition analysis. The polymer obtained by $NaBH_4$ reduction of the periodate-oxidized polysaccharide was methylated, and the noncyclic acetals were hydrolyzed with aq. 90% formic acid to generate a mixture of partially *O*-methylated mono- and di-glycosyl alditols. The partially *O*-methylated oligoglycosyl alditols were *O*-ethylated. The resulting alkylated oligoglycosyl alditols were separated by reverse-phase h.p.l.c. and then characterized by ^{1}H-n.m.r. spectroscopy, g.l.c.–m.s., and glycosyl-linkage composition analysis. The results from the studies described here provide strong evidence that the acidic polysaccharide secreted by *A. radiobacter* (ATCC 53271) has a heptadecasaccharide repeating unit.

INTRODUCTION

Agrobacterium radiobacter (ATCC 53271) produces an extracellular anionic polysaccharide that gives aqueous dispersions which exhibit high viscosity at low concentrations[1]. We report below the structural characterization of the polysaccharide.

RESULTS AND DISCUSSION

Glycosyl-residue and glycosyl-linkage compositions of the polysaccharide. — Glycosyl-residue composition analysis showed that the acidic polysaccharide secreted by *A.*

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radiobacter is composed of D-galactosyl and D-glucosyl residues in the ratio 2:15. No glycosyluronic acid residues were detected in the polysaccharide after formation and analysis of the constituent per-*O*-trimethylsilyl methyl ester methyl glycosides. The polysaccharide contains ~ 6 mol.% pyruvic acid. Analysis of the per-*O*-trimethylsilyl methyl ester methyl glycosides by g.l.c.–e.i.m.s. showed the presence of a derivative whose e.i.-mass spectrum was identical to that of the di-*O*-trimethylsilyl methyl ester methyl glycoside of 4,6-*O*-(1-carboxyethylidene)-D-glucose[2].

Attempts to fractionate the polysaccharide by precipitation with hexadecyltrimethylammonium bromide (CTAB)[3,4] and by ion-exchange chromatography on DEAE-Trisacryl were unsuccessful. In each case the single product had a glycosyl-residue composition identical to that of the native polysaccharide.

Glycosyl-linkage composition analysis (Table I, column 1) indicated that the polysaccharide contains two branched glycosyl residues, namely, 2,4-linked and 4,6-linked glucosyl residues. The ratio of the 2,4-linked to the 4,6-linked glucosyl residues (1.0:1.7) indicated that the 2,4-linked glucosyl residue is present about half as often as the 4,6-linked glucosyl residue. However, since the polysaccharide was not completely soluble in methyl sulfoxide, incomplete methylation could not be discounted. Furthermore, there are examples of selected oxygen atoms that are difficult to methylate; for example, position O-2 of the 3-linked glucosyl residues in a polysaccharide produced by *Alcaligenes* sp. (ATCC 31555) was found to be resistant to complete methylation[5].

The *A. radiobacter* polysaccharide was soluble in methyl sulfoxide after partial depolymerization achieved by heating for 1 h at 80° in 50mM trifluoroacetic acid (TFA). The glycosyl-linkage composition of the partially depolymerized polysaccharide (Table I, compare columns 2 and 3) was similar to that of the native polysaccharide. The major effect of partial acid hydrolysis was the cleavage of the *O*-(1-carboxyethylidene) groups resulting in the conversion of 3,4,6-linked glucosyl residues into 3-linked glucosyl residues.

TABLE I

Glycosyl-linkage composition (mol. %) of the acidic polysaccharide secreted by *A. radiobacter* (ATCC 53271)

Glycosyl linkage	*Native polysaccharide*	*Partially acid hydrolyzed polysaccharide*	
		Single methylation[a]	*Double methylation*[b]
T-Glc*p*	18	14	16
3-Glc*p*	10	18	18
4-Glc*p*	16	14	18
6-Glc*p*	12	9	11
2,4-Glc*p*	9	10	7
4,6-Glc*p*	13	15	13
3,4,6-Glc*p*	13	9	5
3-Gal*p*	9	11	12

[a] Polysaccharide partially depolymerized with acid followed by a single methylation. [b] Polysaccharide partially depolymerized with acid followed by a double methylation.

The ^{1}H-n.m.r. spectrum of the polysaccharide (in ^2H_2O at 75°) contained an envelope of signals for anomeric protons between δ 4.30 and 4.75. These signals were assigned to β-linked glycosyl residues[6]. No signals were observed in the region between δ 5.00 and 5.50, which indicated that none of the glycosyl residues present in the polysaccharide were α-linked. A broad signal at δ 1.45 was assigned to the methyl protons of the *O*-(1-carboxyethylidene) groups[6].

The results of both glycosyl-linkage composition analysis and ^{1}H-n.m.r. spectroscopy point to a polysaccharide repeating unit composed of ~ 17 β-linked glycosyl residues containing two *O*-(1-carboxyethylidene) groups attached to O-4 and O-6 of two 3-linked glucosyl residues.

Formolysis of the methylated polysaccharide. — Fragmentation of the methylated polysaccharide by heating with 90% formic acid for 30 min at 80° cleaved sufficient amounts of the glycosyl linkages to give good yields of di- and tri-glycosyl fragments.

H.p.l.c. of the alkylated oligoglycosyl alditols. — The products resulting from formolysis of the methylated polysaccharide were converted to their partially methylated, partially ethylated oligoglycosyl alditols[6,7] and separated by reversed-phase h.p.l.c. using isocratic elution with aq. 50% acetonitrile (Fig. 1).

Structural characterization of the partially methylated, partially ethylated oligoglycosyl alditols. — The components in each of the h.p.l.c. fractions ([a]–[q] of Fig. 1) were characterized by ^{1}H-n.m.r. spectroscopy, g.l.c.–c.i.m.s., g.l.c.–e.i.m.s., and glycosyl-linkage composition analyses. Some of the h.p.l.c. fractions contained more than one alkylated oligoglycosyl alditol. However, seventeen partially methylated, partially ethylated oligoglycosyl alditols were unambiguously structurally characterized.

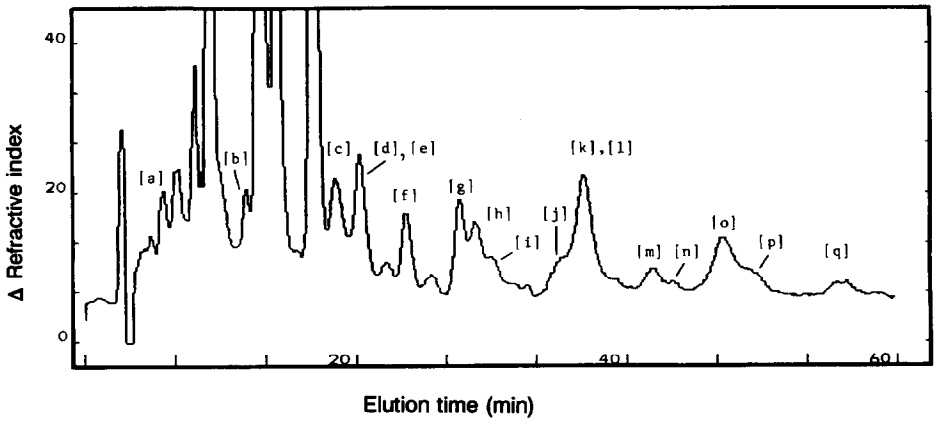
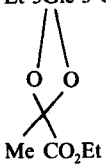


Fig. 1. Reversed-phase h.p.l.c. elution profile of the partially methylated, partially ethylated, oligoglycosyl alditols derived from the oligoglycosyl fragments produced by partial fragmentation of the methylated acidic polysaccharide derived from the polysaccharide secreted by *A. radiobacter* (ATCC 53271). Each alkylated oligoglycosyl alditol that was subsequently structurally characterized was assigned a letter that identifies the order in which it eluted from the h.p.l.c. column. The structures of the characterized fragments are shown in Tables II and III. The large peaks eluting between 9 and 18 min were not characterized, as they are degradation products generated by the reaction procedures.

TABLE II

Structural characteristics of alkylated monoglycosyl alditols obtained from the methylated, hydrolyzed, reduced (NaB^2H_4), and ethylated *A. radiobacter* (ATCC 53271) polysaccharide

Oligosaccharide structure[a]	*Fragment*	*G.l.c. retention time (min)*	*C.i.-m.s. [M + 18]$^+$* (m/z)	*E.i.-m.s. fragment ions* (m/z)					
				aA_1	*aA_1-32*	*aA_1-46*	*aA_1-74*	*$aldJ_1$*	*$aldJ_2$*
Et-4Glc-3Gal	[c]	10.8	n.d.[b]	253 (5)[d]	201 (28)	n.p.[c]	n.p.	324 (77)	264 (22)
Glc-2Glc 4 ↑ Et	[d]	10.3	531	219 (3)	187 (24)	n.p.	n.p.	338 (18)	278 (1)
Et-3-Glc-6Glc Et ↓ 6	[f]	10.7	531	233 (9)	201 (12)	187 (25)	n.p.	338 (5)	264 (14)
Et-4Glc-4Glc	[g]	10.9	545	247 (8)	215 (23)	187 (3)	n.p.	324 (8)	264 (30)
Et-6Glc-6Glc 4 ↑ Et	[h]	11.7	545	233 (4)	201 (15)	187 (2)	n.p.	338 (3)	278 (7)
Et-4Glc-4Glc 2 ↑ Et	[i]	10.7	545	233 (6)	201 (33)	187 (9)	n.p.	338 (18)	278 (3)
Et-4Glc-3Gal 2 ↑ Et	[l]	10.9	545	247 (2)	215 (25)	201 (7)	n.p.	324 (6)	264 (20)

Et-3Glc-3-Glc 	[m]	12.7	601	303 (7)	271 (15)	257 (20)	229 (35)	338 (8)	264 (35)

[a] Et-4Glc-3Gal is equivalent to 4-*O*-ethyl-2,3,6-tri-*O*-methyl-D-Glc*p*(1→3)-1,5-di-*O*-ethyl-2,4,6-tri-*O*-methyl-D-Gal-ol, etc. [b] Not determined. [c] Ion not detected. [d] Values in parentheses are the relative intensities of the fragment ions.

TABLE III

Structural characteristics of the alkylated di- and tri-glycosyl alditols obtained from the methylated, hydrolyzed, reduced (NaB^2H_4), and ethylated *A. radiobacter* (ATCC 53271) polysaccharide

Oligosaccharide structure[a]	*Fragment*	*G.l.c. retention time (min)*	*C.i.-m.s.* $[M+18]^+$ (m/z)	*E.i.-m.s. fragment ions* (m/z)										
				aA_1	aA_1-32	aA_1-46	aA_1-74	$aldJ_1$	$aldJ_2$	$aldJ_0$	baA_1	$baldJ_1$	$baldJ_2$	$bcaldJ_1$
Et (↓6) Et (↓2) Et-4Glc-4Glc-4Glc	[a]	16.5	n.d.[b]	247 (2)[d]	215 (54)	201 (15)	n.p.[c]	338 (2)	278 (50)	n.p.	451 (1)	542 (1)	482 (2)	n.p.
Et-3Glc-3Glc-6Glc (O O / Me CO_2Et)	[b]	19.0	805	303 (64)	271 (18)	257 (18)	229 (50)	n.p.	264 (38)	310 (25)	507 (3)	542 (1)	468 (1)	n.p.
Et-4Glc-4Glc-3Gal	[j]	16.4	735	233 (10)	201 (45)	187 (20)	n.p.	324 (8)	264 (38)	n.p.	437 (2)	528 (1)	468 (2)	n.p.
Et-3Glc-3Glc-6Glc	[k]	17.3	735	233 (10)	201 (20)	187 (80)	n.p.	n.p.	264 ()	310 (8)	437 (3)	n.p.	n.p.	n.p.
Et (↓6) Et-4Glc-4Glc-4Glc	[n]	16.4	n.d.	247 (4)	215 (52)	201 (17)	n.p.	324 (8)	264 (70)	n.p.	451 (1)	528 (2)	468 (1)	n.p.

Et ↓ 4 Et-6Glc-6Glc-4Glc	[o]	16.1	749	233 (8)	201 (25)	187 (65)	n.p.	324 (8)	264 (30)	n.p.	451 (2)	542 (7)	482 (2)	n.p.
Et-3Glc-6Glc-6Glc 4 ↑ Et	[p]	16.9	749	233 (12)	201 (25)	187 (65)	n.p.	338 (8)	278 (24)	n.p.	437 (2)	556 (7)	n.p.	n.p.
Et-4Glc-4Glc-3Gal 2 ↑ Et	[q]	16.5	749	233 (8)	201 (65)	187 (15)	n.p.	324 (10)	264 (35)	n.p.	451 (2)	n.p.	n.p.	n.p.
Et-3Glc-6Glc-6Glc-4Glc 4 ↑ Et	[e]	20.4	953	233 (12)	207 (25)	187 (55)	n.p.	324 (8)	264 (35)	n.p.	437 (2)	542 (8)	482 (1)	760 (2)

[a] Et-4Glc-3Gal is equivalent to 4-*O*-ethyl-2,3,6-tri-*O*-methyl-D-Glc*p*(1→3)-1,5-di-*O*-ethyl-2,4,6-tri-*O*-methyl-D-Gal-ol, etc. [b] Not determined. [c] Ion not detected.
[d] Values in parentheses are the relative intensities of the fragment ions.

The ^{1}H-n.m.r. spectra of 13 alkylated oligoglycosyl alditols ([c], [d], [e], [f], [g], [h], [j], [k], [l], [m], [o], [p], and [q], data not shown) contained signals for anomeric protons in the region δ 4.20–4.80. The vicinal coupling constants ($J_{1,2}$) for the signals of the anomeric protons were between 7.0–7.8 Hz, which established that the anomeric linkages of the alkylated oligoglycosyl alditols were all β-linked. The results of ^{1}H-n.m.r. analyses of the alkylated oligoglycosyl alditols are consistent with the results of ^{1}H-n.m.r. analysis of the intact polysaccharide, which also indicated that all the glycosyl residues are β-linked.

Ammonia c.i.-mass spectra of the alkylated oligoglycosyl alditols contained intense ions that correspond to the $[M + NH_4]^+$ ions of the mono-, di-, and tri-glycosyl alditol derivatives (see Tables II and III). These pseudomolecular ions gave information about the molecular weight and number of *O*-ethyl groups in the oligoglycosyl derivatives.

The nomenclature of Kochetkov and Chizhov[8] is used to describe the e.i.-mass spectra of the alkylated oligoglycosyl alditols. Thus, the terminal, non-reducing hexosyl residue of an alkylated diglycosyl alditol is designated as residue "a", the internal hexosyl residue as residue "b", and the alditol fragment as "ald"[9]. The ions from the A and J series are particularly important, for these ions provide information about the location of *O*-ethyl groups in the alkylated oligoglycosyl alditols and thus the points of attachment in the polysaccharide of other glycosyl residues.

Fragment ions at *m/z* 219, 233, and 247 correspond to the aA_1 ions of terminal, non-reducing, hexosyl residues that contain zero, one, or two *O*-ethyl groups, respectively. A terminal, non-reducing residue in the polysaccharide would have no *O*-ethyl group attached in the alkylated oligoglycosyl alditol. An unbranched internal glycosyl residue in the polysaccharide would have one *O*-ethyl group attached when it is in a terminal, nonreducing position in the alkylated oligoglycosyl alditol, the *O*-ethyl group indicating the point of attachment of another glycosyl residue in the polysaccharide. A branched glycosyl residue in the polysaccharide would have two *O*-ethyl groups when it is in a terminal, non-reducing position in the alkylated oligoglycosyl aldiol, and, again, each *O*-ethyl group indicates the point of attachment of a glycosyl residue in the polysaccharide.

The A_1 ions of the alkylated oligoglycosyl alditols can lose methanol (*m/z* 32) or ethanol (*m/z* 46) to yield A_2 ions. Alkyl groups are eliminated more readily[8] from O-3 than from O-4, and from O-4 more readily than from O-6. The intense ion at *m/z* 201 in the mass spectrum of the alkylated monoglycosyl alditol [c] (see Table II) corresponds to the elimination of methanol from the aA_1 ion and shows that the *O*-ethyl group on residue "a" is not located at O-3. In contrast, the intense ion at *m/z* 187 in the e.i.-mass spectrum of the monoglycosyl alditol [f] (see Table II) represents the elimination of ethanol from the aA_1 ion and establishes that the *O*-ethyl group is located on O-3 of residue "a".

The number of *O*-ethyl groups attached to the alditol of the alkylated oligoglycosyl alditols is delineated by $aldJ_2$ ions[6]. An ion at *m/z* 264 is present if the alditol contains two *O*-ethyl groups, confirming that the glycosyl residue of the polysaccharide from which that alditol was derived was not branched (see [c], [f], [g], [l], and [m], Table II). An $aldJ_2$ ion is present at *m/z* 278 if the alditol contains three *O*-ethyl groups, which would

establish that the glycosyl residue of the polysaccharide from which that alditol was derived was branched (see [d], [h], and [i], Table II). The presence of an *O*-ethyl group on O-3 of residue "a" of the monoglycosyl alditol derivatives is established by an $aldJ_1$ ion[6] at *m/z* 338 ($aldJ_2$ ion at *m/z* 264) (see *e.g.*, [f], Table II).

Evidence for the existence of a branched 2,4-linked glucosyl residue in the polysaccharide was provided by characterizing two alkylated monoglycosyl alditol fragments ([d] and [l], Table II) and one alkylated diglycosyl alditol fragment ([a], Table III). The e.i.-mass spectrum of [d] contained ions at *m/z* 219 (aA_1) and 278 ($aldJ_2$) and established that it had a terminal, nonreducing hexosyl residue (that is, it contained no *O*-ethyl groups) attached to a branched hexitol residue (that is, it contained three *O*-ethyl groups). The point of attachment to the alditol could not be determined from the e.i.-mass spectrum of [d]. However, glycosyl-linkage composition analysis of [d] showed the presence of a terminal, nonreducing glucosyl residue and 2,4-linked glucitol.

The A series of fragment ions in the e.i.-mass spectrum of [l] (see Table II) established that residue "a" was branched (that is, contained two *O*-ethyl groups). The $aldJ_2$ ion at *m/z* 264 gave the mass of the alditol and established that it was a linear residue (that is, contained two *O*-ethyl groups). The presence of an $aldJ_1$ ion at *m/z* 324 indicated that residue "a" is substituted at O-3 with an *O*-methyl group. The e.i.-mass spectrum of [l] in combination with its glycosyl-linkage composition established that the 2,4-linked glucosyl residue is attached to O-3 of the 3-linked galactosyl residue.

The mass of residue "a" of [a] (see Table III) was obtained from the aA_1 ion at *m/z* 247, which showed that residue "a" was a branched hexosyl residue (that is, contained two *O*-ethyl groups). The intense ion at *m/z* 215 corresponds to the fragment ion $aA_1 - 32$, which resulted from the elimination of methanol from O-3. The presence of $aldJ_2$ fragment ions at *m/z* 278 indicated that the alditol was branched (that is, contained three *O*-ethyl groups); the baA_1 ion at *m/z* 451 indicated that residue "b" contained only *O*-methyl groups. Characterization of alkylated diglycosyl alditol [a] by analysis of its constituent alkylated alditol acetates[7] established that a 4,6-linked hexosyl residue was present at the nonreducing end, a 4-linked unbranched hexosyl residue at the internal position, and 2,4-linked glucitol at the reducing end.

The $[M + NH_4]^+$ ions at *m/z* 601 and 805, respectively, in the c.i.-mass spectra of alkylated oligoglycosyl alditols [m] (Table II) and [b] (Table III) confirmed that the ketosidically linked 1-carboxyethylidene residue was retained during formolysis and that the carboxyl group of the 1-carboxyethylidene residue was ethyl-esterified. The mass of the alkylated glycosyl residue "a" containing the ethyl-esterified *O*-(1-carboxyethylidene) group was obtained from the aA_1 ion in the e.i.-mass spectrum. An aA_1 ion at *m/z* 303 (see [m] Table II and [b] Table III) established that "a" is a hexosyl residue containing one *O*-ethyl group in addition to an ethyl-esterified *O*-(1-carboxyethylidene) group. The $aldJ_2$ ion at *m/z* 264 in the e.i.-mass spectrum of [m] established that the hexitol was branched (that is, contained two *O*-ethyl groups). The $aldJ_1$ ion at *m/z* 338 indicated that the *O*-ethyl group on residue "a" was attached to O-3. Thus, the masses of the A and J fragment ions confirmed that the *O*-(1-carboxyethylidene) groups are linked to a 3-linked hexosyl residue. Glycosyl-linkage analysis of [m] showed the presence of

1,4,5,6-tetra-*O*-acetyl-3-*O*-ethyl-2-*O*-methylglucitol, confirming that the pyruvylated glucosyl residue is linked through O-3.

The J series of fragment ions[8] were important in deducing the sequence of the glycosyl residues in alkylated diglycosyl alditol [b] (see Table III) which contained an ethyl-esterified *O*-(1-carboxyethylidene) group. The presence of the $aldJ_0$ ion[10] at *m/z* 310 (see Table III) indicated that the internal hexosyl residue (residue "b") of [b] is 3-linked. The A series of fragment ions and the $baldJ_1$ ion at *m/z* 542 (see Table III) indicated that residue "a" contained an ethyl-esterified *O*-(1-carboxyethylidene) group as well as an *O*-ethyl group at O-3. The point of attachment to the alditol could not be determined from the e.i.-mass spectrum of [b]. However, glycosyl-linkage analysis of [b] established the presence of a 6-linked glucitol.

Characteristics of the glycosyl sequence of the A. radiobacter *acidic polysaccharide.* — A total of 17 partially methylated, partially ethylated oligoglycosyl alditols (Tables II and III) were isolated from the products from the partial formolysis of the methylated polysaccharide and structurally characterized. These overlapping fragments provide strong evidence for the entire sequence of glycosyl residues in the repeating unit of the polysaccharide (Fig. 2). Alkylated mono- and di-glycosyl alditols [a], [c], [i], [g], [j], [l], [n], and [q] showed that the backbone of the polysaccharide is composed of six, β-linked glucosyl residues and two galactosyl residues. One of the

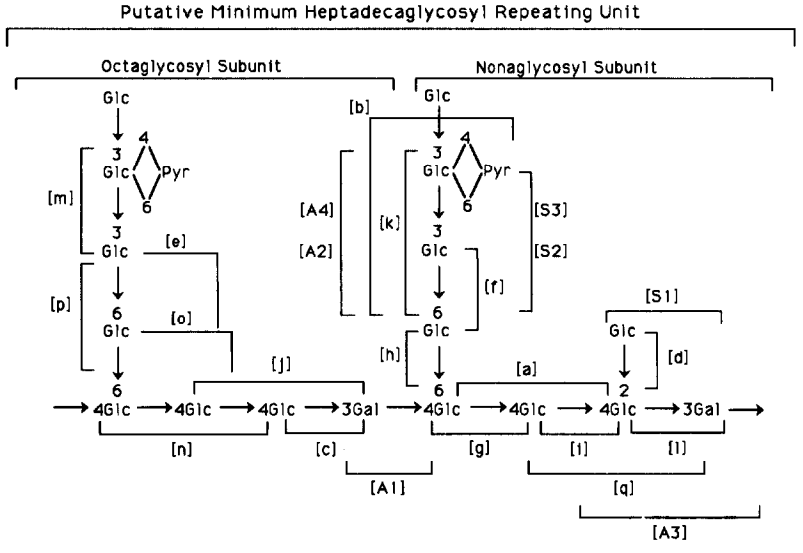


Fig. 2. Proposed structure of the heptadecasaccharide repeating unit of the polysaccharide produced by *A. radiobacter* (ATCC 53271). Symbols [a]–[q] correspond to the mono-, di-, and tri-glycosyl alditol fragments released by partial acid hydrolysis of the methylated polysaccharide; symbols [S1]–[S3] correspond to the diglycosyl alditol fragments released by Smith degradation of the polysaccharide; symbols [A1]–[A4] correspond to mono- and di-glycosyl alditol fragments released by formolysis of the methylated polymer obtained after $NaBH_4$ reduction of the periodate-oxidized polysaccharide. The structures of the oligoglycosyl fragments are shown in Tables II–V.

backbone glucosyl residues is linked through both O-2 and O-4. Fragments [l] and [q] established that the 2,4-linked glucosyl residue is attached to O-3 of one of the two 3-linked galactosyl residues of the backbone. Fragment [d] established that a terminal, nonreducing glucosyl residue was linked to O-2 of the 2,4-linked glucosyl residue. The remaining 3-linked galactosyl residue is substituted at O-3 with an unbranched 4-linked glucosyl residue (fragments [c] and [j]).

The backbone of the polysaccharide contained two glucosyl residues linked through both O-4 and O-6. The positions of the two 4,6-linked glucosyl residues of the backbone were defined by fragments [a] and [n]. Fragment [a] showed that one of the 4,6-linked glucosyl residues was separated from the 2,4-linked glycosyl residue by a single 4-linked glucosyl residue, while fragment [n] established that the other 4,6-linked glucosyl residue had two consecutive 4-linked glucosyl residues at its reducing end (Fig. 2).

The sequence of glycosyl residues in the side chain attached to O-6 of the 4,6-linked glucosyl residue was defined by fragments [b], [e], [f], [h], [m], [o], and [p]. Attachment of the 6-linked glucosyl residue to O-6 of the 4,6-linked glucosyl residue was shown by fragments [e], [h], [o], and [p]. The 6-linked glucosyl residue attached to the branched 4,6-linked glucosyl residue was substituted through O-6 by a 3-linked glucosyl residue (fragments [b], [e], [f], and [p]); the 3-linked glucosyl residue was itself substituted through O-3 by another 3-linked glucosyl residue containing the 1-carboxyethylidene group linked at O-4 and O-6 (fragments [b], [k], and [m]).

Most of the *O*-(1-carboxyethylidene) groups were removed from the polysaccharide by partial depolymerization with 50mM TFA (Table I, column 3); this accounts for the existence of fragment [k]. The *O*-(1-carboxyethylidene) groups that remain after partial aqueous acid hydrolysis of the polysaccharide are stable to treatment with 90% formic acid. Fragments [b] and [m] demonstrated that the *O*-(1-carboxyethylidene) was located on O-4 and O-6 of a glucosyl residue that had another glycosyl residue attached to O-3.

The structure of the backbone of the polysaccharide presented in Fig. 2 contains two 3-linked galactosyl residues linked to the 4,6-linked glucosyl residues. However, no alkylated oligoglycosyl alditol containing a galactosyl residue attached to the 4,6-linked glucosyl residue was found. This can be attributed to the fact that 80% of the glycosidic linkages of the methylated 3-linked galactosyl residues were hydrolyzed during treatment of the methylated polysaccharide with 90% formic acid (data not shown). Therefore, the results obtained did not conclusively prove that the 3-linked galactosyl residues were linked to O-4 of the 4,6-linked glucosyl residues. Furthermore, partial formolysis of the methylated polysaccharide hydrolyzed 70% of the methylated terminal, nonreducing glucosyl residues (data not shown), which accounts for the fact that no alkylated oligoglycosyl alditol was found that contained a 4,6-*O*-(1-carboxyethylidene)-substituted glucosyl residue which was also substituted through O-3 with a terminal, nonreducing glucosyl residue. Therefore, it was still possible that the 3-linked galactosyl residues were linked to O-3 of the 3-linked 4,6-*O*-(1-carboxyethylidene) glucosyl residues. The points of attachment of the 3-linked galactosyl residues were determined, and the anomeric configuration of their glycosidic linkages were confirmed by subjecting the polysaccharide to Smith degradation.

Smith degradation of the polysaccharide[11]. — The polysaccharide was subjected to Smith degradation to determine whether the 3-linked galactosyl residues were attached to the 4,6-linked glucosyl residues. The 3-linked galactosyl residues, which do not contain vicinal hydroxyl groups, are resistant to periodate oxidation. However, the 4-, 6-, and 4,6-linked glucosyl residues, which are oxidized by periodate, were susceptible to Smith degradation. The aldehyde groups produced by the periodate were reduced with $NaBH_4$ to form the corresponding noncyclic acetals. The noncyclic acetals, which were acid labile[11], were hydrolyzed at room temperature with 0.5M TFA. This procedure is designed to generate oligoglycosyl alditols terminated at their reducing end with erythritol or glycerol. Erythritol is the expected oxidation product of 4- and 4,6-linked glucosyl residues, while glycerol is the expected oxidation product of 6-linked glucosyl residues.

Separation and characterization of the products of Smith degradation. — The diglycosyl alditols produced by Smith degradation of the polysaccharide were separated and purified by semi-preparative, high-pH anion-exchange chromatography with pulsed amperometric detection (h.p.a.e.–p.a.d.). Three diglycosyl alditols ([S1]–[S3], see Fig. 3) were isolated and characterized by ^{1}H-n.m.r. spectroscopy and by glycosyl-residue and glycosyl-linkage composition analyses. The diglycosyl alditols were also methylated and characterized by g.l.c.–m.s. (see Table IV).

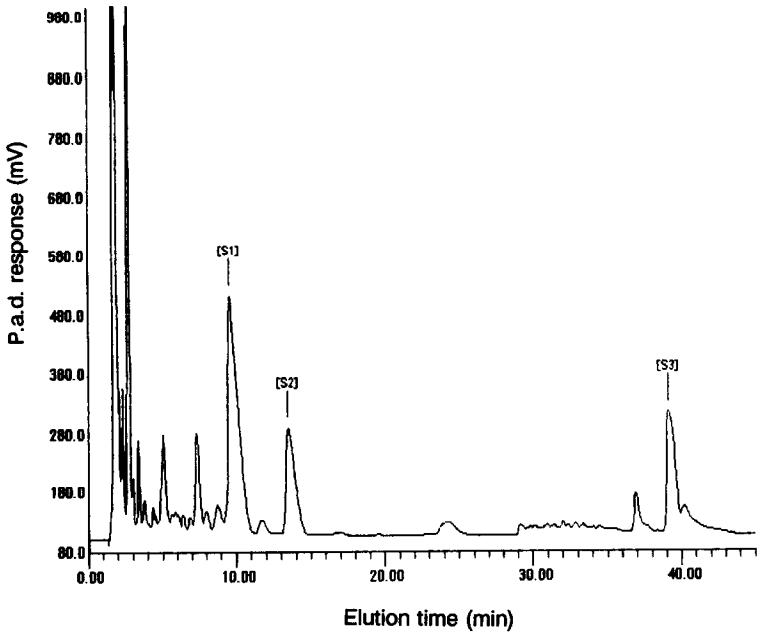
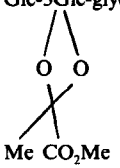


Fig. 3. Semi-preparative h.p.a.e.–p.a.d. elution profile of the diglycosyl alditols derived by Smith degradation of the acidic polysaccharide secreted by *A. radiobacter* (ATCC 53271). Each diglycosyl alditol that was structurally characterized was assigned a letter that identifies where it eluted from the h.p.l.c. column. The structures of the identified fragments are shown in Table IV.

TABLE IV

Structural characteristics of the methylated diglycosyl alditols obtained from the Smith-degraded and methylated *A. radiobacter* (ATCC 53271) polysaccharide

Oligosaccharide structure[a]	*Oligosaccharide fragment*	*H.p.a.e. retention time (min)*	*C.i.-m.s. [M + 18]*$^+$ (m/z)	*E.i.m.s. fragment ions* (m/z)							
				aA_1	aA_2	$aldJ_1$	$aldJ_2$	$aldJ_0$	$baldJ_1$	$baldJ_2$	baA_1
Glc-3Gal-Ery-ol	[S1]	9.5	604	219 (6)[b]	187 (44)	207 (3)	147 (6)	193 (0)	411 (1)	351 (1)	423 (1)
Glc-3Glc-glycerol	[S2]	13.5	560	219 (12)	187 (56)	163 (12)	103 (18)	149 (0)	367 (2)	307 (3)	423 (1)
Glc-3Glc-glycerol 	[S3]	39.5	616	275 (14)	243 (13)	163 (7)	103 (32)	149 (1)	n.p.[c]	307 (2)	n.p.

[a] Glc-3Gal-erythritol is equivalent to 2,3,4,6-tetra-*O*-methyl-D-Glc*p*(1→3)-2,4,6-tri-*O*-methyl-Gal*p*(1→3)-1,2,4-tri-*O*-methyl-D-Ery-ol. [b] Values in parentheses are the relative intensities of the fragment ions. [c] Ion not detected.

The ^{1}H-n.m.r. spectra of diglycosyl alditols [S1]–[S3] contained signals for anomeric protons in the region δ 4.40–4.90 with $J_{1,2}$ coupling constants between 7.4–7.8 Hz, establishing that the diglycosyl alditols derived from the polysaccharide by Smith degradation contained only β-linked hexosyl residues. The ^{1}H-n.m.r. spectrum of [S3] also contained a signal at δ 1.46, which was assigned to the methyl protons of a 4,6-*O*-[(*S*)-1-carboxyethylidene] group[12].

The e.i.-mass spectrum of the methylated diglycosyl alditol derived from [S1] contained an $aldJ_2$ ion at *m/z* 147 which established that its "ald" was a tetritol. Glycosyl-residue composition analysis of [S1] showed that the tetritol was erythritol. Erythritol can be derived from either a 4- or a 4,6-linked glucosyl residue. The linkage to erythritol could not be determined from the e.i.-mass spectrum of the methylated diglycosyl alditol. Glycosyl-linkage composition analysis of [S1] showed that the methylated diglycosyl alditol contained terminal, nonreducing glucosyl and 3-linked galactosyl residues. The methylated alditol acetate derived from the erythritol residue was too volatile to be analyzed by the glycosyl-linkage composition analysis procedure. However, the analyses of [S1] (see Table IV) did establish that the glucosyl residue attached to the 3-linked galactosyl residue was, in the polysaccharide, resistant to periodate oxidation and, therefore, it must have been the branched 2,4-linked glucosyl residue.

Structural analysis of the diglycosyl alditol ([S3], see Table IV) confirmed that the (*S*)-1-carboxyethylidene group was ketosidically linked to a glucosyl residue. The presence of the aA_1 ion at *m/z* 275 confirmed that the *O*-(1-carboxyethylidene) group was located on a glucosyl residue that was resistant to periodate oxidation, *i.e.*, a 3-linked glucosyl residue. The $aldJ_2$ ion at *m/z* 103 established that the "ald" was a triol, and glycosyl-residue composition analysis showed that it was indeed glycerol. The glycerol could only have been derived from a 6-linked hexosyl residue. Glycosyl-linkage composition analysis of [S3] showed terminal, nonreducing glucosyl and 3-linked glucosyl residues to be present and, in combination with ^{1}H-n.m.r. and mass spectral analyses, established the glycosyl sequence of the diglycosyl alditol (see Table IV).

The diglycosyl alditol [S2] had the same glycosyl sequence as [S3] except that the 4,6-*O*-[(*S*)-1-carboxyethylidene] group was not present. No evidence was provided by this experiment to determine whether the 4,6-*O*-[(*S*)-1-carboxyethylidene] group was cleaved during the workup of the sample or whether some of the 3-linked glucosyl residues of the polysaccharide were not substituted with *O*-(1-carboxyethylidene) groups.

Glycosyl sequence of the product obtained by periodate oxidation and reduction of the polysaccharide[13]. — The isolation and characterization of [S1] (see Table IV) as one of the products of Smith degradation of the acidic polysaccharide secreted by *A. radiobacter* indicated that a glucosyl residue that was resistant to periodate oxidation–reduction was attached to O-3 of the β-D-galactosyl residue and that the 3-linked galactosyl residue was linked to erythritol. However, the erythritol in [S1] could be derived from either a 4-linked or 4,6-linked glucosyl residue. To distinguish between these two possibilities the polymer obtained by periodate oxidation followed by $NaBH_4$ reduction of the polysaccharide was methylated and treated with aq. 90% formic acid

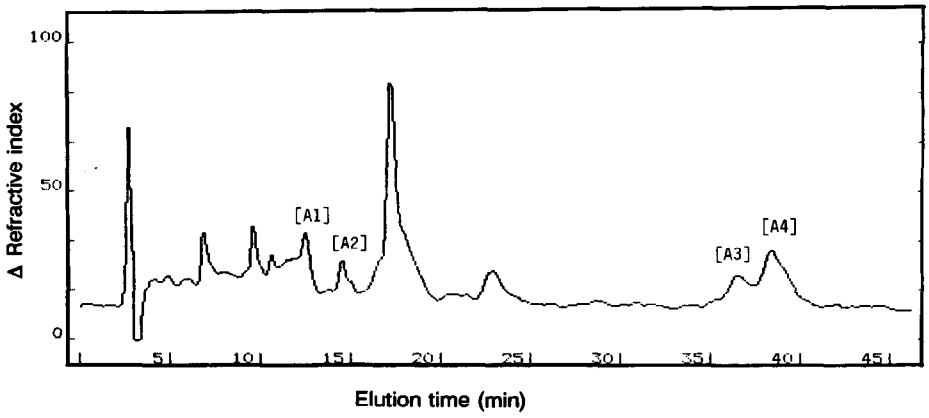


Fig. 4. Reversed-phase h.p.l.c. elution of the partially methylated, partially ethylated oligoglycosyl alditols obtained from fragmentation of the periodate-oxidized, $NaBH_4$-reduced, methylated polymer derived from the acidic polysaccharide secreted by *A. radiobacter* (ATCC 53271). Each alkylated oligoglycosyl alditol that was structurally characterized was assigned a letter that identifies the order in which it eluted from the h.p.l.c. column. The structures of the identified fragments are shown in Table V. The large peak eluting at 17.5 min is a degradation product from the reaction and was not characterized.

for 1 h at 45° to selectively hydrolyze the noncyclic acetals[13]. The resulting partially methylated oligoglycosyl alditols were *O*-ethylated. An erythritol residue derived from a 4,6-linked glucosyl residue would be substituted with two *O*-ethyl groups, whereas an erythritol derived from a 4-linked glucosyl residue would be substituted with only one *O*-ethyl group[13].

Separation and characterization of the alkylated oligoglycosyl alditols formed after formolysis and O*-ethylation of the methylated, periodate-oxidized and reduced polysaccharide.* — The alkylated oligoglycosyl alditols were separated by reversed-phase h.p.l.c., and components in the h.p.l.c. fractions ([A1]–[A4], see Fig. 4) were analyzed by ^{1}H-n.m.r. spectroscopy, g.l.c.–m.s. (c.i. and e.i.), and glycosyl-linkage composition analysis.

The ^{1}H-n.m.r. spectra of [A1]–[A4] contained signals for anomeric protons in the region δ 4.30–4.90 with $J_{1,2}$ coupling constants between 7.0 and 7.8 Hz, which was consistent with the presence of β-linked glycosyl residues. No signals could be attributed to the anomeric protons of α-linked glycosyl residues.

The e.i.-mass spectrum of [A1] contained an aA_1 ion at *m/z* 233 which showed that residue "a" contained one *O*-ethyl group (Table V). The $aldJ_2$ ion at *m/z* 175 established that the erythritol contained two *O*-ethyl groups (Table V) and, therefore, originated from a 4,6-linked glucosyl residue. The presence of the $aldJ_1$ ion at *m/z* 249 indicated that the hexosyl residue was ethylated at O-3. Glycosyl-linkage composition analysis of [A1] showed the presence of 1,5-di-*O*-acetyl-3-*O*-ethyl-2,4,6-tri-*O*-methylgalactitol. The point of substitution of the erythritol could not be determined, as the alkylated erythritol derivative was too volatile to be analyzed by this procedure. However, the presence of two ethyl groups on the erythritol and the results of the mass spectral analyses confirmed that the 3-linked galactosyl residues were connected to 4,6-linked glucosyl residues.

TABLE V

Structural characteristics of the alkylated mono- and di-glycosyl alditols obtained from the periodate-oxidized, reduced, methylated, formolyzed, and ethylated *A. radiobacter* (ATCC 53271) polysaccharide

Oligosaccharide structure[a]	*Fragment*	*G.l.c. retention time (min)*	*C.i.-m.s.* $[M+18]^+$ (m/z)	*E.i.-m.s. fragment ions* (m/z)							
				aA_1	aA_1-32	aA_1-46	$aldJ_1$	$aldJ_2$	$aldJ_0$	$baldJ_1$	$baldJ_2$
Et ↓ 1 Et-3Gal-3Ery-ol 2 ↑ Et	[A1]	10.7	442	233 (1)[c]	201 (2)	187 (3)	249 (15)	175 (8)	n.p.[b]	n.p.	n.p.
Et ↓ 2 Et-3Glc-3Glc-3glycerol	[A2]	19.6	588	233 (10)	201 (17)	187 (33)	177 (10)	147 (11)	163 (1)	395 (8)	321 (3)
Et ↓ 1 Et-4Glc-3Gal-3Ery-ol 2 2 ↑ ↑ Et Et	[A3]	21.9	660	247 (14)	215 (100)	201 (30)	235 (16)	175 (18)	221 (1)	439 (5)	379 (1)
Et ↓ 2 Et-3Glc-3Glc-3glycerol O O Me CO_2Et	[A4]	23.8	658	303 (50)	271 (15)	257 (25)	177 (10)	117 (28)	163 (1)	395 (5)	321 (4)

[a] Et-3Gal-3(1,2-Et)-Ery-ol is equivalent to 3-*O*-ethyl-2,4,6-tri-*O*-methyl-D-Gal*p*-(1→3)-1,2-di-*O*-ethyl-4-*O*-methyl-Ery-ol. [b] Ion not detected. [c] Values in parentheses are the relative intensities of the fragment ions.

The structures of [A2] and [A4] (Table V) agreed with the results of partial formolysis of the methylated polysaccharide that established the sequence of the consecutive 3-linked glucosyl residues in the side chain of the polysaccharide.

The e.i.-mass spectrum of [A3] contained an aA_1 ion at *m/z* 247 (see Table V), which established that residue "a" was branched, that is, it contained two *O*-ethyl groups. The presence of the aA_1-32 and $baldJ_1$ ions at *m/z* 215 and 439, respectively, established that residue "a" was not *O*-ethylated at position 3. The $aldJ_2$ ion at *m/z* 175 indicated that the erythritol contained two *O*-ethyl groups, that is, it was derived from a 4,6-linked glucosyl residue. Glycosyl-linkage composition analysis of [A3] showed the presence of 1,5-di-*O*-acetyl-2,4-di-*O*-ethyl-3,6-tri-*O*-methylglucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol, which, in combination with the results of ^{1}H-n.m.r. and mass spectral analyses, established that, in [A3], the 4-linked glucosyl residue attached to the 3-linked galactosyl residue was substituted at O-2 (see Fig. 1). These analyses confirmed the results obtained from partial formolysis of the methylated polysaccharide and Smith degradation of the unsubstituted polysaccharide that established that approximately 50% of the 4-linked glucosyl residues attached to 3-linked galactosyl residues were also substituted at O-2 (see Fig. 1.).

The presence of "$aldJ_l$" type fragment ions in the e.i.-mass spectra of the alkylated diglycosyl alditols obtained by alkylation of the products of Smith degradation. — The alkylated diglycosyl-glycerol and -erythritol derivatives obtained by alkylation of the products of Smith degradation ([S1]–[S3]) and the products of periodate oxidation reduction of the polysaccharide ([A2]–[A4]) were all shown to contain, by glycosyl-residue composition analysis, an internal 3-linked glycosyl residue (residue "b"). Therefore, it was expected that the e.i.-mass spectra of [S1]–[S3] and [A2]–[A4] would contain an $aldJ_0$ ion, considered diagnostic for 3-linked hexosyl residues[10]. The $aldJ_0$ ion at *m/z* 193 was not present in the e.i.-mass spectrum of the methylated diglycosyl alditol derived from [S1]; rather, an ion at *m/z* 207, corresponding to an $aldJ_1$ ion, was present (Table IV). The $aldJ_0$ fragment (*m/z* 149) was absent or very weak in the e.i.-mass spectra of the methylated diglycosyl alditols derived from [S2] and [S3], but an ion at *m/z* 163 corresponding to the $aldJ_2$ fragment was present (see Table IV). The $aldJ_0$ ions at *m/z* 163 or 221 were also very weak in the e.i.-mass spectra of [A2]–[A4], but the $aldJ_2$ ions were relatively intense (Table V).

Similar fragmentation patterns have been previously observed. The e.i.-mass spectrum of the methylated diglycosyl-erythritol derivative [Rha-(1→3)-Gal-(1→2)-erythritol], obtained by periodate oxidation of the acidic polysaccharide secreted by *Serratia marcescens*, has been shown[14] to contain an $aldJ_0$ ion of low intensity at *m/z* 193; the corresponding $aldJ_1$ fragment ion at *m/z* 207 was not reported[14]. However, the e.i.-mass spectrum of a methylated diglycosyl-erythritol derivative [Rha-(1→3)-Gal-(1→2)-erythritol], obtained by periodate oxidation and subsequent reduction of the polysaccharide secreted by *Klebsiella* serotype K18, has been reported[15] to contain an intense $aldJ_1$ fragment ion at *m/z* 207. The mechanism is not known by which the expected $aldJ_0$ and the observed $aldJ_1$ and $aldJ_2$ fragment ions are produced from the alkylated Smith degradation products obtained after periodate oxidation and reduction of the *A. radiobacter* polysaccharide.

Determination of the ring forms of the glycosyl residues. — Examination of the constituent alditol acetates of the methylated polysaccharide and the alkylated oligoglycosyl alditols showed that all the glycosyl residues are in the pyranose form. Glycosyl-linkage analysis (Table I) established that 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol accounted for all the terminal, nonreducing glucosyl residues. The pyranose form of the 3-linked 4,6-*O*-[(*S*)-1-carboxyethylidene] glucosyl residue was established by the fact that it is substituted with a 1-carboxyethylidene group attached to O-4 and O-6 (see alkylated oligoglycosyl alditols [b] and [m]. The pyranose forms of the remaining glycosyl residues were established[6] by determining the positions of *O*-ethylation of the alkylated alditol acetate derivatives of each residue when the residue was situated at the reducing end of an alkylated oligoglycosyl alditol (compare the alkylated oligoglycosyl alditols [c], [f], [g], [h], and [i]).

Treatment of the polysaccharide with a phage endo*glycanase that hydrolyzes a* R. meliloti *1021 polysaccharide that is structurally related to the polysaccharide secreted by the* A. radiobacter *species.* — The results described above provided strong evidence that the acidic polysaccharide secreted by *A. radiobacter* (ATCC 53271) is composed of a heptadecasaccharide repeating unit (Fig. 2). Evidence that the acidic polysaccharide was not a mixture of polysaccharides was obtained by purification of the polysaccharide by precipitation with CTAB[3,4] and by ion-exchange chromatography. The single product in each case had a glycosyl-residue composition identical with that of the native polysaccharide. Nevertheless, the chemical analyses did not prove conclusively that the *A. radiobacter* polysaccharide was, in fact, a single polysaccharide with a 17 glycosyl-residue repeating unit. There remained a possibility that *A. radiobacter* was producing an as yet inseparable mixture of two polysaccharides, one with an octasaccharide repeating unit and the other with a nonasaccharide repeating unit (see Fig. 2). The glycosyl sequences of the octa- and nona-saccharide repeating units would be identical, except that the nonasaccharide would contain a terminal, nonreducing glucosyl residue linked to O-2 of a 4-linked glucosyl residue (Fig. 2), that is, it would have two branches.

In an attempt to answer this question, the polysaccharide secreted by *A. radiobacter* was treated with bacteriophage M12 that contains an *endo*glycanase known to hydrolyze the glycosidic linkage between 4-linked β-D-glucosyl residues and 3-linked β-D-galactosyl residues in the backbone of the acidic polysaccharide produced by *Rhizobium meliloti* 1021 (ref. 16). The structure of the backbone of the polysaccharide secreted by *R. meliloti* 1021 (ref. 6) is identical to the backbone of the octasaccharide portion of the polysaccharide secreted by *A. radiobacter* (see Fig. 2). If *A. radiobacter* (ATCC 53271) produced two polysaccharides, the polysaccharide with the eight glycosyl-residue repeating unit would have a glycosyl sequence identical to the polysaccharide produced by *R. meliloti* 1021 and, therefore, would be depolymerized by the phage *endo*glycanase.

The polysaccharide secreted by both *A. radiobacter* and *R. meliloti* 1021 were treated with the phage *endo*glycanase. The products were desalted and analyzed by h.p.a.e.–p.a.d. (data not shown). The polysaccharide produced by *R. meliloti* 1021 was depolymerized into a mixture of its eight glycosyl-residue repeating unit (60%) and

dimers of its eight glycosyl-residue repeating unit (40%). These repeating units were shown, by f.a.b.-m.s., to contain *O*-acetyl, *O*-succinyl, and *O*-(1-carboxyethylidene) groups (data not shown). In contrast, the *A. radiobacter* polysaccharide (or polysaccharides) was (or were) not depolymerized by the phage *endo*glycanase. The solution containing the *A. radiobacter* polysaccharide remained viscous following treatment with the phage *endo*glycanase, and no oligoglycosyl subunits were detected by h.p.a.e.–p.a.d. The inability of the phage *endo*glycanase to hydrolyze the *A. radiobacter* polysaccharide provides additional evidence that this polysaccharide exists as a single molecular species with alternating octaglycosyl and nonaglycosyl sequences.

CONCLUSIONS

The structure of the repeating unit of the polysaccharide secreted by *A. radiobacter* (ATCC 53271) is similar to the structure of the repeating unit of the polysaccharide secreted by *R. meliloti* 1021 (ref. 6). However, structural analysis of the *A. radiobacter* polysaccharide has provided evidence that this polymer is composed of a heptadecasaccharide rather than an octasaccharide repeating unit. The heptadecasaccharide repeating unit of the *A. radiobacter* polysaccharide contains a terminal nonreducing β-D-glucosyl group attached to O-2 of every second 4-linked glucosyl residue that is linked to O-3 of the galactosyl residue (Fig. 2).

The fact that the polysaccharide produced by *A. radiobacter* (ATCC 53271) is not depolymerized by the phage *endo*glycanase that cleaved the *R. meliloti* 1021 polysaccharide provides evidence that the *A. radiobacter* polysaccharide is composed of alternating octa- and nona-saccharide sequences, which together constitute the heptadecasaccharide repeating unit. If the polysaccharide possessed several consecutive octasaccharide sequences equivalent to the repeating units of the *R. meliloti* 1021 polysaccharide, the phage *endo*glycanase would cleave these sequences and thereby reduce the molecular weight and viscosity of the *A. radiobacter* polysaccharide. We found no evidence that the phage *endo*glycanase could hydrolyze any of the glycosidic bonds in the *A. radiobacter* polysaccharide. We concluded that the presence of two side chains on the nonasaccharide portion of the seventeen glycosyl-residue repeating unit changes the conformation of the polysaccharide so that the octasaccharide portion no longer has the conformation required to be a substrate for the phage *endo*glycanase.

There is one caveat to this conclusion. The *O*-[(*S*)-1-carboxyethylidene] group of the *A. radiobacter* polysaccharide is located on a 3-linked glucosyl residue of the tetraglycosyl side chain (Fig. 2), while the *O*-[(*S*)-1-carboxyethylidene] group of the *R. meliloti* 1021 polysaccharide is located on the terminal, nonreducing glucosyl residue of the tetraglycosyl side chain[6]. This difference would not be expected to alter the ability of the phage *endo*glycanase to cleave between the 4-linked glucosyl residue and the 3-linked galactosyl residue of the backbone, as the phage enzyme(s) even cleaves this sequence in a polysaccharide (secreted by a mutant strain of *R. meliloti* 1021) that has only a diglycosyl repeating unit[16].

The *A. radiobacter* (ATCC 53271) polysaccharide is, to our knowledge, the first

one with evidence of a repeating unit composed of as many as seventeen glycosyl residues. The largest bacterial polysaccharide repeating unit previously characterized was composed of eleven glycosyl residues[17]. Another unusual feature of the *A. radiobacter* polysaccharide is that three of the glucosyl residues in the repeating unit are branched. Two are 4,6-linked glucosyl residues, and one is a 2,4-linked glucosyl residue. An enzyme capable of depolymerizing the polysaccharide into its repeating unit should make it possible to unambiguously answer the question of whether the polysaccharide produced by *A. radiobacter* has a heptadecasaccharide repeating unit.

EXPERIMENTAL

Production and isolation of the polysaccharides secreted by A. radiobacter *(ATCC 53271).* — *A. radiobacter* (ATCC 53271) was grown in liquid medium (100 mL), pH 7.2, containing D-glucose (2.5%), L-glutamic acid (0.02%), potassium phosphate (0.5%), magnesium sulfate (0.02%), calcium chloride (0.005%), and trace amounts of biotin, pantothenic acid, and manganese. The cultures were grown on a rotary shaker (200 r.p.m.) until the D-glucose was consumed (6–8 days). The fermentation broth was treated with 2-propanol (2 vols.), and the resulting precipitate, which contained the polysaccharide, was collected by centrifugation. A solution of the precipitate in water was reprecipitated by the addition of 2-propanol (2 vols.). This process was repeated four times. A solution of the polysaccharide in water was centrifuged (40 000*g*) for 1 h and then precipitated again by the addition of 2-propanol. The polysaccharide preparation contained no protein as determined by the Lowry method[18].

Glycosyl-residue composition analysis. — The glycosyl-residue composition of the polysaccharide was determined by g.l.c. analysis[19] of the alditol acetate and per-*O*-trimethylsilyl methyl glycoside derivatives.

Determination of the absolute configuration of the glycosyl residues[20]. — The polysaccharide (1 mg) was treated for 1 h at 120° with 2M TFA. The TFA was removed under a stream of air, and the residue was treated for 16 h at 80° with *S*-(+)-2-butanol–HCl. The *S*-(+)-2-butyl glycosides so obtained were *O*-trimethylsilylated and analyzed by g.l.c.

Identification of pyruvylated glycosyl residues[2]. — Glycosyl residues containing *O*-(1-carboxyethylidene) residues were released from the polysaccharide by treatment with M HCl in dry methanol for 16 h at 80°. The products were *O*-trimethylsilylated and examined by g.l.c.–e.i.m.s.

Partial acid hydrolysis of the polysaccharide. — The polysaccharide (50 mg) in water (100 mL) was heated to 80°, TFA was added to 50mM, and the solution was maintained for 1 h at 80°. The cooled solution was dialyzed against deionized water and then freeze-dried (yield 43 mg).

Methylation analysis[19]. — The native and acid-treated (50mM TFA, 1 h, 80°) polysaccharides (500 μg) in Me_2SO (200 μL) were treated with potassium dimethylsulfinyl anion (100 μL of 2.0M) for 8 h. The solutions were cooled to 4°, MeI (100 μL) was added, and the mixtures were stirred for 6 h at 20°. Excess MeI was evaporated, and the

methylated polysaccharide was dialyzed and freeze-dried. The procedure was repeated to ensure complete methylation.

Glycosyl-linkage composition analysis. — Glycosyl-linkage analyses of the methylated polysaccharide (500 μg) and the alkylated oligoglycosyl alditols were performed as previously described[19].

Determination of the conditions for partial formolysis[7]. — Portions (250 μg) of the methylated polysaccharide were treated with 90% formic acid for 30, 45, and 90 min at 85°. Following reduction (NaB^2H_4) of the reducing termini that had been exposed by partial formolysis, the remaining glycosidic linkages were fully hydrolyzed (2M TFA, 1 h 120°). The resulting partially methylated glycoses were reduced with $NaBH_4$, then acetylated and analyzed as their partially methylated alditol acetates by g.l.c.–c.i.m.s. (ammonia reagent gas).

Preparation of partially methylated, partially ethylated oligoglycosyl alditols. — The polysaccharide (30 mg) that had been heated in 50mM TFA for 1 h at 80° was methylated and subjected to treatment with aq. 90% formic acid for 30 min at 85°. The formic acid was removed under a stream of air, and the reducing ends of the partially methylated oligosaccharides were converted, by reduction with NaB^2H_4, to oligoglycosyl alditols. The partially methylated oligoglycosyl alditols were ethylated as described[7]. The mixtures of partially methylated, partially ethylated oligoglycosyl alditols (3 mg) were separated by h.p.l.c. (Bio Rad 700T liquid chromatograph) on a Zorbax ODS column (250 × 4.6 mm) eluted with aq. 50% acetonitrile at 0.8 mL/min. The eluate was monitored with a Hewlett–Packard 1037A differential refractometer, and the components were collected manually.

Smith degradation of the polysaccharide[11]. — A solution of the polysaccharide (50 mg) in 50mM sodium periodate (100 mL) was kept in the dark for 48 h at 20°. Excess periodate was then destroyed by addition of ethylene glycol (3 mL), and the solution was dialyzed. Sodium borohydride (400 mg) was added, and the solution was kept for 8 h at 20°. Excess borohydride was destroyed by the addition of glacial acetic acid, and the solution was dialyzed and freeze-dried. The periodate treatment was repeated to ensure complete oxidation of those glycosyl residues of the polysaccharide that possessed vicinal hydroxyls (yield 27 mg).

A solution of the periodate-oxidized and $NaBH_4$-reduced polysaccharide (10 mg) in 10mM imidazole·HCl (pH 7 (1 mL), was chromatographed on a column (1 × 5 cm) of DEAE-BioGel A by elution with 10mM imidazole·HCl, pH 7 (30 mL), and then M imidazole·HCl, pH 7 (50 mL). The eluates were dialyzed and freeze-dried, yielding 700 μg of neutral material (10 mM imidazole eluant) and 8.8 mg of the oxidized and reduced acidic polysaccharide (M imidazole eluant). The modified acidic polysaccharide (8 mg) in 0.5M TFA (1 mL) was kept for 48 h at 20°. The solution was concentrated to dryness under a stream of air, and the remaining TFA was removed by washing the residue with methanol (3 × 500 μL). A solution of the residue in water (600 μL) was filtered (0.2 μm Nylon-66 membrane), and portions (200 μL) fractionated by h.p.a.e. chromatography on a semi-preparative CarboPac 1 column (9 × 250 mm, Dionex Corp., Sunnyvale, CA) by elution at 5 mL/min with 100mM NaOH (0–20 min) followed by a 0–100mM NaOAc

gradient in 100mM NaOH (20–35 min), and finally 100mM NaOAc in 100mM NaOH (35–45 min). The eluant was monitored with a pulsed amperometric detector (Dionex Corp., Sunnyvale, CA) at 30 μA sensitivity. Fractions were collected manually. The fractions were desalted (5 mL/cartridge) using OnGuard H cartridges (Dionex Corp.), concentrated to 1 mL, and freeze-dried.

The glycosyl-residue compositions of the oligoglycosyl alditols purified by h.p.a.e. were determined by g.l.c. of their alditol acetates[19]. The underivatived oligoglycosyl alditols were analyzed by ^{1}H-n.m.r. spectroscopy, and the methylated oligoglycosyl alditols were analyzed directly by g.l.c.–m.s. (e.i. and NH_4-c.i.). The glycosyl-linkage compositions were obtained by g.l.c.–e.i.m.s. analysis of their partially methylated alditol acetates.

Determination of the sequence of glycosyl residues in the periodate oxidized, $NaBH_4$-reduced polysaccharide[13]. — A solution of the periodate-oxidized and $NaBH_4$-reduced polysaccharide (9 mg) in Me_2SO (1 mL) was methylated by the Hakomori procedure[19]. The methylated product was isolated by dialysis against aq. 50% EtOH and freeze-dried. A solution of the methylated product in aq. 90% formic acid was kept for 1 h at 45°. The solution was concentrated to dryness under a stream of air, and the residue was washed with MeOH (3 × 500 μL). A solution of the residue in Me_2SO (500 μL) was ethylated[20], and the resulting alkylated oligoglycosyl alditols were purified using Spice C_{18} cartridges (Rainin Instrument Co., Woburn, MA). The oligoglycosyl derivatives were separated by reverse-phase h.p.l.c. on a Zorbax ODS column (4.6 × 250 mm) by elution with aq. 50% acetonitrile at 0.8 mL/min. The eluant was monitored with a differential refractive-index detector, and fractions were collected manually. The partially purified, alkylated oligoglycosyl alditols were characterized by g.l.c.–m.s. (e.i. and NH_4-c.i.), by ^{1}H-n.m.r. spectroscopy, and by glycosyl-linkage composition analysis.

Propagation of the R. meliloti *bacteriophage.* — The bacteriophage M12 was propagated[16] in cultures (100 mL) of *R. meliloti* 7094 (an EPS^- mutant) grown in modified Bergersen's medium[21], pH 7, supplemented with 500μM $CaCl_2$. The phage lysate (110 mL) was filtered (0.2 μm membrane), concentrated to 10 mL, dialyzed against 1mM K_2HPO_4, pH 7, containing 500μM $CaCl_2$, and stored at 4°. Bacterial plaque assays[21] showed that the concentrated phage solution contained ~ 10^9 plaque-forming units/mL.

Treatment of the A. radiobacter *and* R. meliloti *1021 polysaccharides with the phage* endo*glycanase.* — Solutions of each of the polysaccharides (1 mg) in 1mM K_2HPO_4 (5 mL), pH 7, containing 500μM $CaCl_2$ were mixed with the concentrated phage solution (1 mL) and incubated for 48 h at 30° under toluene. The solutions were dialyzed (1000 mol. wt. cut-off membrane) at 4° against water and freeze-dried.

Analysis of the products of phage endo*glycanase treatment.* — Solutions (600 μL) containing the phage and phage-treated polysaccharide (~ 1 mg) in water were filtered (0.2 μm membrane), and portions (20 μL) of the filtrate were chromatographed and analyzed by h.p.a.e.–p.a.d. on a CarboPac 1 column (4.6 × 250 mm). The polysaccharide and any oligoglycosyl repeating units were eluted at 1 mL/min with 100mM NaOH containing 100mM NaOAc (0–10 min) followed by a 100mM–500mM NaOAc gradient in 100mM NaOH (10–30 min), and finally 500mM NaOAc in 100mM

NaOH (30–45 min). The eluant was monitored with a pulsed amperometric detector (Dionex Corp.) at 1 μA sensitivity.

Mass spectrometry. — A Hewlett–Packard 5985 or 5987 g.l.c.–m.s. instrument was used. Spectra were obtained in the c.i. (NH_4-c.i.) or e.i. mode. The alkylated oligoglycosylalditols were separated by g.l.c. in a 15-m DB-1 column, using on-column injection. The g.l.c. was programmed to remain at 80° for 2 min, then to rise from 80° to 200° at 30°/min intervals, and, finally, to 340° at 10°/min.

^{1}H-N.m.r. spectroscopy. — Bruker AM 250 or AM 500 n.m.r. spectrometers were used. Spectra of the polysaccharides were obtained at 250 MHz in ^2H_2O (75°) containing 0.1% sodium 3-(trimethylsilyl) (2,2,3,3-$^2H_4$)-propanoate as internal standard (δ = 0.0). Spectra of the oligoglycosyl alditols were obtained at 500 MHz in ^2H_2O (20°). Chemical shifts are reported in p.p.m. relative to internal acetone (δ 2.225 downfield from external Me_4Si δ = 0.0). Spectra of the alkylated oligoglycosyl alditols were obtained at 500 MHz in $(C^2H_3)_2CO$ containing 0.1% Me_4Si. Chemical shifts are reported in p.p.m. downfield from internal Me_4Si (δ = 0.0).

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