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A Structural Comparison of the Acidic Extracellular Polysaccharides from *Rhizobium trifolii* Mutants Affected in Root Hair Infection[1]

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ABSTRACT

The structures of the acidic extracellular polysaccharides (EPSs) from several *R. trifolii* mutants were compared by examining their compositions and their sugar linkages as determined by methylation analysis. These mutant strains were derived from the wild-type *R. trifolii* ANU843 and were unable to induce normal root hair curling (Hac- phenotype) or nodulation response (Nod- phenotype) in clover plants. These strains included several transposon Tn5-induced Nod-mutants, strain ANU871, which possesses a 40 to 50 kilobase deletion of the resident Sym plasmid, and strain ANU845 which is missing the Sym plasmid (pSym-). Strains ANU845(pSym-) containing either plasmid pRt150 or pBR1AN were also used. The recombinant plasmid pRt150 restores only root hair curling capacity to ANU845 while plasmid pBR1AN (an *R. trifolii* pSym) restores both root hair curling and nodulation capacity to this strain. Our composition and methylation results show that the EPSs from all these strains have the same glycosyl and pyruvyl linkages. Thus we suggest that neither the *nod* genes involved in root hair curling nor the entire pSym encodes for the arrangement of glycosyl or pyruvyl residues in these EPSs. Whether or not the *nod* genes dictate the location of acetyl or β-hydroxybutyrate substituent groups remains to be determined.

Rhizobia are gram-negative soil bacteria which form a nitrogen-fixing symbiotic relationship with legumes. This symbiotic relationship can be quite specific in which one species of *Rhizobium* will infect one genus of the *Leguminosae*. Early steps in *Rhizobium*-legume interaction involve the binding of rhizobia to the root hairs, root hair curling, and then infection thread formation (5, 9). Since the initial interaction between the symbiont *Rhizobium* and its host legume occurs at the surfaces of these two organisms, surface molecules are thought to play a role in determining some of these initial events. Specifically, the major surface polysaccharides of rhizobia are thought to be involved in their binding to the host root hair (5, 9). In addition, supernatants from rhizobia cultures are also known to deform and curl root hairs (29). The major surface polysaccharides of rhizobia are the extracellular polysaccharides. EPSs[3] in the form of capsules called capsular polysaccharides and the outer-membrane lipopolysaccharides. Presently there are reports which implicate a role for each one of these major polysaccharides in the symbiotic infection process (5, 9).

The acidic EPS structures from several strains of *R. trifolii* (3, 15, 23), *R. leguminosarum* (23), *R. meliloti* (2, 15, 16, 20), and *R. phaseoli* (13, 14) have been reported. These reports show that all the acidic EPSs consist of oligosaccharide repeating units. These structure studies reveal that: (a) strains of a single species such as *R. phaseoli* can produce EPSs with different structures (13, 14), and (b) strains of different species, *e.g. R. trifolii* and *R. meliloti* (15) or *R. trifolii*, *R. leguminosarum*, and *R. phaseoli* (13, 23) can produce EPSs with identical structures. This report compares the structures of the major acidic EPSs from several *R. trifolii* mutants which are altered in their ability to induce root hair curling and are defective in nodulation (Nod-).

The mutants studied in this report are affected in the early infection event known as root hair curling. Table I gives a decription of these mutants. The genes responsible for this symbiotic event are located on a 5.3 kb *BgI* II fragment on the resident symbiotic plasmid (pSym) of strain ANU843. Insertion of the transposon Tn5 into this 5.3 *BgI* II DNA fragment or deletion of this region causes strain ANU843 to be defective in both root hair curling ability (Hac-) and in nodulation ability (Nod-) (28). The 5.3 kb *BgI* II fragment has been isolated and cloned. Introduction of this fragment (plasmid pRt150) into the pSym- strain ANU845 induces an exaggerated root hair curling response (Hac++) without inducing nodule formation. In contrast, introduction of plasmid pBR1AN, a self-transmissible *R. trifolii* pSym, can restore both clover root hair curling and nodulation ability to pSym- derivatives of *R. trifolii* and *R. leguminosarum* (12). The acidic EPSs of all these types of ANU843 derivatives were compared. Under the conditions used, our results show that the acidic EPSs from these strains have the same glycosyl and pyruvyl linkages.

MATERIALS AND METHODS

***Rhizobium* Strains.** The *Rhizobium* strains are as described in Table I.

Growth Conditions. The *R. trifolii* strains were grown in a yeast extract mannitol media (6) in 500 mL batches on a rotary shaker at room temperature (about 25°C). The *R. leguminosarum* 128C53sm(r)rif(r) strain was grown in defined mannitol media

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[3] Abbreviations: EPS, extracellular polysaccharide; ΔpSym, resident symbiotic plasmid; pSym, Sym plasmid.

Table I. *Bacterial Strains and Plasmids*

Bacterial strains	Relevant Characteristics	Source or Reference
R. trifolii		
ANU843	Wild-type, Hac+Nod+	B. Rolfe
ANU845	pSym derivative of ANU843.	Djordjevic *et al.*, 1983
ANU871	ΔpSym 40–50 kb deletion, Hac−Nod−.	Schofield *et al.*, 1983
ANU851	Tn5: *nod* Hac−Nod−.	Schofield *et al.*, 1983
ANU2274	Tn5: *nod* Hac−Nod−.	P. R. Schofield
ANU2252	Tn5: *nod* Hac−Nod−.	P. R. Schofield
ANU845(pRt150)[a]	Hack++Nod−.	M. A. Djordjevic
ANU845(pBR1AN)[b]	Hac+Nod+.	Djordjevic *et al.*, 1983
R. leguminosarum		
128C53	Sm(r)Rif(r)Nod+	P. Albersheim, Univ. of Colorado

[a] Plasmid pRt150 (K_m) has a 5.3 kb *BglI* II fragment cloned into the *Inc* Q multicopy vector pKT230 (4). Plasmid pRt150 was mobilized to strain ANU845 using a triparental mating, and plasmid pRK2013 (11) as a helper plasmid. [b] Plasmid pBR1AN (K_m) is a self-transmissible *R. trifolii* pSym.

as previously described (27). The growth of the bacteria was followed by measuring the *A* at 620 nm. The bacteria were harvested at early stationary phase. At the time of harvest the 620 nm *A* for all strains was 1.5. All preparations were examined by Gram-staining and by plating on media with appropriate antibiotic markers. Strains containing Tn5, pBR1AN, or pRt150 are resistant to kanamycin. There was no evidence of contamination.

Isolation of the EPSs. The bacteria were harvested by centrifugation at 13,000*g* for 15 min. The supernatants, 1 L for each strain, were each concentrated by rotary evaporation at 45°C to about 200 mL. The EPSs were precipitated by adding three volumes of ethanol. The precipitates were spooled out of the ethanol solution using a stirring rod, dissolved in water, dialyzed, and freeze-dried. The EPSs were further purified by precipitation with cetyltrimethylammonium bromide (CTAB) (23). Details of these EPS purification procedures have been previously described (23).

Analytical Techniques. The hexose compositions were determined by acid hydrolysis, reduction of the monosaccharides to alditols using $NaBH_4$, preparation of the alditol acetates and analysis by gas GC (1). GC was done using a column packed with SP2330 (Supelco). Uronic acid, acetate and pyruvate were determined colorimetrically by previously described procedures (8, 17, 21). Uronic acids were identified by methanolysis of the polysaccharide followed by reduction with $NaBH_4$ and acetylation (13). Increases in glucose showed that all the EPSs contain glucuronic acid.

The sugar linkages were determined by methylation (26) followed by acetylation and combined GC/MS analysis. The various linkages were identified by GC/MS at the NIH Regional Center at Washington University, St. Louis, MO. The hexose identification of the various methylated alditol acetates was accomplished by comparing retention times with those of authentic standards. This was done for the EPS from strain ANU2252 by Mike McNeil, Chemistry Department, University of Colorado, Boulder. Methylation analysis was performed on EPSs, carboxyl-reduced EPSs, and depyruvylated/carboxyl-reduced EPSs. Carboxyl groups were reduced by partially methylating the EPSs followed by reduction with $NaBD_4$ (M. McNeil, personal communication). In this procedure the polysaccharides are treated one time with dimethyl sulfoxide anion, methylated with an excess of methyliodide, and reduced with $NaBD_4$. The now carboxyl-reduced EPSs are then remethylated by the usual procedure (26). Pyruvic acid groups were removed by treating the EPSs with oxalic acid as previously described (13). Removal of the pyruvic acid groups was done prior to reduction of the carboxyl groups and methylation analysis.

RESULTS

The EPS Compositions. Initial experiments indicated that the yield of EPS from the Tn5 insertion mutants, and the ΔpSym deletion mutant, was less than that from the parent and remaining strains shown in Table I. However, when an experiment to compare EPS yields was done in triplicate no significant differences among the strains were observed. The yields varied between 500 and 900 μg/ml of culture with quite large standard deviations. The compositions of the acidic EPSs are given in Table II. Within experimental error, all the EPSs are very similar in their composition. There are some apparent differences in some of the components. For example, strain ANU845 (pRt150) EPS appears to be low in both glucuronic acid and in pyruvate. However, since the methylation data, described below, suggest that all the EPSs are the same with regard to the glycosyl linkages of galactose, glucose, glucuronic acid and pyruvate, it is probable

Table II. *Compositions of the Acidic Extracellular Polysaccharides from Symbiotic Mutants of R. trifolii*

The compositions are given as μmol/10 mg of EPS. The values represent the average and SD for several analyses (a minimum of three) of each sample. Gal, galactose; Glc, glucose; GlcA, glucuronic acid; Pyr, pyruvate; Ac, acetate.

Strain	Gal	Glc	GlcA	Pyr	Ac
ANU843	5 ± 0.1	27 ± 1	7 ± 2	12 ± 1	11 ± 2
ANU851	6 ± 1	26 ± 4	8 ± 1	14 ± 1	9 ± 1
ANU2274	6 ± 0.5	26 ± 0.3	9 ± 1	13 ± 2	9 ± 1
ANU2252	6 ± 0.2	25 ± 3	10 ± 1	16 ± 3	8 ± 1
ANU871	5 ± 0.4	24 ± 1	9 ± 2	14 ± 3	13 ± 1
ANU845	7 ± 0.5	29 ± 4	7 ± 2	17 ± 2	9 ± 0.3
ANU845(pRt150)	5 ± 0.5	25 ± 0.2	6 ± 0.1	9 ± 1	8 ± 1
ANU870(pBR1AN)	6 ± 1	27 ± 2	7 ± 0.2	15 ± 2	10 ± 1

that these composition differences are not significant. It is not yet possible to make any conclusions concerning possible differences in acetyl content since we have not determined the position of these acetyl groups. Uronic acid was identified as glucuronic acid since carboxyl group reduction results in an increase of only glucose. This was confirmed when the EPSs, carboxyl-reduced with $NaBD_4$, were methylated and dideutero-C-6 was found only in the 4-linked glucose derivative.

Methylation Analysis of the EPSs. The types of sugar linkages in the EPSs were compared by methylation analysis of the carboxyl-reduced EPSs and of the depyruvylated/carboxyl-reduced EPSs. The quantitative methylation data for all the strains are given in Table III. The presence of terminal-glucose is much larger in the noncarboxyl-reduced EPSs (data not shown) and is probably produced by β-elimination of the uronic acid residues. The fact that the carboxyl-reduced EPSs still contain detectable levels of terminal glucose suggests that perhaps not all of the uronic acid residues were reduced and therefore there may still have been some β-elimination. Terminal-galactose is present in all samples and is increased after depyruvlation with a corresponding decrease in 4,6-linked galactose. These results indicate that (a) pyruvate groups are attached to the 4,6-position of a terminal galactose and (b) that not all terminal galactose residues have pyruvate attached. In fact, by comparing the terminal-galactose with the 4,6-linked galactose in the carboxyl-reduced EPSs, we can suggest that approximately one-half of the terminal-galactose residues have pyruvate attached. It is presently difficult to be certain of this since quantitation of the 4,6-linked galactosyl residue is made difficult by its incomplete separation (on GC analysis) from the 4,6-linked glucosyl residue. The disappearance of 3,4,6-linked glucose and the corresponding increase in 3-linked glucose upon depyruvylation shows that pyruvate is attached to the 4,6-position of a 3-linked glucose residue in all the EPSs. We do not know why the depyruvylation procedure removes all the pyruvate from the 3-linked glucose but not from the terminal-galactose residues. However, the 4,6-pyruvylated terminal galactose has been shown to be resistant to depyruvylation in other *Rhizobium* EPSs (13, 23). The main feature of these results is the similarity of all the EPS sugar linkages. While there is some quantitative variability in the methylation data from strain to strain, this variability is no greater than that observed among the EPSs from *R. trifolii* 0403 and NA30, *R. leguminosarum* 128C63 and 128C53, and *R. phaseoli* 127K36, all of which have the same structure (13, 23). Thus, it is likely that the variability we observe in the methylation results does not reflect significant differences in EPS structure among these *R. trifolii* strains.

To further compare our data with these previously reported results we isolated and analyzed the EPS from *R. leguminosarum* 128C53sm(r)rif(r) which was selected from the 123C53 wild-type strain. The structure of the EPS from *R. leguminosarum* 128C53 wild-type has been determined (23). The results for the carboxyl-reduced and the depyruvylated/carboxyl-reduced methylated EPS from *R. leguminosarum* 128C53sm(r)rif(r) are also shown in Table III. Comparison with our results for the *R. trifolii* mutants shows that the *R. leguminosarum* EPS and the *R. trifolii* EPSs must be very similar. The difference is that prior to depyruvylation of the *R. leguminosarum* EPS no terminal galactose residues are observed. This confirms the earlier report (23) which shows that all the terminal galactose residues for this EPS are pyruvylated.

DISCUSSION

The results described in this paper strongly suggest that the EPSs from all the *R. trifolii* strains examined have the same structure with regard to their glycosyl and pyruvyl residues. Based on our data and on known EPS structures we can hypothesize that the structure for the EPSs described in this report is the same as that reported for the EPS from *R. leguminosarum* 128C53 (23) with the exception that the latter EPS has all the terminal galactosyl residues pyruvylated while the EPSs in this report have one-half of these residues pyruvylated.

It seems apparent from our results that mutation of the nodulation region of the pSym, deletion of the nodulation region and deletion of the entire pSym does not affect the types of glycosyl and pyruvyl linkages of the EPSs from *R. trifolii* cells grown on laboratory media. Furthermore, the EPS from strain ANU845(pSym-) was not changed after the introduction of plasmids pRt150 or pBR1AN. These plasmids restore root hair curling ability and both root hair curling and nodulation ability, respectively. We suggest that the nodulation region of the pSym does not code for products which determine the structural arrangement of glycosyl or pyruvyl residues in these EPSs. It is possible that these EPSs could vary in the location of their acetyl groups or in the amounts and/or location of β-hydroxybutyric acid residues (18, 19). In addition, changes in CPSs and EPSs

Table III. *Sugar Linkages of the Acidic Extracellular Polysaccharides from Symbiotic Mutants of R. Trifolii and R. Leguminosarum (128C53sm(r)rif(r)*

The compositions are calculated as relative per cent areas of the GC peaks. The values given are for the carboxyl-reduced EPSs. The values in parentheses are for the EPSs which have been depyruvylated prior to carboxyl-reduction and methylation. Tr, trace amounts present.

Glycosyl Residue	Positions of Methyl Groups	Deduced Linkage	843	851	2274	2252	871	845	845 pRt150	845 pBR1AN	R. leg. 128C53
Glucosyl	2,3,4,6	Terminal	3	3	2	2	Tr	Tr	4	Tr	6
			(2)	(5)	(2)	(3)	(3)	(2)	(Tr)	(2)	(4)
Galactosyl	2,3,4,6	Terminal	6	7	6	6	6	4	5	4	0
			(10)	(8)	(8)	(9)	(9)	(10)	(10)	(10)	(4)
Glucosyl	2,4,6	3–	0	0	0	0	0	0	0	0	0
			(10)	(13)	(11)	(11)	(12)	(14)	(13)	(10)	(12)
Glucosyl	2,3,6	4–	56	57	57	58	61	58	57	54	54
			(59)	(58)	(57)	(58)	(58)	(61)	(59)	(58)	(59)
Glucosyl	2,3	4,6–	12	11	13	12	14	12	12	16	13
			(12)	(14)	(14)	(12)	(12)	(13)	(12)	(13)	(11)
Galactosyl	2,3	4,6–	9	8	7	8	10	10	8	9	12
			(5)	(3)	(5)	(5)	(6)	(3)	(4)	(3)	(8)
Glucosyl	2	3,4,6–	14	14	14	15	15	16	14	17	16
			(2)	(0)	(2)	(2)	(2)	(0)	(2)	(0)	(0)

have been detected as a function of growth phase (22), media (25), and the presence of root exudate (7). We are presently analyzing the extracellular material from several mutants which appear defective in EPS production (10, 12) but still nodulate their respective hosts.

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