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Note

Gel-forming capsular polysaccharide of *Rhizobium leguminosarum* and *Rhizobium trifolii**

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Bacteria belonging to the family of the *Rhizobiaceae* produce several different types of cellular and extracellular polysaccharides[1]. Extracellular polysaccharides of the fast-growing rhizobia and agrobacteria are usually excreted into the medium as freely soluble slime. These excreted gums have a remarkably constant chemical and linkage composition within certain groups of rhizobia[2]. Polysaccharides excreted by strains of *R. meliloti* and of *Agrobacterium* lack uronic acid and have D-glucose, D-galactose, pyruvic acid, and succinic acid in the molar ratios 7:1:1:1, whereas the common excretion products of many strains of *R. leguminosarum*, *R. trifolii*, and *R. phaseoli* are composed[3] of D-glucose, D-galactose, D-glucuronic acid, and pyruvic acid in the ratios 5:1:2:2.

Investigation of the production of cell-bound carbohydrates of fast-growing *Rhizobium* cultures revealed that these carbohydrates accumulate towards the end of the exponential phase of growth. In several instances, most of the cell carbohydrates are utilised by the cells on subsequent incubation of the cultures, and turned out to be intracellular glycogen[1]. For several strains of *R. leguminosarum* and *R. trifolii*, a large part of the cell carbohydrate of high molecular weight was not utilised by the cells and it was suggested that this non-metabolisable carbohydrate was present in the form of a capsule or of loose adhering slime. Floc-forming rhizobia and agrobacteria produce a (1→4)-β-D-glucan in the form of cellulose microfibrils around the cells[4]. Apart from freely soluble slime, *Agrobacterium* species produce an insoluble, gel-forming (1→3)-β-D-glucan (curdlan) that can be extracted from the cells with cold 0.5M NaOH and precipitated by neutralisation with acid[5]. Moreover, all species of fast-growing rhizobia and of agrobacteria produce a cyclic (1→2)-β-D-glucan[6] of low molecular weight.

Extraction of cell pellets of several *R. leguminosarum* and *R. trifolii* strains with M NaOH at room temperature released an anthrone-positive material which, on neutralisation of the extract, precipitated as a gel. An alternative method of iso-

*Surface Carbohydrates of *Rhizobium*, Part IV. For Part III, see ref. 3.

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lation of the polysaccharide involved heating the cell suspension at ~100° (boiling-water bath) for 1 h, followed by filtration of the hot suspension through a bacteriological filter. On cooling to room temperature, the clear filtrate solidified to a stable gel, already at a polysaccharide concentration as low as 0.2%. The sol–gel transition temperature lies between 50–55°.

Hydrolysis of the polysaccharide (2M trifluoroacetic acid, 100°, 6 h) released D-galactose, D-glucose, and D-mannose in the molar ratios 4:1:1 (Table I, column

TABLE I

SUGAR ANALYSIS OF THE ORIGINAL AND MODIFIED CAPSULAR POLYSACCHARIDES FROM *Rhizobium leguminosarum* AND *R. trifolii* (VALUES ARE GIVEN FOR STRAIN TA-1)

Sugar component (as alditol acetates)	*Molar ratios*[a]					
	A	*B*	*C*	*D*	*E*	*F*
Glycerol		+				
Threitol		0.7				
Erythritol					+	
D-Mannose	1.0	1.00	1.00	1.00	1.00	0.17
D-Galactose	4.0	0.92	1.03	1.41	0.99	0.34
D-Glucose	1.0	0.97	0.96	0.96	0.14	0.11

[a]Polysaccharide: A, original; B, periodate-oxidised and borohydride-reduced; C, periodate-oxidised, borohydride-reduced, and Smith-degraded (main chain); D, partially periodate-oxidised (0.5 h), borohydride-reduced, and Smith-degraded; E, periodate-oxidised and borohydride-reduced main chain; and F, chromium trioxide-oxidised, acetylated original polysaccharide.

TABLE II

METHYLATION ANALYSIS OF THE ORIGINAL AND MODIFIED CAPSULAR POLYSACCHARIDES OF *Rhizobium trifolii*, STRAIN TA-1

Methylated sugar[a]	T[b]	*Molar ratios*[c]					
		A	*B*	*C*	*D*	*E*	*F*
2,3,4,6-Man	1.00						1.00
2,3,4,6-Gal	1.07	1.80				0.34	
2,4,6-Man	1.54	1.00	1.00	1.00	1.00	1.00	
2,4,6-Gal	1.58	0.96	0.99	1.05	1.11	0.93	0.83
2,3,6-Gal	1.65	0.84					
2,3,6-Glc	1.79			0.88	0.83[d]	0.46	
2,3-Glc	2.98					0.70	
3-Glc	4.58	0.87	0.88				

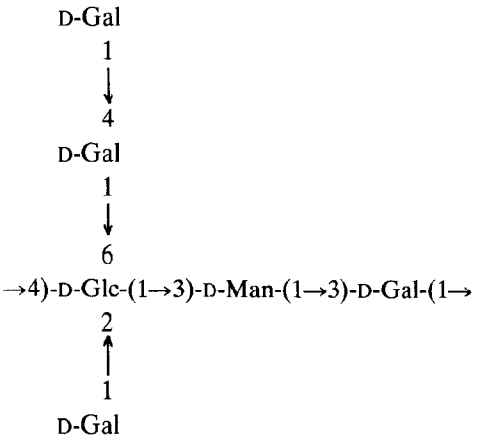
[a]2,3,4,6-Man = 2,3,4,6-tetra-*O*-methyl-D-mannose, *etc.* [b]Retention times of the corresponding alditol acetates relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a fused-silica WCOT-OV-225 column at 190°. [c]Polysaccharide: A, original; B, periodate-oxidised and borohydride-reduced; C, periodate-oxidised, borohydride-reduced, and Smith-degraded; D, periodate-oxidised, borohydride-reduced, methylated, Smith-degraded, and trideuteriomethylated; E, partially periodate-oxidised (0.5 h), borohydride-reduced, and Smith-degraded; F, periodate-oxidised, borohydride-reduced, Smith-degraded main chain. [d]2,6-Di-*O*-trideuteriomethyl-3-*O*-methyl-D-glucose.

A), suggesting a hexasaccharide repeating-unit. No uronic acids, acyl (acetyl, succinyl), or acetal-linked pyruvic-substituents could be detected. Methylation analysis of the polysaccharide (Table II, column A) revealed two D-galactosyl end-groups, one (1→3)-linked D-mannosyl, one (1→3)-linked D-galactosyl and one (1→4)-linked D-galactosyl residue, and a doubly branched D-glucosyl residue linked through O-1,2,4,6.

The polysaccharide consumed 5 mol of periodate per hexasaccharide repeating-unit and produced 2 mol of formic acid within 5 h at room temperature. Borohydride reduction of the resulting polyaldehyde, followed by hydrolysis in 2M trifluoroacetic acid, afforded D-glucose, D-galactose, and D-mannose in the ratios 1:1:1, together with glycerol and threitol (Table I, column B). Methylation of the polyalcohol (Table II, column B) indicated that periodate oxidation had destroyed two D-galactosyl end-groups and one (1→4)-linked D-galactosyl residue. Smith degradation of the polyalcohol (90% formic acid, 40°, 1 h) gave a product that was still macromolecular and non-dialysable, containing equal amounts of D-glucose, D-galactose, and D-mannose (Table I, column C). Methylation analysis of the Smith-degraded polymer (Table II, column C) proved this to be unbranched, consisting of (1→3)-linked D-mannosyl, (1→3)-linked D-galactosyl, and (1→4)-linked D-glucosyl residues. The positions of attachment of the D-galactosyl side-chains were confirmed by methylation of the polyalcohol followed by Smith degradation and trideuteriomethylation of the degraded polymer (Table II, column D). The resulting 2,3,6-tri-*O*-methyl-D-glucose had MeO-2,6 deuterated, indicating thereby the positions of the D-galactosyl and the di-D-galactosyl side-chains. Their relative positions were assessed by methylation analysis of partially periodate-oxidised and Smith-degraded polysaccharide (Table I, column D). Since a D-galactosyl end-group containing a vicinal triol group is considered to be more susceptible to periodate attack than a (1→4)-linked D-galactosyl residue, containing a vicinal diol grouping, any non-oxidised D-galactosyl residue remaining linked to the main chain after such oxidation will indicate the position of the di-D-galactosyl side-chain. The formation of 2,3-di-*O*-methyl-D-glucose on methylation analysis of incompletely periodate-oxidised and Smith-degraded polysaccharide (Table II, column E) indicated that the di-D-galactosyl side-chain occupied position 6 of the main-chain D-glucosyl residue.

The sequence of the hexose residues in the main chain was studied by periodate oxidation of the debranched polysaccharide, as the now (1→4)-linked D-glucosyl residues will be susceptible to periodate oxidation (Table I, column E). Successive reduction and Smith degradation of the polyalcohol afforded a trisaccharide product containing D-mannose, D-galactose, and erythritol, methylation analysis (Table II, column F) of which gave 2,3,4,6-tetra-*O*-methyl-D-mannose and 2,4,6-tri-*O*-methyl-D-galactose, indicating the sequence: →3)-D-Man-(1→3)-D-Gal-(1→4)-D-Glc-(1→ in the main chain.

Chromium trioxide oxidation of the acetylated original polysaccharide followed by sugar analysis (Table I, column F) showed that all sugar residues had



been oxidised and therefore the residues must have been mainly β-linked.

The structure reported here displays some unusual features for a bacterial exopolysaccharide formed from repeating units. It is a neutral heteropolysaccharide with no carbonyl-containing substituents and thus resembles the extracellular polysaccharide gum from a slow-growing *Rhizobium*, strain CB744, of the cowpea group[7]. The structure also contains a doubly branched sugar residue, a rare feature of bacterial polysaccharides. Such features are present[8] in the capsular polysaccharides of *Klebsiella*, types 33 and 38. The proposed structure of the gel-forming polysaccharide, which is common to many, if not all, strains of *R. leguminosarum* and *R. trifolii*, is the first reported for an insoluble, capsular polysaccharide of the fast-growing rhizobia. Thus, bacteria belonging to these genera are able to produce two completely different exopolysaccharides built up from repeating units, namely, an acidic exopolysaccharide (octasaccharide repeating-units[9]) which is excreted into the medium, and an insoluble, capsular neutral polysaccharide (hexasaccharide repeating-units). An analogous situation exists with agrobacteria, which produce a soluble succinoglycan (also formed from octasaccharide units) and insoluble, neutral (1→3)-β-D-glucan (curdlan[10]), and with many enterobacteria, which excrete soluble colanic acid and have specific capsular polysaccharide.

EXPERIMENTAL

The bacterial strains used in this investigation were *R. leguminosarum* (strains F-13, TOM, RCR-1012, and RCR-1044), *R. trifolii* (strains TA-1 and 0403), and *R. phaseoli* (strain 127 K-82). They were cultivated[1] in 0.1% glutamic acid–0.5% D-mannitol–salts medium. The methods for sugar and methylation analysis have also been described[2,11].

Preparation of gel-forming polysaccharide. — Washed cell pellets (~1 g) from 1 L of culture were suspended in M NaOH (100 mL), stirred for 1 h at room

temperature, and then stored in a refrigerator overnight. Extracted cells were removed by centrifugation and the supernatant solution was neutralised with acetic acid. The precipitated gel was collected by centrifugation, washed twice with water, dialysed, and freeze-dried. In this way, strains 1044, TA-1, and F-13 yielded 200–300 mg of product, containing >95% of hexose (calculated as D-galactose) as determined by the anthrone–sulphuric acid method. Strains 1012, 0403, TOM, K-82, and several other strains yielded smaller amounts of a similar product.

Periodate oxidation. — Polysaccharide (100 mg) was dissolved in water (50 mL) at 60°. To the stirred solution was added 0.03M sodium metaperiodate (50 mL), and the mixture was immediately cooled to room temperature. At intervals, samples (0.1 mL) were diluted to 25 mL and the periodate consumption was measured spectrophotometrically[12] at 223 nm. Liberation of formic acid was measured by titration with 0.1M NaOH after reduction of unreacted periodate with ethylene glycol. Oxidation proceeded rapidly in the dark at 20° and, after 8 h, an excess of ethylene glycol was added, and the oxidised product was isolated by dialysis and then reduced overnight with sodium borohydride (200 mg). The excess of reductant was decomposed with acetic acid, and the mixture was dialysed and freeze-dried.

Smith degradation of the product (84.8 mg) was carried out for 1 h at 40° in 90% formic acid (40 mL). Formic acid was evaporated *in vacuo*, and a solution of the residue in water was dialysed and freeze-dried. A portion (33.1 mg) of the product (43.1 mg) was dissolved in 15mM $NaIO_4$ (40 mL). After 4 days in the mixture was worked-up as described above. Part (10 mg) of the product (30 mg) was subjected to sugar analysis, and another part (10 mg) was Smith-degraded at 40° for 1 h in 90% formic acid (5 mL). The solution was concentrated *in vacuo* and freeze-dried, and the residue was subjected to methylation analysis.

Chromium trioxide oxidation. — This reaction was carried out as described by Lindberg and Lönngren[13].

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