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METHYLATION ANALYSIS OF ACIDIC EXOPOLYSACCHARIDES OF *Rhizobium* AND *Agrobacterium*

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ABSTRACT

Exopolysaccharides of *Agrobacterium tumefaciens* and *Rhizobium meliloti*, containing D-glucose, D-galactose, pyruvic acid, and *O*-acetyl groups in the approximate proportions 6:1:1:1.5, were analysed by methylation. They were found to contain the following main structural units (all β-glycosidic): chain residues of (1→3)-linked D-glucose (24%), (1→3)-linked D-galactose (15%), (1→4)-linked D-glucose (20%), and (1→6)-linked D-glucose (18%); (1→4,1→6)-linked branching residues of D-glucose (12%), and terminal D-glucose residues substituted at positions 4 and 6 by pyruvate (11%). Uronic acid-containing exopolysaccharides of *Rhizobium leguminosarum*, *R. phaseoli*, and *R. trifolii* contained D-glucose, D-glucuronic acid, D-galactose, pyruvic acid, and *O*-acetyl groups in the approximate proportions 5:2:1:2:3. Methylation gave identical patterns of methylated sugar components, from which the following structural elements were deduced: chain residues of (1→3)-linked D-glucose substituted at positions 4 and 6 by pyruvate (13%), (1→4)-linked D-glucose (32%), and (1→4)-linked D-glucuronic acid (20%); (1→4,1→6)-linked branching residues of D-galactose and/or D-glucose (13%), and terminal D-glucose and/or D-galactose residues substituted at positions 4 and 6 by pyruvate (13%).

INTRODUCTION

During quantitative chemical analysis of exopolysaccharides[1] of 21 strains of *Rhizobium* and *Agrobacterium* from 5 taxonomic groups, a distinctive difference was observed between the groups of *R. leguminosarum*, *R. phaseoli*, and *R. trifolii*, and the groups of *R. meliloti* and *A. tumefaciens*. The first three groups of *Rhizobium* polysaccharides all had the same range of D-glucose (48–61%), D-galactose (8–14%), D-glucuronic acid (14–19%), pyruvate (10–15%), and acetyl contents (7–13%). *R. meliloti* and *A. tumefaciens* polysaccharides showed a much higher content of D-glucose (76–83%) and contained less than 1% of D-glucuronic acid; the pyruvate content (3–8%) was lower than with the first three groups of rhizobia.

The chemical structure of these polysaccharides has now been examined by methylation analysis, in combination with periodate oxidation and fragmentation of the polysaccharides by partial hydrolysis.

METHODS

Organisms. — Organisms which were used in the methylation study were from the collection of the Laboratory of Microbiology at Wageningen, as follows: *Rhizobium leguminosarum* PRE (R-7), *R. phaseoli* Bokum (R-21), *R. trifolii* K-8 (R-38), *R. meliloti* K-24 (R-13), *R. meliloti* A-148 (R-15), *Agrobacterium tumefaciens* A-8, and *A. tumefaciens* A-10.

Cultivation of organisms. — Bacterial strains were kept on agar slants of the following compositions: yeast extract, 1; mannitol, 10; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.25; NaCl, 0.1; $CaCO_3$, 3; agar, 12 g/l (*Rhizobium* strains), and yeast extract, 7; D-glucose, 10; agar, 12 g/l (*Agrobacterium* strains). Growth of large quantities of bacteria took place in a fully synthetic medium of the following composition: D-glucose, 10; $(NH_4)_2SO_4$, 1; K_2HPO_4, 1; $MgCl_2$, 0.2; $CaCO_3$, 2.5 g/l; biotin, 10 μg/l; thiamine, 100 μg/l; trace elements: $FeCl_3 \cdot 6H_2O$, 2.5; H_3BO_3, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.01; $CoCl_2 \cdot 6H_2O$, 0.01; $CuSO_4 \cdot 5H_2O$, 0.01; $MnCl_2$, 1; $Na_2MoO_4 \cdot 2H_2O$, 0.01 mg/l. For strain *R. leguminosarum* PRE, 10 mg of methionine and 10 mg of uracil were added per litre of medium as necessary growth-factors (private communication of A. W. S. M. van Egeraat).

Bacteria were precultivated in 100 ml of the synthetic medium contained in 300-ml Erlenmeyer flasks on a rotary shaker at 25° for 2 days. They were then transferred into 1 litre of the same medium in a Kluyver flask and aerated at 25° for 2–3 days.

Preparation of exopolysaccharides. — Cells were separated from the culture by centrifugation at 25,000 *g* and 20° for 30 min. To the clear, viscous supernatant liquid was added a solution of cetylpyridinium chloride[2] (1.5 g/l of culture). After settling of the jelly-like polysaccharide complex, the supernatant liquid was decanted, and the residual complex was centrifuged and washed with water. It was decomposed by stirring in 200 ml of 5% aqueous sodium chloride. To the resulting viscous solution, two volumes of ethanol were added to precipitate the polysaccharide which was then dissolved in 200 ml of water, dialysed against running tap-water for 3 days, and freeze-dried. Yields: 0.5–1 g/l of culture.

Component analysis of polysaccharides. — Exopolysaccharides were hydrolysed in closed ampoules with 0.5M sulfuric acid at 100° for 16 h. D-Glucose in the hydrolysate was measured with D-glucose oxidase (Kabi, Stockholm, Sweden); D-glucose and D-galactose were separated as their alditol acetates by g.l.c. on an OV-225 column at 200°, and their ratio determined. Uronic acid, pyruvate, and *O*-acetyl were determined as described previously[1].

Partial hydrolysis. — Polysaccharide (100 mg) in 10 ml of 0.5M sulfuric acid was heated at 100° for 50 min. The hydrolysate was neutralised with saturated, aqueous barium hydroxide, centrifuged, and concentrated *in vacuo*. Separations were carried out on paper (solvent *B*) and by carbon–Celite chromatography.

Methylation analysis of acidic polysaccharides. — Prior to methylation, polysaccharides were converted into their free-acid form by deionisation on a column of Amberlite IR-120 (H^+) resin, and freeze-dried.

Polysaccharide (10 mg; dried at 70° for 2 h) was dissolved in 2 ml of dry methyl sulfoxide in a serum bottle under nitrogen. The free-acid form of the polysaccharide immediately dissolved. Methylsulfinyl sodium solution[3] (1.5 ml; 1M) was added by using a syringe; for complete formation of alkoxide, the mixture was stirred on a magnetic stirrer at room temperature for 4 h. Methyl iodide (0.5 ml) was then added from a syringe, with external cooling by ice–water. Stirring was continued at room temperature for 2 h. The resulting solution was dialysed overnight in cellophane against running tap-water, and lyophilised.

The methylated polysaccharide (5 mg) was first treated with 3 ml of 90% formic acid at 100° for 4 h and then evaporated in *vacuo* at 40°. Hydrolysis was continued in 3 ml of 0.25M sulfuric acid at 100° for 16 h. The hydrolysate was neutralised with saturated, aqueous barium hydroxide and centrifuged. The hydrolysate of methylated sugars was reduced with 20 mg of sodium borohydride for at least 4 h. The mixture was then neutralised with acetic acid and deionised with Amberlite IR-120(H^+). After concentration *in vacuo*, boric acid was removed by codistillation with methanol (2 × 5 ml of methanol), and the residue was converted into alditol acetates by treatment with acetic anhydride–pyridine (1:1, 4 ml) at 100° for 20 min. The reaction mixture was concentrated to dryness *in vacuo*, and the residue was dissolved in chloroform and analysed by g.l.c.[4].

Periodate oxidation. — Polysaccharide (100 mg) was oxidized in 100 ml of 15mM sodium metaperiodate, with stirring, in the dark. Samples (10 ml) were taken at different times for spectrophotometric determination (223 nm) of periodate consumption, and for potentiometric determination of formic acid produced[5].

Separation techniques. — All evaporations were carried out in a rotary evaporator at bath temperatures not exceeding 40°.

Paper chromatography of methylated sugars was carried out on Whatman No. 3MM paper with 1-butanol–ethanol–water (4:1:5, upper phase; solvent *A*). Monosaccharides and oligosaccharides were separated on Whatman No. 3MM paper with 1-butanol–pyridine–benzene–water (5:3:1:3, upper phase; solvent *B*). Larger quantities of polysaccharide fragments were separated on a carbon–Celite (1:1) column by using an alcohol gradient[6]. Smith-degradation products were separated on Whatman No. 1 paper with pyridine–ethyl acetate–water (2:5:7, upper phase; solvent *C*). Chromatograms were sprayed with *p*-anisidine hydrochloride for the detection of reducing sugars, and with benzidine–sodium metaperiodate for the detection of alditols.

G.l.c. of alditol acetates[7] was effected with a Becker Unigraph-F, type 407, gas chromatograph equipped with a flame-ionisation detector; nitrogen flow-rate of 30 ml/min; stainless-steel column (200 cm × 4 mm) containing 3% of OV-225 on Chromosorb W-HP (100–120 mesh), and operating at 200° for alditol acetates and at 185° for partially methylated alditol acetates.

Methylated sugar components were identified by comparison of the *T* values of the corresponding alditol acetates with those of authentic *O*-methyl sugars, and by their mass spectra. Mass spectra were recorded with an AEI MS 902 mass spectrometer at an inlet temperature of 200° and an ion-source temperature of 130°.

RESULTS

Exopolysaccharides were prepared from cultures of bacteria grown in a fully synthetic, liquid medium. Component analyses of polysaccharide preparations used throughout this investigation are summarised in Table I.

TABLE I

COMPONENT ANALYSIS OF POLYSACCHARIDE PREPARATIONS

Strain	*Component* (%)[a]				
	D-*Glucose*	D-*Galactose*	*Uronic acid*	*Pyruvate*	*Acetyl*
R. leguminosarum PRE	50	10	19	12	9
R. phaseoli Bokum	59	8	16	11	6
R. trifolii K-8	51	9	20	11	9
R. meliloti K-24	70	14	1	6	9
A-148[b]	75	13	0	6	6
A, tumefaciens A-8	76	12	1	7	4
A-10	77	12	0	7	4

[a]Weight (%) calculated on the sum of all components determined. [b]Small amounts of D-mannose were present.

Acidic polysaccharides of Rhizobium meliloti *and* Agrobacterium tumefaciens. — The approximate, relative proportions of D-glucose, D-galactose, and pyruvic acid residues in polysaccharides of *R. meliloti* and *A. tumefaciens* were 6:1:1; acetyl contents were more variable (1–2 moles). On treatment of the polysaccharides with 0.25M sulfuric acid at 100° for 50 min, all pyruvate was liberated, whereas only small amounts of reducing sugars were set free. Partial hydrolysis yielded a number of oligosaccharides which were separated by chromatography on a carbon–Celite column. The isolated di- and tri-saccharides could be cleaved by emulsin β-D-glucosidase, yielding only D-glucose. On comparing the R_F values on paper (solvent *B*) with known reference compounds, four of the fragments could be identified as laminaribiose, laminaritriose, cellobiose, and gentiobiose, respectively.

Exopolysaccharides of *Rhizobium meliloti* (strains K-24 and A-148) and of *Agrobacterium tumefaciens* (strains A-8 and A-10) were methylated with methylsulfinyl sodium and methyl iodide in methyl sulfoxide, according to Hakomori[8]. The fully methylated polysaccharides were hydrolysed, and the resulting methylated sugars were converted into their alditol acetates and analysed by g.l.c. Typical chromatograms on an OV-225 column are given in Fig. 1.

Chromatograms of methylated sugars from polysaccharides of *R. meliloti* and *A. tumefaciens* were very similar, both qualitatively and quantitatively (Table II). 2,3,4,6-Tetra-*O*-methyl-D-glucose (peak *T*) was present in amounts of only 1–2%, and is derived from unsubstituted, non-reducing end-groups in the polymer. Peaks *A* and *B* were identified as 2,4,6-tri-*O*-methyl-D-glucose and 2,4,6-tri-*O*-methyl-D-

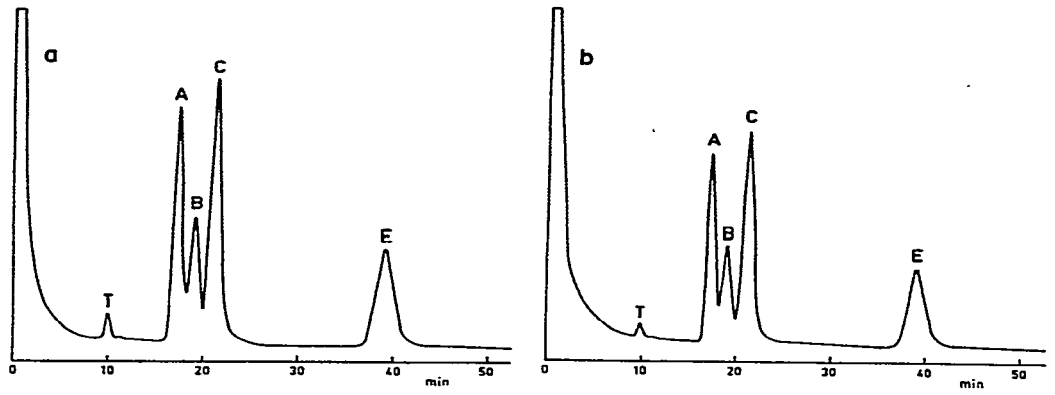

Fig. 1. Chromatograms obtained in the methylation analysis of the exocellular polysaccharides from *A. tumefaciens* A-8 (a) and *R. meliloti* K-24 (b).

galactose, respectively, indicating (1→3)-linked D-glucose and D-galactose chain-residues. The amount of 2,4,6-tri-*O*-methyl-D-galactose corresponded with the D-galactose content of the polysaccharide. Consequently, the material in the other peaks must be derived from D-glucose. 2,3,4-Tri-*O*-methyl-D-glucose and 2,3,6-tri-*O*-methyl-D-glucose (peak *C*) could not be resolved on the OV-225 column (*T* values 2.08 and 2.12, respectively). The corresponding (1→4)- and (1→6)-linked D-glucose chain-residues were differentiated by periodate oxidation. Peak *E* was identified as 2,3-di-*O*-methyl-D-glucose. The occurrence of 2,3-di-*O*-methyl-D-glucose (22%), in the absence of an equivalent amount of 2,3,4,6-tetra-*O*-methyl-D-glucose, can be explained by assuming that half the amount of the di-*O*-methyl sugar is derived from (1→4,1→6) D-glucose branching residues and half from terminal D-glucose residues, to which are attached pyruvic acid acetals in positions 4 and 6.

TABLE II

METHYL ETHERS OBTAINED ON METHYLATION ANALYSIS OF THE ACIDIC POLYSACCHARIDES OF *R. meliloti* AND *A. tumefaciens*

Peak	*Sugars*	T[a]	*Mole %*			
			K-24	*A-148*	*A-8*	*A-10*
T	2,3,4,6-Tetra-*O*-Me-Glc	1.00	1	0.5	2	0.5
A	2,4,6-Tri-*O*-Me-Glc	1.72	24	26	25	24
B	2,4,6-Tri-*O*-Me-Gal	1.90	15	13	15	14
C	2,3,4-Tri-*O*-Me-Glc + 2,3,6-Tri-*O*-Me-Glc	2.12	37	39	36	39
E	2,3-Di-*O*-Me-Glc	3.90	23	22	22	23

[a] *T*, Retention times of the corresponding alditol acetates, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

The polysaccharides from *A. tumefaciens* (strain A-10) and *R. meliloti* (strain K-24) were oxidized with periodate at 25°. After 100 h, 0.81 mole of periodate was consumed and 0.18 mole of formic acid released per mole of hexose. The periodate-oxidised polysaccharide was reduced with sodium borohydride, and the resulting polyalcohol was hydrolysed with sulfuric acid (Smith degradation). On chromatography of the hydrolysate in solvent *C*, a large proportion (40–50%) of periodate-resistant hexose residues were recovered; D-galactose was found to be completely periodate-resistant, thereby proving its (1→3)-linked position in the polysaccharide. Furthermore, glycerol (14–15 mole%) and erythritol (35–40 mole%) were found, representing residues which are linked (1→6) and (1→4), respectively.

Uronic acid-containing polysaccharides of Rhizobium leguminosarum, R. phaseoli *and* R. trifolii. — Methylation of the uronic acid-containing polysaccharides was followed by reduction with sodium borohydride and remethylation to give a fully methylated, neutral polysaccharide[3]. This procedure has the advantage that, on hydrolysis of the final product, no acid-resistant aldobiouronic acid residues are formed. Moreover, methylated uronic acid residues are converted into the parent hexoses, which can be chromatographed and analysed as partially methylated alditol acetates (Figs. 2 and 3).

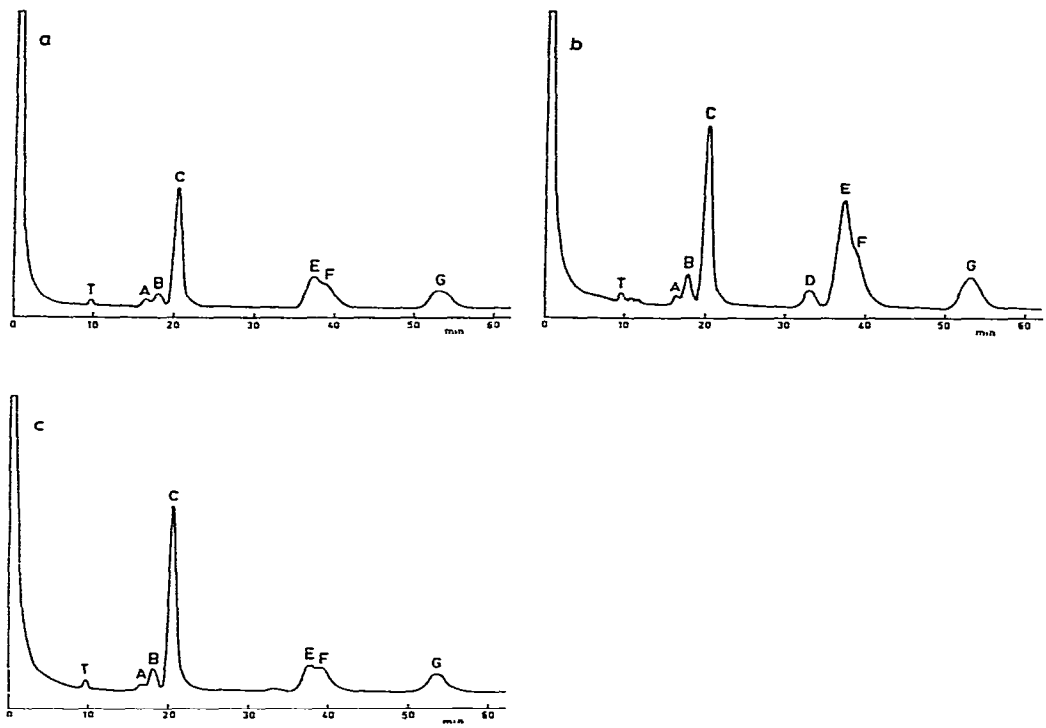

Fig. 2. Chromatograms obtained in the methylation analysis of the exocellular polysaccharide from *R. leguminosarum* PRE. a, Initially methylated (I); b, methylated and reduced (II); c, methylated, reduced, and remethylated (III).

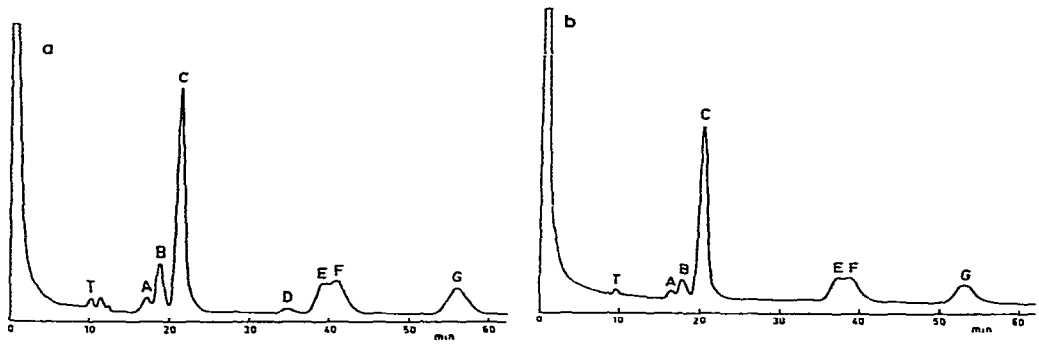

Fig. 3. Chromatograms obtained in the methylation analysis of the exopolysaccharides from *R. phaseoli* Bokum (a) and *R. trifolii* K-8 (b). Methylated, reduced, and remethylated polysaccharide.

Chromatograms of fully methylated, neutral-polysaccharide hydrolysates of *Rhizobium leguminosarum*, *R. phaseoli*, and *R. trifolii* were nearly identical and contained four main peaks (*C*, *E*, *F*, and *G*) and a number of minor components. 2,3,6-Tri-*O*-methyl-D-glucose (peak *C*) was derived from (1→4)-linked chain-residues of D-glucose and from originally (1→4)-linked D-glucuronic acid residues after they had been reduced and remethylated. Di-*O*-methylhexoses (peaks *E* and *F*) were not completely separated by g.l.c., but could be separated more fully on paper (solvent *A*). They were eluted from the paper and converted into their alditol acetates. Demethylation[9] with BBr_3 showed that *E* was derived from D-glucose, and *F* from D-galactose. Mass spectrometry[10] of the two di-*O*-methyl derivatives gave identical mass spectra which corresponded with a 2,3-substitution pattern. Half of the di-*O*-methylhexose fraction has to be interpreted as being derived from branching points at positions 1, 4, and 6, the other half being derived from terminal hexose residues substituted at positions 4 and 6 by pyruvate. On remethylation of the methylated and reduced polysaccharide (*R. leguminosarum*, strain PRE; Table III, Fig. 2), ~20 mole% of 2,3-di-*O*-methyl-D-glucose in the methylated and reduced polysaccharide was converted into an equivalent amount of 2,3,6-tri-*O*-methyl-D-glucose in the fully methylated, neutral polysaccharide. This is conclusive evidence that ~20 mole% of D-glucuronic acid is present as (1→4)-linked chain-residues. Peak *G* was identified as 2-*O*-methyl-D-glucose by demethylation to D-glucose and by mass spectrometry. Therefore, this mono-*O*-methylhexose has a (1→3)-linked position in the polysaccharide chain, at the same time being substituted at positions 4 and 6 by pyruvate. This makes a total proportion of 25 mole% of pyruvate-linked hexose residues (Table III), in accordance with the analytical values for pyruvate of Table I. Minor components found in all chromatograms included 2,3,4,6-tetra-*O*-methyl-D-glucose and -galactose (peak *T*), indicating unsubstituted, non-reducing end-groups; 2,4,6-tri-*O*-methyl-D-glucose (peak *A*) and 2,4,6-tri-*O*-methyl-D-galactose (peak *B*), indicating (1→3)-linked chain-residues of D-glucose and D-galactose; and, in some chromatograms, an unidentified peak *D*.

TABLE III

METHYL ETHERS OBTAINED ON METHYLATION ANALYSIS[a] OF THE URONIC ACID-CONTAINING POLYSACCHARIDE OF *Rhizobium leguminosarum* (STRAIN PRE)

Peak	*Sugars*	T[b]	*Mole %*		
			I	*II*	*III*
T	2,3,4,6-Tetra-*O*-Me-Glc	1.00	1.5	1	1
	2,3,4,6-Tetra-*O*-Me-Gal	1.12	—	—	—
A	2,4,6-Tri-*O*-Me-Glc	1.71	2	1	1
B	2,4,6-Tri-*O*-Me-Gal	1.88	5	5	6
C	2,3,6-Tri-*O*-Me-Glc	2.10	43	32	53
D	N.i.[c]	3.44	—	5	—
E	2,3-Di-*O*-Me-Glc	3.88	20	31	12
F	2,3-Di-*O*-Me-Gal	4.06	12	12	13
G	2-*O*-Me-Glc	5.52	16	13	13

[a]I, Initially methylated polysaccharide; II, methylated and reduced polysaccharide; III, methylated, reduced, and remethylated polysaccharide. [b]See Table II. [c]Not identified.

Periodate oxidation of the polysaccharide from *Rhizobium leguminosarum* (strain PRE) was essentially complete in ~6 days. Periodate uptake was 1 mole per mole of hexose, with no significant release of formic acid. Smith degradation of the periodate-oxidised polysaccharide gave mainly erythritol, D-glucose, and D-galactose, with traces of glycerol.

DISCUSSION

The neutral salts of the acidic polysaccharides, in particular the uronic acid-containing polysaccharides, were only poorly soluble in methyl sulfoxide, and subsequent methylation was incomplete. Therefore, polysaccharides were deionised on a cation exchanger. With the free-acid forms, which were freely soluble in methyl sulfoxide, the single step methylation procedure of Hakomori gave, in most instances, fully methylated products in nearly quantitative yields.

Titration of the deionised polysaccharides with sodium hydroxide indicated that the carboxyl groups of the uronic acid and pyruvic acid residues were in the free-acid forms. The pyruvate substituents were not removed under the strong alkaline conditions of the methylation reaction and therefore must be present as *O*-(1-carboxyethylidene) groups linked to sugar residues by acetal bonds[11].

Exopolysaccharides of Rhizobium meliloti *and* Agrobacterium tumefaciens. Isolation of β-(1→3)-, β-(1→4)-, and β-(1→6)-linked di- and tri-saccharides after partial hydrolysis of *R. meliloti* and *A. tumefaciens* polysaccharides showed that glycosidic linkages are largely, if not entirely, of the β-D configuration. Methylation analysis, combined with the results of periodate oxidation, gave the main structural elements summarised in Table IV. These polysaccharides contain the same structural units as the extracellular polysaccharide of *R. meliloti*, strain U 27[12], which was

analysed by Björndal *et al.*[13]. Their values of the relative amounts of methylated sugars were only slightly different. Also, they inferred a slightly higher proportion of D-glucose in the polysaccharide, with a component composition of D-glucose–D-galactose–pyruvate–*O*-acetyl 7:1:1:1. According to these authors, the *O*-acetyl groups did not occupy a unique position in the polysaccharide, but were found in position 6 of both the (1→3)- and (1→4)-linked chain-residues of D-glucose.

TABLE IV

PRINCIPAL STRUCTURAL FEATURES OF EXOPOLYSACCHARIDES OF *Rhizobium* AND *Agrobacterium*

Mode of linkage	*Molar proportions of sugar residues*	
	Type I	*Type II*
	R. meliloti *and* A. tumefaciens	R. leguminosarum, R. phaseoli, *and* R. trifolii
Terminal groups	4,6-*O*-(1-Carboxyethylidene)-D-glucose (11%)	4,6-*O*-(1-Carboxyethylidene)-D-glucose or -D-galactose (13%)
Chain residues		
(1→3)	D-Glucose (24%); D-galactose (15%)	4,6-*O*-(1-Carboxyethylidene)-D-glucose (13%); D-galactose (6%)
(1→4)	D-Glucose (20%)	D-Glucose (32%); D-glucuronic acid (20%)
(1→6)	D-Glucose (18%)	
Branching residues		
(1→4,1→6)	D-Glucose (12%)	D-Galactose or D-glucose (13%)

Production of acidic polysaccharides of the *R. meliloti* and *A. tumefaciens* type was not restricted to strains of these two species within the family of *Rhizobiaceae*, but was also observed with a number of slime-producing Gram-negative rods of the *Pseudomonas* type isolated from activated sludge (unpublished results). With the strains of *A. tumefaciens* available from the laboratory collection, only acidic polysaccharides were produced in large amounts, and no appreciable quantities of neutral polysaccharides were found in the culture fluid. It is noteworthy that neutral polysaccharides of the β-(1→2)-D-glucan type, also called crown-gall polysaccharides[14], have been found as the principal polymer among the excreted products of a number of phytopathogenic *Agrobacteria*, being the only organisms that have yet been shown to produce this type of polysaccharide[15]. In addition to extracellular, soluble slime, some of our *A. tumefaciens* strains (A-8 and A-10) and also some *Rhizobium* strains (*R. trifolii* K-8) formed electron-microscopically visible fibrils of cellulose around the cells, holding them together in a flocculent mass[16].

Exopolysaccharides of Rhizobium leguminosarum, R. phaseoli *and* R. trifolii. On hydrolysis of the methylated derivative of the capsular polysaccharide synthesized by *Rhizobium radicicolum* (Clover Bartel "A" strain), Schlüchterer and Stacey[17]

obtained approximately equimolecular parts of 2,3,6-tri-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-D-glucose, and 2,3-di-*O*-methyl-D-glucuronic acid. Part of the last constituents were shown to originate from a cellobiouronic acid derivative. The presence of a di-*O*-methyl sugar without any 2,3,4,6-tetra-*O*-methyl-D-glucose, which could not be explained by them, is now readily understood by the fact that terminal groups of the polysaccharide are substituted by pyruvate.

Methylated polysaccharide hydrolysates of the uronic acid-containing polysaccharides described in this report (Type II of Table IV) have two di-*O*-methylhexoses, namely 2,3-di-*O*-methyl-D-glucose and 2,3-di-*O*-methyl-D-galactose in approximately equal quantities. On the basis of the methylation analysis, it was not possible to decide which of these was derived from a branching point, and which sugar came from a substituted end-group. The presence of 2-*O*-methyl-D-glucose in the hydrolysate is explained by the fact that part of the pyruvate is attached to (1→3)-linked chain-residues of D-glucose.

A number of bacterial polysaccharides having a differing degree of substitution by pyruvate groups are now known. In some cases, the positions of the pyruvate substituents are well-established. Polysaccharides with a relatively low content of pyruvate (4–8% by weight) usually have the groups attached to terminal hexose residues. *Xanthomonas campestris*[11], *Rhizobium meliloti*[13,18], and *Agrobacterium tumefaciens* have pyruvate attached to end-groups of D-glucose, and *Corynebacterium insidiosum*[19] and *Enterobacteriaceae*[20] to end-groups of D-galactose. The uronic acid-containing polysaccharides of *R. leguminosarum*, *R. phaseoli*, and *R. trifolii* with 12% of pyruvate, described herein, have their groups linked partly to terminal hexose residues, and partly to chain residues. Examination of exopolysaccharides of a number of bacteria isolated from activated sludge led to a group of organisms with up to 20% of pyruvate groups, all of which are linked to chain residues of the essentially linear polymer (unpublished data).

By comparison, types I and II polysaccharides (Table IV) can be considered as having the same basic structure of an equally branched polymer of sugar residues which are linked principally (1→3) and (1→4). Nearly all end-groups in both types of polysaccharides are substituted by pyruvate. Differences between the two types are the presence of a considerable number of D-glucuronic acid and of pyruvate-substituted (1→3)-linked D-glucose chain-residues in type II, and the presence of (1→6)-linked D-glucose chain-residues in type I polysaccharides, which appear to be absent in type II polysaccharides.

Within each group of polysaccharides, there is a remarkable constancy of component composition and of structural properties. On the basis of methylation analysis, no specificity can be found towards different species of *Rhizobium* in each group. Therefore, the concept put forward by Fåhraeus and Ljunggren[12] that the high specificity of infective properties of *Rhizobium* to host plants is determined by their capsular polysaccharide structure cannot be substantiated by the results of this investigation.

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