Production and Chemical Composition of Extracellular Polysaccharides of *Rhizobium*

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Five cultures of *Rhizobium meliloti* (J7017, 202, 204, 207, 209) and one of *Rhizobium trifolii* (J60) produced water-soluble polysaccharides containing glucose, galactose and pyruvic acid in a molar ratio of 7:1:1 and some succinic and acetic acids. These were identified as succinoglycan-like polysaccharides on the basis of their components, methylation analysis and fragmentation with two specific β -glycanases. One culture of *R. meliloti* (IFO 13336) produced water-soluble polysaccharide containing glucose, galactose, glucuronic acid and acetic acid in a molar ratio of 5:1:1:2, and an unidentified component. Two cultures of *R. meliloti* (201, 206) produced water-soluble polysaccharides containing glucose, galactose, mannose and glucuronic acid in a molar ratio of 4:2:3:1 and 4:1:2:1, respectively, and some pyruvic acid. *Rhizobium trifolii* IFO 13337 and *R. japonicum* IFO 13338 produced water-soluble polysaccharides containing glucose, galactose, glucuronic acid, pyruvic acid and acetic acid in a molar ratio of 6:1:1:2:1. Two isolates from the stock culture of *R. trifolii* J60 produced large amounts of the water-insoluble polysaccharide curdlan. This is the first report in *Rhizobium* of the occurrence of curdlan and of spontaneous mutation in ability to produce succinoglycan-like polysaccharide and curdlan.

INTRODUCTION

The extracellular polysaccharides of *Rhizobium* strains are of interest because they are thought to play a role in determining the specificity of the bacteria for their host legume plants. Various attempts have been made to relate the extracellular polysaccharide composition of *Rhizobium* strains to their host specificity. All strains of a given *Rhizobium* species, except *R. japonicum*, appear to produce similar polysaccharides (Dudman, 1976). *Rhizobium trifolii*, *R. leguminosarum* and *R. phaseoli* apparently produce identical polysaccharides (Zevenhuizen, 1973), while the polysaccharides of *R. meliloti* are identical to those of *Agrobacterium tumefaciens* (Zevenhuizen, 1973) and *Agrobacterium radiobacter* (Hisamatsu *et al.*, 1978). We thought it interesting to continue studies on the role of bacterial polysaccharides in interaction with the host plant because *A. tumefaciens*, which produces extracellular polysaccharide, causes crown gall tumours in plants.

The polysaccharide of one strain of *R. meliloti* (U27) was studied by Jansson *et al.* (1977). Comparative studies (Harada *et al.*, 1979) showed that the water-soluble polysaccharides from *R. meliloti* U27, *Alcaligenes faecalis* var. *myxogenes* 10C3 and *Agrobacterium radiobacter* have the same structure, except for the ratio of their contents of the succinyl or acetyl radical: the sugar moiety is shown in formula I.

We now report the composition of the extracellular polysaccharides of a number of *Rhizobium* strains and the quantity of polysaccharide formed during growth.

Formula I

METHODS

Strains. *Rhizobium meliloti* IFO 13336, *R. trifolii* IFO 13337 and *R. japonicum* IFO 13338 were obtained from the Institute for Fermentation, Osaka, Japan; *R. meliloti* J7017 and *R. trifolii* J60 were supplied by Professor Y. Maruyama, University of Tokyo, Japan; *R. meliloti* strains 201, 202, 204, 206, 207 and 209 were supplied by Professor M. Yatazawa, Nagoya University, Japan.

Media. Strains were maintained on slopes of yeast extract/mannitol agar medium (Graham, 1965). The medium used to examine polysaccharide production contained (per 100 ml distilled water): glucose, 4 g; $(\text{NH}_4)_2\text{HPO}_4$, 0.15 g; KH_2PO_4 , 0.1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g; NaCl, 1 mg; CaCl_2 , 1 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1 mg; thiamin, 20 μg ; biotin, 2 μg ; CaCO_3 , 0.5 g; pH 7.0. Aniline blue medium (Nakanishi *et al.*, 1974), used to detect colonies forming curdian, contained (per 100 ml distilled water): glucose, 1 g; yeast extract, 0.5 g; aniline blue, 5 mg; agar, 1.5 g; pH 7.0.

Culture conditions for polysaccharide production. The medium (95 ml) in 500 ml conical flasks was inoculated with a seed culture (5 ml) which had been grown in the same medium. The culture was shaken reciprocally at 120 strokes min^{-1} at 30 °C for 5 d.

Separation and analysis of precipitates (cells including cell surface polysaccharide). On centrifuging the culture broth, cells and CaCO_3 were precipitated. The CaCO_3 was removed by washing with 1 M-HCl and then the remaining precipitate was washed with water, dehydrated with acetone and weighed. The amount of sugars in the precipitate was assayed by the phenol/ H_2SO_4 method (Dubois *et al.*, 1956) using glucose as a standard. Nitrogen in the precipitate was assayed with a CHN-Corder, type M72 (Yanagimoto, Tokyo, Japan), and the amount of protein was calculated by multiplying the value by 6.25.

Preparation of water-soluble polysaccharide. Water-soluble polysaccharide was prepared by the method described previously (Misaki *et al.*, 1969) involving precipitation with acetone and cetylpyridinium chloride.

Quantitative analysis of sugars. Water-soluble polysaccharide was hydrolysed in a sealed tube with 1 ml 90% formic acid for 16 h at 100 °C, and then with 0.5 ml 4 M-trifluoroacetic acid for 6 h at 100 °C. The hydrolysate was converted to a mixture of alditol acetates and analysed (Björndal *et al.*, 1967) in a CG7A gas chromatograph (Shimadzu, Tokyo, Japan) fitted with a flame-ionization detector and a column (4 × 200 mm) of 3% (w/w) ECNSS-M on Gas-chrom Q (Applied Science Laboratories) at 190 °C. Xylose was used as an internal standard in these analyses.

Uronic acids were determined by the modified carbazole reaction described by Galambos (1967).

Quantitative analysis of organic acids. Pyruvic acid in water-soluble polysaccharide was assayed by the method of Koepsell & Sharpe (1952). Succinic acid and acetic acid were assayed by liquid chromatography as described previously (Hisamatsu *et al.*, 1978).

Preparation of water-insoluble polysaccharide. The precipitate containing cells (200 mg) was homogenized with 50 ml 0.5 M-NaOH and centrifuged at 20000 rev. min^{-1} for 30 min. The supernatant was neutralized with 1 M-HCl and the precipitate thus formed was collected by centrifugation, washed with water, dehydrated by addition of acetone and then dried *in vacuo*.

Successive digestion of polysaccharides with succinoglycan depolymerase and intracellular endo-(1→6)- β -D-glucanase of *Flavobacterium M64*. A solution of 0.1% (w/v) polysaccharide was stirred in 10 mM-KOH for 5 h at 20 °C under nitrogen to remove acyl groups. Deacylated polysaccharide (1 mg) was dissolved in 200 μl 0.1 M-sodium acetate buffer (pH 5.8) and incubated with 100 μl succinoglycan depolymerase (2 units) for 5 h at 40 °C. The mixture was then boiled for 1 min, mixed with 100 μl intracellular endo-(1→6)- β -D-glucanase (2 units) and incubated for another 5 h. The mixture was boiled again for 1 min and passed through a column (10 × 50 mm) of Amberlite IR-120 (H^+ resin). The effluent was evaporated *in vacuo* and subjected to paper chromatography to examine the hydrolysis products.

Preparation of succinoglycan depolymerase and intracellular endo-(1→6)- β -D-glucanase. Succinoglycan depolymerase and intracellular endo-(1→6)- β -D-glucanase were prepared as described previously (Amemura *et al.*,

1974; Abe *et al.*, 1980). One unit of enzyme activity is defined as the amount of enzyme which liberates 1 μmol aldehyde group as glucose h^{-1} .

Paper chromatography. Samples were applied to Toyo-Roshi no. 50 paper (Toyo, Tokyo, Japan) and developed with a solvent system of 1-butanol/pyridine/water (6:4:3, by vol.). Sugars were detected with AgNO_3 reagent. The solvent used to separate uronic acids was 1-butanol/acetic acid/water (4:1:5, by vol.).

Methylation analysis. Samples (2 mg) were methylated and analysed as described previously (Hisamatsu *et al.*, 1980).

RESULTS AND DISCUSSION

Isolation of colonies on aniline blue plates

Stock cultures of *R. meliloti* strains IFO 13336, J7017, 201, 202, 204, 206, 207 and 209, *R. trifolii* strains IFO 13337 and J60 and *R. japonicum* strain IFO 13338 were suspended in saline and spread on aniline blue plates. After 5 d at 30 °C, colonies of all the strains were slimy. *Rhizobium meliloti* strains 201 and 206 and *R. trifolii* IFO 13337 formed white colonies which stained slightly with the dye, while other strains formed unstained colonies.

Fig. 1. Colonies of *Rhizobium trifolii* J60 after growth at 30 °C for 5 d on aniline blue medium: (a) stock culture; (b) strain J60W; (c) strain J60B1; (d) strain J60B2.

Table 1. *Polysaccharides produced by Rhizobium strains*

Strain	Water-soluble polysaccharide [mg (100 ml) ⁻¹]	Precipitate [mg (100 ml) ⁻¹]		
		Total wt	Protein	Carbohydrate
<i>R. meliloti</i>				
IFO 13336	840	500	140	90
J7017	220	200	126	15
201	255	93	58	11
202	330	450	198	30
204	355	630	221	43
206	450	140	72	11
207	340	730	204	50
209	92	270	179	44
<i>R. trifolii</i>				
IFO 13337	150	280	78	10
J60W	580	310	162	35
J60B1	0	1460	210	1180
J60B2	18	1430	200	1240
<i>R. japonicum</i>				
IFO 13338	600	420	184	11

However, when the thick suspension of *R. trifolii* J60 was plated, many blue colonies were detected in the slimy lawn of white colonies (Fig. 1a); the ratio of blue to white colonies was 1:380. White (J60W) and blue (J60B1 and J60B2) colony types were purified by repeated single colony isolation for further study (Fig. 1). We have previously reported the appearance on aniline blue plates of similar blue colonies from stock cultures of *Alcaligenes faecalis* var. *myxogenes* and several strains of *Agrobacterium* (Amemura *et al.*, 1977; Hisamatsu *et al.*, 1977).

Production of water-soluble polysaccharides and precipitates

All the strains except *R. trifolii* J60B1 produced water-soluble polysaccharide (Table 1). The precipitates from all strains contained between 60 and 220 mg protein. The precipitates from strains J60B1 and J60B2 contained more than 1 g neutral sugars, but those from other strains contained only 10 to 90 mg sugars (Table 1). Although colonies of *R. meliloti* strains 201 and 206 and *R. trifolii* IFO 13337 stained slightly with aniline blue these strains did not seem to produce appreciable amount of curd, because the content of neutral sugars was quite low (10 to 11 mg).

Components of water-soluble polysaccharides

All water-soluble polysaccharides contained glucose and galactose; those of *R. meliloti* strains 201 and 206 also contained mannose, and the soluble polysaccharides from *R. meliloti* IFO 13336, 201 and 206, *R. trifolii* IFO 13337 and *R. japonicum* IFO 13338 contained glucuronic acid (Table 2).

The polysaccharides of *R. meliloti* strains J7017, 202, 204, 207 and 209 and *R. trifolii* J60W had the same molar ratio of glucose, galactose and pyruvic acid (7:1:1). This ratio is the same as that of the polysaccharides of *Alcaligenes faecalis* var. *myxogenes* 10C3, nine strains of *Agrobacterium* which we examined previously (Hisamatsu *et al.*, 1978, 1980) and *R. meliloti* U27 (Jansson *et al.*, 1977). Methylation analysis showed that these polysaccharides were composed of (1→3)-, (1→6)-, (1→4)- and (1→4,1→6)-linked D-glucose residues, (1→3)-linked D-galactose residues and non-reducing terminal D-glucose residues with pyruvic acid in a molar ratio of 2:1:2:1:1:1, i.e. identical to the polysaccharides of *Alcaligenes faecalis* var. *myxogenes* 10C3, *Agrobacterium radiobacter*, *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes* and *R. meliloti* U27 (Hisamatsu *et al.*, 1980).

The polysaccharide of *R. meliloti* IFO 13336 contained glucose, galactose and glucuronic

Table 2. Components of water-soluble polysaccharides of *Rhizobium* strains

Strain	Component (% w/w; molar ratio in parentheses)							$[\alpha]_D^{25}$
	Glucose	Galactose	Mannose	Glucuronic acid	Pyruvic acid	Succinic acid	Acetic acid	
<i>R. meliloti</i> IFO 13336	60.5 (5.2)	11.6 (1.0)	0 (0)	13.7 (1.1)	0 (0)	0 (0)	8.1 (2.1)	-5°
	77.4 (7.0)	11.0 (1.0)	0 (0)	0 (0)	6.8 (1.3)	2.5 (0.3)	2.2 (0.6)	-21°
	37.2 (3.9)	19.0 (2.0)	27.2 (2.9)	10.3 (1.0)	4.3 (0.9)	0 (0)	0 (0)	+48°
	79.6 (6.9)	11.6 (1.0)	0 (0)	0 (0)	5.5 (1.0)	1.5 (0.2)	1.8 (0.5)	-19°
	78.8 (6.9)	11.4 (1.0)	0 (0)	0 (0)	6.0 (1.1)	1.3 (0.2)	1.8 (0.5)	-13°
	50.3 (4.2)	12.0 (1.0)	24.1 (2.0)	9.9 (0.8)	1.9 (0.3)	0.1 (0)	0.3 (0.1)	+48°
	78.1 (6.9)	11.4 (1.0)	0 (0)	0 (0)	6.1 (1.1)	1.4 (0.2)	1.5 (0.4)	-26°
	78.3 (7.0)	11.2 (1.0)	0 (0)	0 (0)	5.4 (1.0)	1.7 (0.2)	2.0 (0.5)	-14°
<i>R. iriifolii</i> IFO 13337	59.1 (5.9)	10.1 (1.0)	0 (0)	13.2 (1.2)	10.2 (2.1)	0 (0)	4.1 (1.2)	-14°
	78.2 (6.8)	11.5 (1.0)	0 (0)	0 (0)	7.0 (1.2)	1.3 (0.2)	0.6 (0.2)	-23°
<i>R. japonicum</i> IFO 13338	60.4 (6.2)	9.8 (1.0)	0 (0)	14.0 (1.3)	8.4 (1.8)	0 (0)	3.5 (1.1)	-12°

acid in a molar ratio of 5:1:1, but no pyruvic acid. The polysaccharides of *R. meliloti* strains 201 and 206 contained mannose and their molar ratios of glucose, galactose, mannose and glucuronic acid were 4:2:3:1 and 4:1:2:1, respectively. These polysaccharides also contained some pyruvic acid. *Rhizobium trifolii* IFO 13337 and *R. japonicum* IFO 13338 produced similar polysaccharides with molar ratios of glucose, galactose, glucuronic acid and pyruvic acid of 6:1:1:2. This ratio is similar to that reported for polysaccharides of other strains of *R. trifolii* (Chaudhari *et al.*, 1973; Sømme, 1974; Jansson & Lindberg, 1979).

All the polysaccharides except that of strain 201 contained succinic acid and/or acetic acid. An unidentified acidic substance that was released by treatment with weak acid was present in the polysaccharide of *R. meliloti* IFO 13336.

Dudman (1976, 1978) reported compositional heterogeneity in the polysaccharides of *R. japonicum*. Our results indicate heterogeneity in the polysaccharides of *R. meliloti* and *R. trifolii*.

Digestion of water-soluble polysaccharides with succinoglycan depolymerase and intracellular endo-(1→6)-β-D-glucanase of Flavobacterium

Previously (Hisamatsu *et al.*, 1978; Harada *et al.*, 1979; Abe *et al.*, 1980) we reported that water-soluble polysaccharides (deacylated) from *Alcaligenes faecalis* var. *myxogenes* 10C3, nine strains of *Agrobacterium* and *R. meliloti* U27 were hydrolysed by successive treatment with succinoglycan depolymerase and intracellular endo-(1→6)-β-D-glucanase to produce two oligosaccharides. By analysing these oligosaccharides we showed that the polysaccharides have the same structure irrespective of their modes of acylation (Hisamatsu *et al.*, 1980). Since these enzymes have high specificity (Amemura *et al.*, 1974; Abe *et al.*, 1980) we tested whether they could hydrolyse the polysaccharides of the various *Rhizobium* strains. The polysaccharides of *R. meliloti* strains J7017, 202, 204, 207 and 209 and *R. trifolii* J60W were hydrolysed by succinoglycan depolymerase, as was the polysaccharide (succinoglycan) of *Alcaligenes faecalis* var. *myxogenes*, to give a single spot of reducing material near the origin of the paper chromatogram. Moreover, these polysaccharides were hydrolysed successively by succinoglycan depolymerase and intracellular (1→6)-β-D-glucanase to give two spots of oligosaccharides which had the same R_F values as those from succinoglycan (R_F 0.27 and 0.11).

Rhizobium meliloti IFO 13336 polysaccharide was hydrolysed by succinoglycan depolymerase (the activity was about 5% of that for succinoglycan) and was also hydrolysed by the intracellular enzyme of *Flavobacterium*, but the pattern of the final products on the paper chromatogram was different (R_F 0.11 and 0).

No other polysaccharides were hydrolysed by these enzymes. These results show that the polysaccharides of *R. meliloti* strains J7017, 202, 204, 207 and 209 and *R. trifolii* J60W may have substantially the same structure as succinoglycan. The polysaccharide of *R. meliloti* IFO 13336 has a different structure, but it also is susceptible to the enzymes, probably because it contains the components →3)-Gal-(1→4)-Glc-(1→ and →6)-Glc-(1→6)-Glc-(1→.

Water-insoluble polysaccharide of R. trifolii J60

A sample of 580 mg of the water-insoluble polysaccharide of *R. trifolii* J60B1 was obtained from 1 g of the precipitate by extraction with 0.5 M-NaOH. The polysaccharide stained with aniline blue, like curdlan from *Alcaligenes faecalis* var. *myxogenes* and strains of *Agrobacterium*. The optical rotation $[\alpha]_D^{20}$ was +12° (c 1 in 1 M-NaOH). After complete hydrolysis of the polysaccharide only glucose was detected by paper chromatography, while after partial hydrolysis glucose, laminaribiose, laminaritriose and higher laminari-oligosaccharides were detected. When a 2% (w/v) suspension of the polysaccharide in water was heated at 90 °C for 10 min, it made a firm gel with a strength of 740 g cm⁻², like that of curdlan from *Alcaligenes faecalis* var. *myxogenes* 10C3K (Maeda *et al.*, 1967). Methylation

analysis established that 2,4,6-tri-*O*-methyl-D-glucose was the predominant product of hydrolysis of the fully methylated polysaccharide. From these results it was concluded that the water-insoluble polysaccharide was curdlan. Curdlan has not previously been shown to be present in *Rhizobium* strains.

Strain J60W mutated spontaneously to make curdlan during prolonged storage (2 months) on yeast extract/mannitol agar slants, as has previously been reported for *Alcaligenes faecalis* var. *myxogenes* (Amemura *et al.*, 1977) and several strains of *Agrobacterium* (Hisamatsu *et al.*, 1977). *Rhizobium trifolii* J60 produces succinoglycan-like polysaccharide as well as curdlan, like *Alcaligenes faecalis* var. *myxogenes* (Harada, 1965, 1977; Harada *et al.*, 1968*a, b*) and *Agrobacterium* strains (Nakanishi *et al.*, 1976; Hisamatsu *et al.*, 1978). It is interesting that spontaneous mutation in the ability to produce these types of polysaccharides occurs in a strain of *Rhizobium* as well as *Alcaligenes faecalis* var. *myxogenes* and *Agrobacterium* strains.

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