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(1→2)-β-D-GLUCAN AND ACIDIC OLIGOSACCHARIDES PRODUCED BY *Rhizobium meliloti*

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ABSTRACT

In addition to cellular glycogen and an acidic exopolysaccharide of high molecular weight, *Rhizobium meliloti* (5 strains) produces a cyclic (1→2)-β-D-glucan (d.p. ~20) that accumulates in the cells, and a pyruvylated and succinylated octasaccharide that is excreted into the medium. The octasaccharide has the same structural elements as the high-molecular-weight exopolysaccharide and is considered to be the repeating unit of this polysaccharide.

INTRODUCTION

Cells of fast-growing species of *Rhizobium*, when growing in carbohydrate-rich media, produce large amounts of polymeric carbon compounds that are either accumulated in the cell or excreted into the medium. In the cell are laid down poly(β-hydroxybutyric acid) and/or glycogen, which function as sources of carbon and energy, and a (1→2)-β-D-glucan of low molecular weight (~4000) having a yet unknown physiological function[1]. Also produced are acidic polysaccharides that are excreted into the surrounding medium. In most studies of exopolysaccharides of *Rhizobium*, preparations were obtained by precipitation with 2–5 vol. of ethanol or acetone, or by complex formation with cetyltrimethylammonium bromide followed by regeneration of the polysaccharide by dissolution in NaCl and reprecipitation with ethanol. In this way, high-molecular-weight, gelatinous precipitates are obtained; these give highly viscous, non-dialysable, aqueous solutions from which a loose, fibrous material can be isolated by freeze-drying. The gums thus obtained have remarkably constant chemical and linkage composition within certain groups of rhizobia. Type I polysaccharides[2] lack uronic acid and have the molar composition D-glucose–D-galactose–pyruvic acid 7:1:1, with various amounts of acyl substituents (succinyl and acetyl). They are common excretion-products of strains of *Rhizobium meliloti*[3] and of *Agrobacterium*[4], and are identical with the so-called succinoglycan of *Alcaligenes faecalis*[5]. Methylation analysis of these polysaccharides revealed the same structural elements for the three groups of bacteria[2,6]. The complete structure was elucidated by chemical degradation[7] and by enzymic breakdown[8]. Type II polysaccharide[2], composed of D-glucose, D-galactose, D-

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glucuronic acid, and pyruvic acid in the ratios 5:1:2:2, is the generally occurring type of exopolysaccharide excreted by many strains of *R. leguminosarum*, *R. phaseoli*, and *R. trifolii*.

Precipitation of the soluble exopolysaccharides by adding up to 5 vol. of ethanol, or by Cetavlon-complexation, does not remove all of the carbohydrate material from supernatants of *Rhizobium* cultures, and further precipitation of carbohydrate material may be achieved by concentrating the supernatant solutions of the first alcohol-precipitation to a small volume and then adding up to 10 vol. of ethanol[1]. In this way, carbohydrate fractions of low molecular weight are obtained, especially with cultures of *R. meliloti*. During gel chromatography on Sephadex G-50, these fractions behave like the (1→2)-β-D-glucan with respect to particle size, and it was therefore suggested that accumulated (1→2)-β-D-glucan was excreted from the cells during growth. Further structural investigation of this material, however, made it clear that, in most cases, it contained all the structural elements of the polysaccharide fractions of high molecular weight from the culture supernatants of *R. meliloti*. A more detailed description of the low-molecular-weight fractions of the excretion products of *R. meliloti* is now reported.

EXPERIMENTAL

Strains of *Rhizobium meliloti* SU-47, SU-234, SU-255, SU-256, and K-24, and of *Agrobacterium tumefaciens* A-8, and the general methods of analysis have been described[1,2].

Isolation of low-molecular-weight oligosaccharides from cultures of R. meliloti. — *R. meliloti* cells were cultivated in a glutamic acid–D-mannitol–salts medium of the following composition: glutamic acid, 1.0; D-mannitol, 5.0; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.04 g per litre; 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; $MnCl_2$, 1.0; $Na_2MoO_4 \cdot 2H_2O$, 0.01 mg per litre; biotin, 10; thiamine, 100 μg per litre of distilled water of pH 7.0. Cultivation took place in 100 mL of medium in 300-mL Erlenmeyer flasks on a rotary shaker at 25° for 5–7 days; larger amounts of cultures were obtained with 1–1.5 L of medium in 5-L Erlenmeyer flasks at 30°, which were inoculated with 100 mL of preculture and cultivated for 7 days on a shaking machine.

For preparation of carbohydrate fractions of high and low molecular weight, cells were centrifuged for 30 min at 40,000*g*. The clear, supernatant fluid was concentrated by vacuum evaporation at 40° to 20% of the original volume. To this were added 3 vol. of ethanol with stirring, and the resulting, gelatinous precipitate of exopolysaccharide of high molecular weight was collected by centrifugation. The supernatant, alcoholic solution was concentrated *in vacuo* to ~5% of the original-culture volume, and 10 vol. of ethanol were added, to give a fine, turbid precipitate. After storage for 1 day in a refrigerator, the material was collected by centrifugation, dissolved in a small amount of water, and further purified and desalted

by gel filtration on a column of Sephadex G-50 equilibrated with water.

Isolation of (1→2)-β-D-glucan from the cells was effected by extraction of the cells with hot, aqueous 75% ethanol as described earlier[1]; curdlan, a gel-forming (1→3)-β-D-glucan, was extracted from the cell pellet with 0.5M NaOH at room temperature and reprecipitated with hydrochloric acid[9].

Gel chromatography of whole supernatant-solutions was performed on a column (2.5 × 37 cm) of Biogel Agarose A-5m, which was equilibrated with 0.1M NaCl–0.01M Na_2HPO_4 at pH 7.0.

Separation of acidic fractions was carried out on a column (2 × 38 cm) of QAE Sephadex equilibrated with water[10]. Material was first eluted with 100 mL of water and then with 800 mL of a gradient of 0→100mM KCl. Fractions (10 mL) were collected, and their sugar content was measured by the anthrone–sulphuric acid method.

Analysis of poly- and oligo-saccharides. — Sugar composition of samples was determined by total hydrolysis in 2M trifluoroacetic acid at 100° for 6 h. The liberated sugars were converted into alditol acetates and separated on a capillary WCOT fused-silica column (25 m × 0.32 mm) of OV-225 at 200°, using a Kipp Analytica system 8200 gas chromatograph. Total hexoses were measured by the anthrone–sulphuric acid method. Using the response factor of 0.7 for equal weights of D-galactose and D-glucose in the anthrone reaction, absolute amounts of D-glucose and of D-galactose in the samples were computed from their ratio as determined by g.l.c.

Succinic acid was determined by g.l.c. in the presence of an internal standard. A known amount of glutaric acid was added to the sample prior to hydrolysis with trifluoroacetic acid. Dried hydrolysates were converted into methyl esters by the action of BF_3-methanol complex (1 mL, 5 min at 80°). After cooling, 2–4 mL of chloroform and 2 mL of water were added to destroy excess of reagent. The chloroform layer (1 μL) was injected into the OV-225 column at 80°. Using the response factor of succinic acid (0.94) towards an equal weight of glutaric acid, the amount of succinic acid was calculated from the peak areas. It was not possible to determine pyruvic acid at the same time because of irreproducible responses of pyruvic acid methyl ester during g.l.c. Absolute amounts of pyruvic acid in the samples were measured, after hydrolysis with M HCl for 3 h at 100°, with 2,4-dinitrophenylhydrazine[11].

Uronic acid was measured by the *m*-hydroxybiphenyl method[12], using D-glucurono-6,3-lactone as standard. Reducing sugars and reducing end-groups of oligosaccharides were measured by the Somogyi–Nelson method, using the appropriate standards. Methylation analysis of poly- and oligo-saccharides was performed as described earlier[2]. Methylated sugars were separated as alditol acetates on a WCOT-OV-225 fused-silica column at 190° and identified by mass spectrometry. ^{1}H-N.m.r. spectra of solutions in deuterium oxide were recorded with a Varian EM-390 instrument at 90 MHz.

TABLE I

PRODUCTION OF CELLULAR (1→2)-β-D-GLUCAN AND OF EXOPOLYSACCHARIDES OF HIGH AND LOW MOLECULAR WEIGHT BY *Rhizobium meliloti*, EXPRESSED AS μg OF CARBOHYDRATE PER ml OF CULTURE

Strain	*Dry weight of cells (μg/ml)*	*Cellular (1→2)-β-D-glucan*[a]	*Supernatant carbohydrates*[a]		
			Total	*Fractions subsequently precipitated with ethanol*	
				5 vol	*10 vol*
R. meliloti SU-47	1505	171	500	165	204
SU-234	1360	112	457	241	152
SU-255	1830	121	320	183	84
SU-256	1270	165	400	297	65
K-24	1240	116	510	287	70
A. tumefaciens A-8	2535[b]	390	245	108	86

[a]Determined by the anthrone–sulphuric acid method and expressed as glucose equivalents. [b]Cell pellet also contained 815 μg of (1→3)-β-D-glucan, which was extracted with 0.5M NaOH (see text).

RESULTS AND DISCUSSION

Rhizobium meliloti strains were cultivated in a glutamic acid (0.1%)–D-mannitol(0.5%)–salts medium. After 5 days of growth at 25°, cells and culture supernatant were separated by centrifugation. (1→2)-β-D-glucan of low molecular weight, which has been found to be generally present in *Rhizobium* cells[13], was extracted from the cells with hot, aqueous 75% ethanol, and its carbohydrate content was determined by the anthrone–sulphuric acid method (Table I). The supernatant solutions from the cultures were fractionated into products of high and low molecular weight by ethanol precipitation (see Experimental), and their carbohydrate contents were determined by the anthrone method. This fractionation procedure removed nearly all of the carbohydrate from the culture supernatant, leaving behind only small amounts of carbohydrate in the last supernatants.

For comparison, a strain of *Agrobacterium tumefaciens* was included in this study, as agrobacteria are known to produce the same types of polysaccharide as *R. meliloti*[2,4,6]. In addition, *Agrobacterium* produces an insoluble, gel-forming (1→3)-β-D-glucan[9]. Following the extraction of (1→2)-β-D-glucan with hot ethanol, the so-called curdlan was extracted from the remaining cell pellet by stirring with 0.5M NaOH at room temperature.

Distribution of the extracellular, soluble carbohydrates of *R. meliloti* cultures according to molecular weight was also demonstrated by gel filtration of whole supernatants on a column of Biogel Agarose A-5m with a fractionation range between molecular weights of 10,000 and 5,000,000. *R. meliloti* SU-47 showed 2 major peaks, one corresponding to material of high molecular weight (several millions) and a larger peak derived from material having mol. wt. $\leq 10^4$ (Fig. 1).

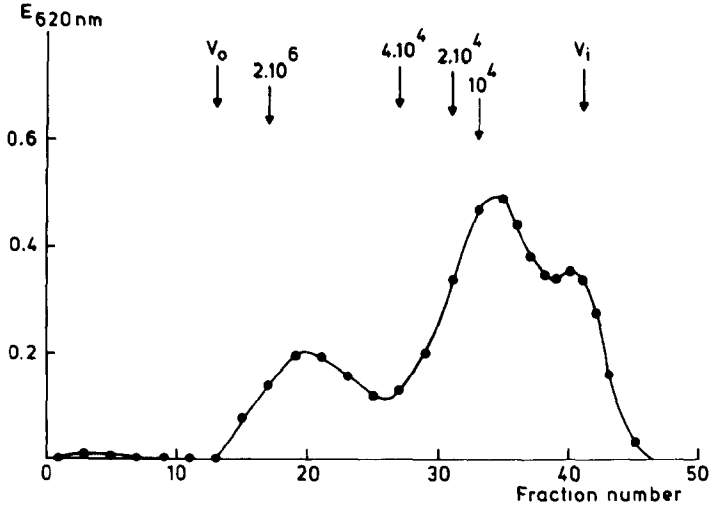


Fig. 1. Gel chromatography of whole culture-supernatants of *R. meliloti* SU-47 on a column of Agarose A-5m equilibrated with 0.1M NaCl. The void volume (V_o) and included volume (V_i) of the column are indicated, as are the elution volumes of dextrans of known molecular weights. Fractions were analysed for neutral hexoses by the anthrone reaction.

Supernatant solutions of other *R. meliloti* cultures displayed similar patterns, but with different ratios for the fractions of high and low molecular weight.

Fractions of low molecular weight were further purified and desalted by gel filtration on a Sephadex G-50 column. Material from *R. meliloti* SU-47 gave a symmetrical elution-pattern with a molecular-weight distribution around 2000, close to the molecular-weight range[1] for (1→2)-β-D-glucan of ~4000.

Component analysis of the fractions of excreted carbohydrates having low molecular weight revealed two types of product (Table II): a neutral saccharide from *R. meliloti* strain SU-234 and *A. tumefaciens* A-8, consisting entirely of D-glucose residues; and acidic oligosaccharides from *R. meliloti*, strains SU-47, SU-255, and SU-256, composed of D-glucose, D-galactose, pyruvic acid, and succinic acid in the ratios 7:1:1:1, and identical in composition with the corresponding fractions of high molecular weight. Succinic acid, which had been overlooked in earlier studies of this polysaccharide[2], was found to be a general constituent of *R. meliloti* and *A. tumefaciens* polysaccharides.

Determination of the reducing power (Somogyi–Nelson) of the neutral glucans did not demonstrate reducing end-groups. Acidic oligosaccharide fractions yielded reducing end-groups corresponding to ~10% (reducing sugar, calculated as D-galactose) of the total amount of hexose units determined by the anthrone–sulphuric acid method.

The presence of charged groups was studied by ion-exchange chromatography on QAE-Sephadex A-50. The glucans of *R. meliloti* SU-234 and of *A. tumefaciens* A-8 passed through the column on elution with water. The oligosac-

TABLE II

COMPOSITION (WEIGHT %) OF EXOPOLYSACCHARIDES OF *Rhizobium meliloti*

Strain	D-*Glc*	D-*Gal*	D-*GlcA*	*Pyruvic acid*	*Succinic acid*
Fractions of high molecular weight					
R. meliloti SU-47	73.3	10.8	0	6.4	9.4
SU-234	56.3	8.3	26.8	8.5	0
SU-255	73.9	11.1	0	6.5	8.4
SU-256	74.3	10.7	0	6.3	8.7
A. tumefaciens A-8	76.4	13.7	0	5.0	4.7
Fractions of low molecular weight					
R. meliloti SU-47	76.2	10.6	0	5.5	7.7
SU-234	100	0	0	0	0
SU-255	78.0	10.7	0	5.6	5.7
SU-256	76.8	10.8	0	5.2	7.2
A. tumefaciens A-8	100	0	0	0	0

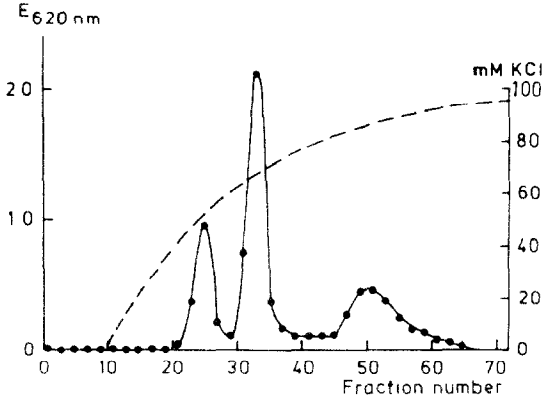


Fig. 2. Anion-exchange chromatography of the fractions of low molecular weight from the culture supernatant of *R. meliloti* SU-47. A sample (100 mg) was applied to a column (2 × 37 cm) of QAE-Sephadex A-50 equilibrated with water and eluted with a gradient of 0→100mM KCl (---). Fractions (10 mL) were analysed for carbohydrate by the anthrone–sulphuric acid method.

charide of *R. meliloti* SU-47 was retained on the column and could be separated into 3 fractions by elution with a KCl-gradient from 0→100mM (Fig. 2). Analysis of the three fractions obtained gave constant ratios for D-glucose, D-galactose, and pyruvic acid of 7:1:1, and increasing amounts of succinic acid (0, 1, and 2 mol) in subsequent fractions[10]. These ratios were also observed in the ^{1}H-n.m.r. spectra of the fractions, from the signals at δ 1.48 (assigned to the methyl protons of pyruvic acid residues) and the multiplets at δ 2.59 (assigned to the methylene protons of the succinyl groups). The presence of signals for *O*-acetyl groups at 2.15 was also observed[6]. The behaviour of the acidic oligosaccharides on ion-exchange chromatog-

TABLE III

METHYLATION ANALYSIS (WEIGHT %) OF EXCRETED POLYSACCHARIDES OF *Rhizobium meliloti*

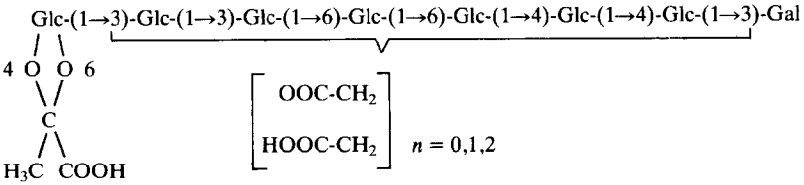
Methylated sugar	T[a]	*Fractions of high molecular weight*		*Fractions of low molecular weight*[b]				
		SU-47	*SU-256*	*SU-47*	*SU-234*	*SU-255*	*SU-256*	*A-8*
1,2,4,5,6-Gal	0.65	0	0	7.1	0	10.1	6.2	0
2,4,6-Glc	1.42	22.7	23.7	25.3	0	27.4	25.7	0
3,4,6-Glc	1.45	0	0	0	100	0	0	100
2,4,6-Gal	1.54	10.7	12.8	1.7	0	3.7	1.2	0
2,3,4-Glc	1.62	14.6	12.4	23.1	0	21.2	25.3	0
2,3,6-Glc	1.69	27.2	26.1	29.0	0	22.7	27.9	0
2,3-Glc	2.73	24.8	24.8	13.6	0	14.8	13.8	0

[a]Retention times (*T*) of the corresponding alditol acetates relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; column: WCOT fused-silica, OV-225, at 190°. [b]Oligomers reduced with $NaBD_4$ prior to methylation.

raphy showed the presence of free carboxyl-groups, namely, those of pyruvate (acetal-linked) and those of succinic acid with one free carboxyl-group and the second group esterified to sugar.

To determine the nature of the reducing end-groups, the oligosaccharides were reduced with $NaBD_4$ and subsequently hydrolysed with 2M trifluoroacetic acid. The liberated sugars and hexitols were converted into fully acetylated derivatives that were subjected to g.l.c. on a WCOT-OV-225 column, to give D-glucose penta-acetate (88%) and galactitol hexa-acetate (12%), thereby proving the D-galactose residues to be the original reducing end-units of the oligomer consisting mainly of the octasaccharide repeating-unit of the polysaccharide[8,14].

Methylation analysis (Table III) of the exocellular glucans produced by *R. meliloti* SU-234 and *A. tumefaciens* A-8 yielded 3,4,6-tri-*O*-methyl-D-glucose without any other methylated sugar. It thus proved the glucans to be identical with the cyclic (1→2)-β-D-glucans that have been isolated from the cells of all fast-growing *Rhizobium* species[13]. Methylation of oligosaccharides of low molecular weight from supernatants of *R. meliloti* SU-47, SU-255, and SU-256 gave a methylated-sugar pattern that indicated the same structural elements as those of the fractions of high molecular weight[2]: (1→3)-, (1→4)-, and (1→6)-β-linked D-glucosyl residues, 3-β-linked D-galactose reducing end-groups, and pyruvate attached to O-4 and O-6 of D-glucosyl end-groups, in full accord with the proposed structure of the octasaccharide repeating-unit[8].



This octasaccharide was first found during depolymerisation of the succinoglycan of *Alcaligenes faecalis* with the aid of a specific, inducible β-glycanase from *Flavobacterium*[15]. Mutants of *A. faecalis* growing in a synthetic medium excreted an identical oligosaccharide. It was also excreted by the original wild-strain if the bacterium was grown in synthetic medium in the presence of such inhibitors as penicillin or bacitracin[16].

Recent investigation of nine strains of *Agrobacterium*[10] showed them to produce curdlan, succinoglycan, a (1→2)-β-D-glucan of low molecular weight, and an octasaccharide repeating-unit. Mutant strains producing high proportions of curdlan without succinoglycan only produced (1→2)-β-D-glucan and no octasaccharide. In our study of *R. meliloti* cultures, all strains were found to produce cellular (1→2)-β-D-glucan, and most of them excreted a succinoglycan-like polysaccharide and its octasaccharide repeating-unit without (1→2)-β-D-glucan. One strain, *R. meliloti* SU-234, producing a different, uronic acid-containing polysaccharide, only excreted (1→2)-β-D-glucan. Cultures of *Agrobacterium tumefaciens* A-8, which produced high proportions of curdlan and small proportions of succinoglycan, had both cellular and exocellular (1→2)-β-D-glucan.

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