Chemical Composition of Exopolysaccharides of *Rhizobium* and *Agrobacterium*

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Hopkins, Peterson & Fred (1930) found glucose and glucuronic acid as constituents of the exopolysaccharides of *Rhizobium meliloti*, *R. trifolii* and *R. leguminosarum*; Schlüchterer & Stacey (1945) investigated the structure of the exopolysaccharide of *R. radicicolum* (Clover Bartel A strain) by methylation. Subsequently, composition was studied in relation to taxonomy (Humphrey & Vincent, 1959; Graham, 1965) and to antigenic properties (Dudman, 1964). Ljunggren & Fåhræus (1959) studied the effect of exopolysaccharides on root-hair invasion by rhizobia, while Clapp, Davis & Waugaman (1962) used *Rhizobium* exopolysaccharides as model compounds for studying the importance of polysaccharides in crumb stability of soils.

Various attempts have been made to relate the exopolysaccharide composition of *Rhizobium* strains with their specificity in cross-inoculation groups. In a qualitative chemical analysis of exopolysaccharides of *Rhizobium leguminosarum*, *R. trifolii* and *R. phaseoli*, Humphrey & Vincent (1959) found both glucose and glucuronic acid, while strains of *R. meliloti* were found to contain only glucose. Besides glucose (66 %), Dudman (1964) detected galactose (12 %) and smaller amounts of unidentified sugars in hydrolysates of *R. meliloti* polysaccharides (two strains). The same components were also found in 10 strains of *R. meliloti* by Amarger, Obaton & Blachère (1967). However, Clapp & Davis (1970) concluded that polysaccharide analysis has only limited value for classification of *Rhizobium*. When testing several cultures of various groups they did not find a direct relationship between host legume and constituent sugars.

METHODS

In this study the exopolysaccharide composition of *Rhizobium* and *Agrobacterium* has been examined, using 21 strains from five taxonomic groups. Strains were taken from the culture collection of the laboratory; they had different origins and were all infective in the host legume of their own group.

Cultivation of organisms. Bacterial strains were kept on slants of the following medium: yeast extract, 0.1 %; mannitol, 1 %; K_2HPO_4 , 0.05 %; $MgSO_4 \cdot 7H_2O$, 0.025 %; NaCl, 0.01 %; $CaCO_3$, 0.3 %; agar, 1.2 %. This medium was also used to grow the organisms in Petri dishes. For larger quantities of bacteria a liquid medium of the following composition was used: mannitol, 1 %; glutamic acid, 0.1 %; K_2HPO_4 , 0.1 %; $MgSO_4 \cdot 7H_2O$, 0.02 %; $CaCl_2$, 0.004 %; biotin, 10 $\mu g./l.$; thiamin, 100 $\mu g./l.$; 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. Salts and other nutrients were dissolved in distilled water and adjusted to pH 6.7 with NaOH. Organisms were grown in 100 ml. of the above synthetic

mannitol-glutamic acid medium, contained in 300 ml. Erlenmeyer flasks, and aerated on a rotary shaker at 25° for 5 days.

Preparation of exopolysaccharides. After cultivation the bacteria were harvested from the plates and suspended in water. The suspensions and liquid cultures were centrifuged at 40,000g for $\frac{1}{2}$ h., followed by dialysis of the supernatant against distilled water. Isolation of the polysaccharide was achieved by adding three volumes of ethanol to the clear supernatant, centrifuging, redissolving in water, and dialysing against running water for 48 h. These procedures did not remove all impurities. With highly viscous polysaccharide solutions it was difficult to remove all bacteria completely by centrifugation. Sometimes dilution with water prior to centrifugation, or filtration over a filter aid (celite) was more successful. The soluble protein content, as determined by the method of Lowry, Rosebrough, Farr & Randall (1951), usually was lower than 1 %. All preparations contained amounts of ash from the bound cations of the acidic groups in the polysaccharide.

Quantitative chemical analysis of exopolysaccharides of Rhizobium and Agrobacterium. Exopolysaccharides were hydrolysed with 0.5 M-H₂SO₄ in sealed ampoules at 100° for 16 h. The hydrolysates were neutralized with barium hydroxide and centrifuged, followed by de-ionization of the supernatant over IR-120 resin (H⁺-form). Component sugars were separated on Whatman no. 1 paper, using *n*-butanol-pyridine-benzene-water, 5:3:1:3 (v/v), and sprayed with *p*-anisidine-HCl.

RESULTS AND DISCUSSION

Paper chromatograms of polysaccharide hydrolysates showed glucose as the main component. Galactose was always present as a minor component, while in some cases small amounts of mannose were found. Non-uronic acid containing polysaccharides (*Rhizobium meliloti* and *Agrobacterium tumefaciens*) gave nearly quantitative yields of reducing sugars with no detectable, incompletely hydrolysed fragments on paper chromatograms. Moreover, uronic acid containing polysaccharides yielded glucuronic acid and aldobiouronic acid(s) in combination with a fast running spot of glucuronolactone. These aldobiouronic acid(s) could not be completely hydrolysed, even after prolonged hydrolysis for 24 h. in M-H₂SO₄. Consequently, the total amount of hexoses, as determined with anthrone, could not entirely be recovered as the sum of free glucose + galactose in the hydrolysate (90 to 95 % recovery). 4-*O*-methylglucuronic acid, which was found in polysaccharide hydrolysates of *R. trifolii*, *R. leguminosarum* and *R. phaseoli* by Graham (1965) and Humphrey (1959) as a fast-running spot on paper chromatograms, could not be found in the present investigation.

Pyruvate and *O*-acetyl were present as non-carbohydrate components in all preparations tested. They were identified by chromatographic analysis of the 2,4-dinitrophenylhydrazones in benzene-ethyl acetate (8:2, v/v), and by chromatography of the hydroxamates in ethyl acetate-acetic acid-formic acid-water (18:3:1:4, v/v) respectively, using the appropriate standards.

The results of the quantitative chemical analyses of exopolysaccharides of *Rhizobium* and *Agrobacterium* are summarized in Table 1. Weight percentages of the components were calculated on the sum of all components determined (glucose + galactose + uronic acid + pyruvate + acetyl). A distinctive difference can be observed between the groups of *Rhizobium leguminosarum*, *R. phaseoli*, and *R. trifolii*, and the groups of *R. meliloti* and *Agrobacterium tumefaciens*. The first three groups of *Rhizobium* polysaccharides all had the same range of glucose (48 to 61 %), galactose (8 to 14 %), glucuronic acid (14 to 19 %), pyruvate (10 to 15 %) and acetyl (7 to 13 %). *R. meliloti* and *A. tumefaciens* polysaccharides showed a much

higher content of glucose (76 to 83 %) and contained less than 1 % glucuronic acid; pyruvate (3 to 8 %) was lower than with the first three groups of rhizobia. Dudman & Heidelberger (1969), who were the first to prove the presence of non-carbohydrate components in *Rhizobium* exopolysaccharides, found 9 to 10 % pyruvic acid and 3 to 4 % acetyl in air dried

Table 1. *Quantitative chemical analysis of exopolysaccharides of Rhizobium and Agrobacterium*

The first number refers to exopolysaccharide of bacteria grown on yeast extract-mannitol-agar plates; the second number, in a liquid mannitol-glutamic acid medium.

Strain	Component (%)				
	Glucose*	Galactose*	Uronic acid†	Pyruvate‡	Acetyl§
<i>Rhizobium leguminosarum</i>					
H VIII	48, 53	11, 10	19, 14	14, 15	8, 8
P-8	48, 61	11, 9	19, 14	13, 10	9, 6
PRE	48, 51	11, 9	19, 19	13, 11	9, 10
310a	51	9	16	15	9
313	46, 49	14, 13	14, 18	15, 11	11, 9
402	49, 50	10, 10	16, 19	15, 13	10, 8
404	49, 50	10, 9	17, 18	14, 13	10, 10
<i>R. phaseoli</i>					
Blink	48, 56	11, 9	16, 17	13, 10	12, 8
460	50, 54	10, 9	17, 21	11, 12	12, 5
Bokum	50, 59	9, 8	18, 16	14, 11	9, 6
<i>R. trifolii</i>					
AR-3	48	10	13	15	14
Coryn KL	49, 50	11, 19	17, 14	13, 10	10, 7
In 2	50, 50	10, 10	18, 19	12, 11	10, 10
K-8	51, 51	11, 9	16, 20	11, 11	11, 9
<i>R. meliloti</i>					
BPE	77, 76	10, 9	0, 1	5, 7	8, 7
K-24	75, 76	8, 8	2, 1	6, 6	9, 9
A-145	83, 79	8, 9	0, 1	3, 6	6, 5
A-148	74, 78	12, 9	0, 1	5, 6	9, 6
<i>Agrobacterium tumefaciens</i>					
A-8	78, 80	10, 10	0, 2	7, 6	5, 2
A-9	77, 76	10, 11	0, 1	8, 7	5, 5
A-10	77, 81	11, 10	0, 0	8, 6	4, 3

* Glucose and galactose in the hydrolysate were measured with glucose oxidase and galactose oxidase reagents (Kabi, Stockholm, Sweden) respectively.

† Uronic acid content was measured with the modified carbazole reaction (Bitter & Muir, 1962) using glucuronolactone as a standard and corrected for interference by hexoses.

‡ Pyruvate was measured according to a modification of the method of Sloneker & Orentas (Sutherland, 1969).

§ O-acetyl measurements were carried out according to Hestrin (1949) using glucose penta-acetate as a standard.

samples of *R. trifolii* polysaccharides. These results are in agreement with the present data when expressed on a dry weight basis. They also found a much lower pyruvic acid content for *R. meliloti* (approx. 5 %).

Quantitative data on sugar composition were until recently very scarce. Amarger, Obaton & Blachère (1967) found a close similarity in percentage composition of polysaccharides of 10 strains of *Rhizobium meliloti*. After hydrolysis in 0.5 M-H₂SO₄ at 100° for 6 h., the percentage of the total sugars varied from 82 to 86 % for glucose, 13 to 16 % for

galactose and 0.4 to 1.2 % for glucuronic acid, suggesting a homogeneity of the polysaccharides of this group of *Rhizobium*.

On the contrary, Clapp & Davis (1970) found wide variations in chemical composition between 18 strains of *Rhizobium* of different groups. They found as many differences within a single host legume group as between groups. Uronic acid, after separation from neutral sugars on anion exchange resin, ranged from 0 to 22 %. No direct relationship between uronic acid content and host legume was found. Moreover, their ratios of total carbohydrate/uronic acid showed a wide range, in contrast with the nearly constant ratio of 3 to 4 for the uronic acid containing preparations of Table 1. Their preparations were obtained by cultivation of the bacteria in complex yeast extract-mannitol medium. In the present investigation organisms were grown in a synthetic medium (mannitol-glutamic acid) or on agar surfaces of a complex medium (yeast extract-mannitol) in order to avoid contamination of the polysaccharide preparations by polymeric carbohydrate of the medium. Hexoses and uronic acids were directly determined in the polysaccharide hydrolysates with anthrone and carbazole respectively, and corrected for interference by the other component.

Concluding, it can be said that *Rhizobium leguminosarum*, *R. phaseoli* and *R. trifolii* form a homogeneous and indivisible group, differing from *R. meliloti* and *Agrobacterium tumefaciens*, which were very similar. These results are consistent with earlier reports in which taxonomy was studied by application of computer techniques (Graham, 1964) and by DNA base composition and flagellation (DeLey & Rassel, 1965).

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