Extracellular Polysaccharides of Rhizobium Strains Associated with Lotus Species

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SUMMARY

The monosaccharide composition of extracellular polysaccharides isolated from 101 strains of rhizobia, principally associated with the lotus group of legumes, have been examined. Polysaccharides from 43 fast-growing, acid-producing strains (17 lotus) gave a substantially consistent monosaccharide pattern of glucose, uronic acid and galactose with little or no mannose. Polysaccharides from lucerne and goat's rue strains (four) of this class of rhizobia differed in that they contained almost no uronic acid. Fifty-eight slow-growing, non-acid-producing strains (52 lotus) were also investigated. On centrifuging, 36 of them gave a mucilaginous clot of cells and polysaccharide and required dilute alkali treatment to separate the polysaccharide. No polysaccharide was produced by four non-acid-producing strains. Polysaccharides from the non-acid-producing rhizobia gave much more complex monosaccharide patterns. Glucose and mannose were nearly always present in large amounts; galactose and uronic acid were frequently absent while fucose, rhamnose and three unidentified sugars were occasionally present. Electron micrograph studies showed extracellular material which, in strains giving a clot, was not removed by centrifuging. Monosaccharide composition of the polysaccharides appeared to be correlated with the division into acid- and non-acid-producing classes of rhizobia. Both classes of rhizobial strains associated with Lotus species conformed to this correlation apart from one strain which was intermediate.

INTRODUCTION

The monosaccharide compositions of the extracellular polysaccharides produced by a large number of rhizobial strains have been reported (see Graham, 1965; Amarger, Obaton & Blachère, 1967). Nearly all of the polysaccharides so described have, however, been isolated from fast-growing, acid-producing strains (Fred, Baldwin & McCoy, 1932). With these rhizobia the diluted culture fluid is easily centrifuged to remove cells leaving polysaccharide in solution. In contrast, few polysaccharides produced by slow growing, non-acid-producing rhizobial strains have been examined. One reason for this may be that the polysaccharides from these strains are usually more difficult to isolate by diluting the cultures and centrifuging (Graham, 1965).

Norris (1965) showed that within the group of rhizobia nodulating Lotus species, both acid- and non-acid-producing strains occur, associated with different host species. We have been interested in the rhizobia associated with Lotus species growing in New Zealand, and an extensive collection of strains (both acid- and non-acid-producing) isolated from or able to nodulate these legumes has been built up by one of us (R.M.G.). Since published work on rhizobial polysaccharides includes few strains

able to nodulate *Lotus* species the monosaccharide composition of the extracellular polysaccharides produced by a number of strains from our collection of *lotus* rhizobia have, therefore, been determined as part of a study of the classification of these bacteria. The majority of the organisms tested were non-acid-producing strains, many of them giving insoluble gums not easily separated from the bacteria. A simple alternative method for isolating these polysaccharides was, therefore, devised. To compare the results obtained in the present study with published results the monosaccharide composition of the extracellular polysaccharides from 32 *Rhizobium* strains, principally acid-producing, associated with genera other than the *lotus* group were also examined.

METHODS

Organisms.

Of 101 rhizobia isolates, 69 (17 acid-producers and 52 non-acid-producers) nodulated *Lotus* species. There were nine isolates of clover (*Trifolium* spp.) rhizobia, two isolates each of lucerne (*Medicago sativa* L.), pea vetch (*Pisum*, *Vicia*), garden bean (*Phaseolus vulgaris* L.), goat's rue (*Galega officinalis* L.), *Sophora tomentosa* L., peanut (*Arachis hypogaea* L.) and chick pea (*Cicer arietinum* L.) rhizobia; one isolate each from *Acacia dealbata* Link., crown vetch (*Coronilla varia* L.), cowpea (*Vigna*) and six isolates from New Zealand indigenous legumes (*Carmichaelia* and *Sophora* spp.). Strain numbers of these organisms are given in Tables 1, 2, and 3. All isolations were made at Palmerston North except for the following organisms. Strains CB 562, CB 569, CB 745, CB 756, CB 782, CB 935, CB 1189 and CB 1199 were from Dr D. O. Norris, C.S.I.R.O., Brisbane, Queensland, Australia. Strains CC 809a, CC 811, CC 814s, CC 856 and CC 1005 were from Mr J. Brockwell, C.S.I.R.O., Canberra, Australia. Strains SU 202, TA 1, WU 95 and WU 425 were from Dr C. A. Parker, University of Western Australia, Perth. Strains SU 343, SU 391 and CC 511 were from Dr R. A. Date, U-DALS, New South Wales, Australia. Strain 3 G 161 was from Dr U. M. Means, U.S.D.A., Beltsville, Maryland, U.S.A. Strains PDD lucerne (NZP 4008), SU 47, 1346 (NZP 5087), 1314 (NZP 5088), W 72 (NZP 5251) and CC 829 (NZP 2021) were from Mr A. Hastings, D.S.I.R., Auckland, New Zealand, and birdsfoot trefoil 461 Canada strain (NZP 2227) and F.J.N. (NZP 2154) were from Mr G. G. Taylor, Fruitgrowers Chemical Co. Ltd, Nelson, New Zealand.

All strains were checked for freedom from contamination and ability to nodulate host plants. Strains were maintained on slopes of yeast mannitol agar medium (Graham, 1965). For polysaccharide production the organisms were grown in the same medium (200 ml.) without agar in conical flasks (1 l.) for 10 days at 28° in a Gallenkamp orbital incubator.

Isolation of extracellular polysaccharide. Cultures (150 ml.) diluted with an equal volume of water were centrifuged at 3000g for 30 min. and the cell-free solution decanted from the pellet. Ethanol (3 vol.) was added to the supernatant with stirring and, after standing overnight at 2°, the precipitated polysaccharide was harvested by centrifuging, dissolved in water (100 ml.), dialysed for 2 to 3 days against tap water and freeze-dried. While this procedure was satisfactory with all of the acid-producing and some of the non-acid-producing strains, many of the latter strains gave on dilution and centrifuging a large viscous clot, with the bacteria dispersed through it, filling about half the liquid volume. The following modification was therefore used with cultures of all non-acid-producing strains which on a preliminary centrifuging of culture (50 ml.) formed this clot. Undiluted culture fluid (150 ml.) was made approximately 0.5 N with

10 N-NaOH (7 to 8 ml.), stirred for 30 min., centrifuged to remove organisms and immediately acidified with 50 % (v/v) acetic acid. Polysaccharides were not precipitated on acidification but still required ethanol (3 vol.) for precipitation. As isolated, the polysaccharides contained 70 to 80 % carbohydrate (anthrone+carbazole values) and were not further purified.

Carbohydrate analyses. Total neutral hexoses and methyl pentoses in the polysaccharides were measured by an anthrone method (Bailey, 1967) using a glucose standard. Total uronic acids were measured by the carbazole-sulphuric acid method of Dische as described by Montreuil & Spik (1963) using a glucuronic acid standard. Control experiments showed that pyruvate (Dudman & Heidelberger, 1969) did not interfere in this reaction. Neutral hexoses interfere slightly in the carbazole reaction and a correction was therefore applied to each uronic acid value. This correction was calculated from a glucose-carbazole standard and the neutral hexose value obtained by anthrone for each polysaccharide. No attempt was made to differentiate between glucuronic acid and 4-*O*-methyl glucuronic acid both of which occur in these polysaccharides (Humphrey & Vincent, 1959; Graham, 1965). As in most other studies of rhizobial polysaccharides the neutral monosaccharide portion of any acid-resistant aldobiouronic acid liberated during hydrolysis was not identified.

Individual neutral monosaccharides present in polysaccharide hydrolysates were measured by quantitative paper chromatography (Wilson, 1959) using acid-washed paper and aniline hydrogen phosphate spray reagent. Two samples of each polysaccharide were usually hydrolysed. Results were calculated against the appropriate monosaccharide standards except for X_1 , X_2 and X_3 which were calculated as glucose. Polysaccharides (10 mg.) were hydrolysed with 1 N- H_2SO_4 (2 ml.) for 4 h. at 100°. The hydrolysates were neutralized with $BaCO_3$, filtered and concentrated to a small volume (1 ml.). Chromatograms were developed with either solvent (1) ethyl acetate + water + pyridine (12 + 4 + 5, by vol.) or (2) *n*-butyl acetate + water + ethanol + pyridine (8 + 1 + 2 + 2, by vol.). Both of these solvents clearly separate glucose, galactose and mannose from one another in mixtures, uronic acids remain at the starting line and uronic acid lactones run off the paper. Other solvents used were (3) ethyl acetate + acetic acid + formic acid + water (18 + 3 + 1 + 4, by vol.), and (4) *n*-butanol + ethanol + water (4 + 1.1 + 1.9, by vol.). Details of spray reagents (aniline hydrogen phosphate, *p*-anisidine HCl, naphthoresorcinol, ninhydrin, thiobarbiturate and diphenylamine aniline) were given by Bailey (1969).

Electron microscopy. Bacterial suspensions were sprayed onto carbon-coated collodion films, shadowed with platinum off carbon electrodes and examined in a Philips EM200 electron microscope. Bacterial suspensions were prepared from 10 day cultures or from isolated bacteria. Cultures were diluted 1:10 in order to minimize the effect of salts and used directly. Bacteria were centrifuged (3000g for 30 min.) from acid-producing cultures after diluting 1:1 or from non-acid-producing cultures after alkali treatment and resuspended in the same amount of distilled water as the original culture. Cultures of non-acid-producing strains containing clots were also centrifuged directly and culture fluid decanted from the clot which was then redispersed in distilled water to its original volume.

RESULTS

Extracellular polysaccharides from acid-producing rhizobia

The neutral monosaccharide and uronic acid compositions of the extracellular polysaccharides produced by 43 strains of acid-producing rhizobia are given in Table 1. They show a substantially similar pattern for the polysaccharides from rhizobia nodulating a wide range of host legumes. The only exceptions appear to be the lucerne and goat's rue rhizobia which produced polysaccharides differing mainly in their lack of uronic acid.

Table 1. *Monosaccharide composition of extracellular polysaccharides from acid-producing strains of rhizobia*

Host legume and strain number	Total neutral hexoses (%) [*]	Yield (g./l. of culture)	Monosaccharides (%) [†]			
			Uronic acid	Galactose	Glucose	Mannose
Goat's rue NZP 5067, 5068	54 to 58	1.5	0 to 0.5	26 to 33	66 to 71	0 to 2.7
Lucerne NZP 4008	56	1.1	—	21	75	4
Lucerne SU 47	30	0.24	1	13	54	32
<i>Carmichaelia</i> spp. NZP 5061, 5083	46 to 65	1.6 to 2.1	15 to 30	16 to 33	49 to 65	Trace
<i>Sophora</i> spp. NZP 5055						
<i>Lotus</i> spp. NZP 2034, 2037, 2042, 2048, 2079, 2085, 2093, 2147, 2150, 2171, SU 343, CC 809a	47 to 55	1.4 to 2.0	20 to 30	21 to 24	50 to 60	—
<i>Lotus</i> spp. NZP 2014, 2024, 2077, CC 856, 811	45 to 59	1.0 to 1.5	20 to 40	18 to 22	40 to 60	2 to 5
Chick pea CB 1189, 1199	42 to 53	0.8 to 1.2	23 to 35	13 to 14	51 to 57	7 to 9
Clover NZP 514, 5039, 5098, 5107, CB 782, SU 202, WU 95, TA 1	42 to 52	0.6 to 1.0 [‡] 0.9 to 2.8	30 to 40	15 to 28	34 to 45	2 to 5
Bean NZP 5097						
Pea vetch NZP 5225, SU 391						
Crown vetch 3G 161						
<i>Sophora</i> spp. NZP 5057						
<i>Carmichaelia</i> spp. NZP 5080, 5105						
Bean CC 511	35	0.6	48	18	27	7
Clover NZP 5063	36	0.8	31	34	32	3

* Percentage of neutral hexoses as anhydro-glucose by anthrone in freeze-dried polysaccharide corrected for moisture.

† Results calculated as percentage of the sum of the measured individual neutral monosaccharides in polysaccharide hydrolysate + corrected total uronic acid; — or 0 = clearly absent.

‡ Yields for clover strains only.

After 10 days growth all of the strains gave cultures with low pH values (4.0 to 4.5) and all produced polysaccharide.

As a check on the use of alkali with cultures of non-acid-producing rhizobia, washed organisms from 20 cultures of the acid-producing strains were extracted with alkali and the extracts processed. Polymer yields were only 0.01 to 0.02 g. from the organisms in 1 l. of culture. Hydrolysates showed glucose and rhamnose (trace) on chromatograms in all cases and occasionally traces of galactose and mannose. There was never any sign of fucose or the unknowns X₁, X₂ or X₃ of Table 2.

Table 2. *Monosaccharide composition of extracellular polysaccharide from non-acid-producing strains of rhizobia*

Host plant and strain numbers	Total neutral hexoses (%) (as in Table 1)	Yield (g./l. of culture)	Monosaccharides (% as in Table 1)						
			Uronic acid	Galactose	Glucose	Mannose	Fucose	Rham- nose	Unknown
			(a) Lotus strains						
NZP 2076, 2088, 2100, 2182, 2257,* 5091, 5144	32 to 55	0.3 to 0.7	0 to 1	16 to 21	36 to 48	30 to 45	—	—	—
NZP 2075,* 5026,* 5274*	11 to 42	0.11 to 0.7	—	0 to 3	0 to 9	88 to 100	—	—	—
NZP 2170, 2175, 2240	37 to 45	0.4	2 to 4	18 to 24	36 to 45	34 to 38	—	—	—
NZP 5059/2*	23	0.4	10	15	40	35	—	—	—
NZP 2243*	12	0.2	7	—	48	45	—	—	—
NZP 2227*	44	0.8	18	18	53	11	—	—	—
WU 425*	18	0.2	22	7	17	54	—	—	—
NZP 2021f, 2089, 2180, 2193, 2200, 2254, CC 8148	40 to 56	0.7-1.4	27 to 39	—	41 to 49	18 to 25	—	—	—
NZP 5042*	18	0.12	33	—	31	36	—	—	—
NZP 2186, 2192, 5052	61 to 68	0.7 to 1.0	21 to 23	—	36 to 39	11 to 14	—	25 to 32	—
NZP 2247,* 5025,* 5251/2*	23 to 38	0.2 to 0.3	0 to 2.5	31 to 35	26 to 30	30 to 40	3 to 6	—	—
NZP 5113	44	0.8	—	23	42	22	12	—	—
NZP 2087, 2141, 2151, 2181,* 5032, 5033, 5035, 5044, 5074, 5087, 5088*	42 to 55	0.5 to 0.8	22 to 40	6 to 15	26 to 39	8 to 20	—	—	10 to 17X ₁
NZP 2128,* 2154, 2183, 2189	37 to 51	0.2 to 0.6	16 to 25	20 to 40	19 to 28	12 to 28	—	—	5 to 19X ₂
NZP 2073*	28	0.6	1.5	36	27	33	—	—	2.5X ₂
(b) Non-Lotus strains									
Peanut CB 935*	40	0.4	0.5	41	30.5	23	—	5	—
Peanut CB 745	42	—	23	7	45	15	—	—	10X ₁
Acacia dealbata NZP 5073	53	0.6	26	—	35	14	—	25	—
Sophora tomentosa CB 569	11	0.3	2.5	—	—	9	—	—	—
Sophora tomentosa CB 562*	25	0.2	18	—	—	32	—	50	—
Cowpea CB 756	54	0.4	20	9	37	20	1	—	13X ₃

* Cultures did not give typical mucilagenous clot on centrifuging; alkali not used in isolation.

Extracellular polysaccharides from non-acid-producing rhizobia

The neutral monosaccharide and uronic acid composition of the polysaccharides isolated from cultures of 54 strains of non-acid-producing rhizobia, 48 of which were associated with *Lotus* species, are given in Table 2 together with an indication of clot production. Four strains (NZP2221, 2245, 2246; CC1005) gave no clot or extracellular polysaccharide. There was a wide variation possible in polysaccharide composition which appeared to extend beyond the *Lotus* group. Several consistencies in monosaccharide composition are evident. All 12 polymers containing unknown X_1 have a common monosaccharide composition as does one group of seven galactose-free polymers. High mannose content generally correlates with very low uronic acid levels while high uronic acid levels correlate with lower amounts of mannose. Four out of six polymers containing rhamnose also have a similar monosaccharide pattern. The fact that 26 of the 54 polysaccharides from non-acid-producing rhizobia are entirely free from galactose or uronic acid suggests that if these polysaccharides are mixtures at least the mixtures do not contain any of the usual polysaccharide produced by most acid-producing rhizobia.

After 10 days growth these non-acid-producing organisms gave cultures within the pH range 5.5 to 7.5 which generally contained less polysaccharide than the acid-producing ones. Polysaccharides isolated with alkali from clot-producing cultures appeared to be more difficult to redissolve in water than those from acid-producing rhizobia. The anthrone values suggested that occasionally these crude preparations contained very little actual polysaccharide and these cases were nearly always ones of very low yields of material.

The possibility existed that alkali was extracting an additional or different polysaccharide, which might explain some of the variations listed in Table 2. Polymer extracted by alkali from six strains of bacteria yielding no clot was therefore examined, including two which gave no polysaccharide in their culture fluid. In five cases (NZP2221, 2227, 5059/2, 5251/2 and CC1005) alkali extraction did not contribute additional polysaccharide. With strain (NZP5274), however, alkali treatment extracted a polysaccharide whose composition (high galactose, glucose, mannose and rhamnose but no uronic acid) clearly differed from the composition of the polysaccharide in the cell-free culture fluid (Table 2). In some cases, therefore, alkali may have extracted additional polysaccharide although the fact that many non-clot producers gave extracellular polysaccharides of the same composition as the clot producers supports the view that in many cases this is not so. None of the extracellular polysaccharides in Table 2 extracted with alkali are likely to be the lipopolysaccharides extracted with phenol at 60° by Graham & O'Brien (1968) as these always contained rhamnose and glucosamine.

Effect of culture age on polysaccharide composition

The fast-growing acid-producing rhizobia gave good yields of polysaccharide after 10 days incubation. In view of the much slower growth and lower polysaccharide yields of the non-acid-producing rhizobia, cultures of two of these strains (NZP2021f and 2087) were processed for polysaccharide after 3, 6, 10 and 16 days growth. The proportions of monosaccharides in these polysaccharides showed little change with growth after 3 days except that there was a gradual increase in the proportion of X_1 in

the NZP2087 polymer at the expense of glucose and uronic acid. Consequently it is assumed that 10 day cultures yield polysaccharide representative of each non-acid-producing strain.

Identity of monosaccharides

The monosaccharides recorded as glucose, galactose, mannose, fucose and rhamnose behaved like authentic specimens on papers developed with solvents (1), (2), (3) or (4) respectively and reacted similarly with aniline hydrogen phosphate and anisidine HCl. Unknowns X_1 and X_2 moved at clearly different rates from these latter sugars with R glucose values respectively of 1.26 and 1.62 in solvent (1) and 1.70 and 2.41 in solvent (2). The R glucose values of the other sugars in these solvents were: in solvent (1), 1.12 mannose, 0.87 galactose, 1.41 fucose, 1.87 rhamnose; in solvent (2), 1.50 mannose, 0.80 galactose, 2.20 fucose and 3.80 rhamnose. X_1 and X_2 gave the colour reactions of simple, reducing aldohexoses with aniline hydrogen phosphate, anisidine HCl, and diphenylamine-aniline sprays. Their reactions or non-reactions with these and sprays of naphthoresorcinol, ninhydrin or thiobarbiturate indicated that they were not pentoses, ketoses, amino sugars, 2- or 6-deoxy aldohexoses or γ or δ lactones. Rates of movement in solvents (1) and (2) and colour reactions with aniline hydrogen phosphate suggested that they were not monosaccharide uronic acid lactones.

Neither X_1 nor X_2 were fermented by yeast and treatment of the hydrolysates with yeast followed by ion-exchange resins yielded solutions free of all other sugars. When freeze-dried solids from these solutions were treated with boron trichloride (Allen, Bonner, Bourne & Saville, 1958) X_1 and X_2 remained unchanged suggesting that they were not *O*-methyl aldohexoses and their nature remains to be established. Unknown X_3 gave the colour reaction of a methyl pentose but moved clearly faster (R glucose, 2.15) than rhamnose (R glucose, 1.87) in solvent (1).

Electron microscopy of organisms and associated polysaccharide

Electron micrographs prepared directly from cultures of acid-producing (NZP2014, 2024) and non-acid, clot-producing (NZP2076, 5032) strains showed extracellular, possibly mucilaginous material, associated with the bacteria. This extracellular material seemed to have a different appearance on the micrographs of the clot-producing organisms as compared with the non-clot-producing ones. Other electron micrographs showed that the acid-producing cultures gave clean organisms by simple dilution and centrifugation, whereas redispersion of the centrifuged clot from the non-acid-producing cultures gave the same electron micrograph as the original culture. After alkali treatment, however, these latter clot-producing organisms gave bacteria free from extracellular material. Micrographs prepared from cultures of non-acid-, non-clot-producing strains (NZP2247, 2257; WU25) showed minimal or zero amounts of extracellular material of either physical type. We believe that the electron microscope results are consistent with the extracellular material being the polysaccharide isolated in the present study.

Polysaccharide composition in relation to effectiveness patterns in Lotus strains rhizobia

Effectiveness patterns of the lotus rhizobia in nitrogen fixation with various species of *Lotus* and other genera (*Ulex*, *Cytisus*, *Ornithopus* and *Lupinus*) occurring in New

Zealand are complex. A detailed study of these patterns obtained with the strains used in the present work is in progress. The following points appear relevant to polysaccharide composition.

All of our strains effective in nitrogen fixation with *Lotus corniculatus* L. and ineffective with *L. pedunculatus* Cav. are acid-producers, except for strains CC1005 and NZP2227. These two were classed as non-acid-producers as they did not produce acid under the conditions defined by Norris (1965). Strain CC1005 yielded no polysaccharide, a result which is in agreement with its assignment to the non-acid producers. Strain NZP2227 gave no clot and yielded polysaccharide (0.89 g./l.) showing the typical monosaccharide composition for polymer from an acid-producing strain except for a rather high mannose value. Thus this strain might be better placed within the acid-producers.

Eleven isolates included in this study formed ineffective nodules with *Lotus pedunculatus*, *L. hispidus* Desf. and *L. corniculatus*, but were effective with gorse (*Ulex europaeus* L.) and broom (*Cytisus scoparius* L.). Of these, six (NZP2141, 2151, 5032, 5033, 5035 and 5044) gave polysaccharides of the same monosaccharide composition, including X₁, but the remaining five (NZP2170, 2186, 5042, 5052 and 5144) gave a variety of different patterns in their polysaccharides, and the other strains giving X₁ all differed in their effectiveness with various host species.

Among non-acid-producing strains that are at least partially effective with *Lotus pedunculatus* or *L. hispidus* there is an apparent correlation between effectiveness with one or other of these species and the presence or absence of uronic acid in the polysaccharide, provided strains effective with both species are excluded. This is shown in Table 3.

Table 3. *Uronic acid content of polysaccharide from non-acid-producing rhizobia effective with Lotus species*

Effectivity on		Number of strains giving polysaccharide with	
<i>Lotus pedunculatus</i>	<i>Lotus hispidus</i>	High uronic acid	Low or nil uronic acid
High	Partial or nil	NZP2021f, 2089, 2154, 2181, 2189, 2193, 2254	None
Partial	High	NZP2192, 5074	NZP2075, 2076, 2088, 2175, 2240, 2243, 5025, 5091, 5113
High	High	NZP2087, 2183, 5087, CC8148	NZP2100, 2182, 2247, 2257
Partial	Partial	NZP2128, 2180, 2200, 5059/2, 5088, WU425	NZP2073, 5026, 5251/2, 5274

DISCUSSION

Individual monosaccharide values recorded in the present work may be low, particularly in high uronic acid polysaccharides, as no allowance is made for any monosaccharide present in the hydrolysate as aldobiouronic acid. The following conclusions appear to be independent of this limitation.

In general the polysaccharides from acid-producing rhizobia possess a remarkably similar monosaccharide composition except for the lack of uronic acid in poly-

saccharides from lucerne and goat's rue rhizobia. Amager *et al.* (1967), investigating lucerne rhizobia, and Humphrey & Vincent (1959) working with a wide range of acid-producing strains, found no uronic acid only in lucerne rhizobial polysaccharides. This apparent correlation may be fortuitous as Clapp & Davis (1970) have reported a high uronic acid polysaccharide from a lucerne strain and a uronic acid-free polysaccharide from a clover rhizobium strain. It is perhaps relevant that Graham (1964), on the basis of a large number of tests not including polysaccharide composition, separated lucerne rhizobia (*Rhizobium meliloti*) from all other fast-growing acid-producing rhizobia he studied which he grouped under *R. leguminosarum*.

Polysaccharides produced by the non-acid-producing strains (Table 2) are, in monosaccharide composition, quite different as a class from polysaccharides produced by acid-producing rhizobial strains (Table 1). Galactose is often absent (16 out of 54), mannose is always present as a major component, while fucose, rhamnose and three unidentified probable sugars may be present. Uronic acids are also commonly absent or very low (19 out of 54). These polysaccharides also differ from those of acid-producing rhizobia in the frequent (36 out of 54 strains) occurrence of the polysaccharide as a diffuse mucilagenous clot after centrifuging the cultures.

Concerning rhizobial relationships within the lotus rhizobia, evidence from extracellular polysaccharides, apart from two possible exceptions, clearly supports their separation into the acid-producing and non-acid-producing groups. The acid-producing lotus rhizobia give polysaccharides not distinguishable in monosaccharide content from a number of other acid-producing rhizobial groups quite unrelated symbiotically. In the non-acid-producing rhizobia no clear distinctions could be drawn between the polysaccharides of the lotus rhizobia and the few non-lotus strains studied, both groups giving a great variety of monosaccharide patterns.

Norris (1965) suggested that non-acid-producing rhizobia represented the primitive type, and that acid-producing rhizobia were more advanced. However, the finding of close similarity of monosaccharide pattern in different cross-inoculation groups within the acid-producers, compared with the wide range of patterns in the non-acid-producers, is not easy to reconcile with Norris's suggestion of evolution of the different acid-producing groups independently from non-acid-producing rhizobia. The possibility is not excluded, however, that the acid-producers arose from the non-acid-producers and later became specialized in host range.

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