THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 266, No. 15, Issue of May 25, pp. 9556-9564, 1991
Printed in U.S.A.

Distribution of *O*-Acetyl Groups in the Exopolysaccharide Synthesized by *Rhizobium leguminosarum* Strains Is Not Determined by the Sym Plasmid*

(Received for publication, November 1, 1990)

Hayo C. J. Canter Cremers‡§, Michael Batley¶, John W. Redmond¶, Andre H. M. Wijfjes‡, Ben J. J. Lugtenberg‡, and Carel A. Wijffelman‡

From the ‡Department of Plant Molecular Biology, Leiden University, Nonnensteeg 3, 2311 VJ Leiden, The Netherlands and the ¶School of Chemistry, Macquarie University, Sydney, 2109 New South Wales, Australia

The patterns of *O*-acetylation of the exopolysaccharide (EPS) from the Sym plasmid-cured derivatives of *Rhizobium leguminosarum* bv. *trifolii* strain LPR5, *R. leguminosarum* bv. *trifolii* strain ANU843 and *R. leguminosarum* bv. *viciae* strain 248 were determined by ^{1}H and ^{13}C NMR spectroscopy. Beside a site indicative of the chromosomal background, these strains have one site of *O*-acetylation in common, namely residue b of the repeating unit (Fig. 1).

The *O*-acetyl esterification pattern of EPS of the Sym plasmid-cured derivatives of strains LPR5, ANU843, and 248 was not altered by the introduction of a *R. leguminosarum* bv. *viciae* Sym plasmid or a *R. leguminosarum* bv. *trifolii* Sym plasmid. The induction of *nod* gene expression by growth of the bacteria in the presence of *Vicia sativa* plants or by the presence of the flavonoid naringenin, produced no significant changes in either amount or sites of *O*-acetyl substitution. Furthermore, no such changes were found in the EPS from a *Rhizobium* strain in which the *nod* genes are constitutively expressed. The substitution pattern of the exopolysaccharide from *R. leguminosarum* is, therefore, determined by the bacterial genome and is not influenced by genes present on the Sym plasmid. This conclusion is inconsistent with the suggestion of Philip-Hollingsworth *et al.* (Philip-Hollingsworth, S., Hollingsworth, R. I., Dazzo, F. B., Djordjevic, M. A., and Rolfe, B. G. (1989) *J. Biol. Chem.* 264, 5710–5714) that *nod* genes of *R. leguminosarum* bv. *trifolii*, by influencing the acetylation pattern of EPS, determine the host specificity of nodulation.

In the past few years, attention has been paid to the possibility that rhizobial exopolysaccharide (EPS)[1] is involved in nodulation. Several authors described the isolation of mutants affected in the synthesis of EPS that also showed defects in nodulation ability. Leigh *et al.* (1985) described numerous Exo$^-$ mutants of *Rhizobium meliloti* that all induce non-nitrogen-fixing nodules. Diebold and Noel (1989) described the isolation of Exo$^-$ mutants of *R. leguminosarum* bv. *phaseoli* and *R. leguminosarum* bv. *viciae*. Since the *R. leguminosarum* bv. *phaseoli* Exo$^-$ mutants induced nitrogen-fixing nodules on beans, while comparable mutants of *R. leguminosarum* bv. *viciae* failed to nodulate their host plants, they concluded that the requirements for EPS in nodulation depend on the cross-inoculation group concerned (Diebold and Noel, 1989).

More direct evidence for a role in nodulation of EPS was described by Djordjevic *et al.* (1987), who reported that purified EPS from *R. leguminosarum* bv. *trifolii* strain ANU843 could restore the nitrogen fixation ability of a mutant deficient in the synthesis of EPS. Further evidence for a biological role of EPS in nodulation was described by Skorupska *et al.* (1985). They described that nodulation could be inhibited by incubation of pea roots with EPS prior to inoculation with *R. leguminosarum* bv. *viciae*. In their system, deacetylated EPS did not block nodulation.

That the pattern of *O*-acetylation in the EPS could be important for the outcome of the nodulation process was also described by Philip-Hollingsworth *et al.* (1989b), who reported that the acetylation pattern of the EPS isolated from *R. leguminosarum* bv. *viciae* strain 300 is influenced by a plasmid harboring several nodulation genes isolated from *R. leguminosarum* bv. *trifolii*. They suggested that changes in the *O*-acetylation pattern determine the host specificity of nodulation.

In this paper we describe the distribution of the *O*-acetyl and 3-hydroxybutanoyl substituents in EPS synthesized by several *R. leguminosarum* biovar strains.

MATERIALS AND METHODS[2]

RESULTS

Basic Chemical Structure of the EPS of R. leguminosarum—The structure of the EPS of *R. leguminosarum* bv. *trifolii* strains LPR5 and ANU843, as well as *R. leguminosarum* bv. *viciae* strain 248 has been reported (McNeil *et al.*, 1986; Hollingsworth *et al.*, 1988; Canter Cremers *et al.*, 1991 respectively; Fig. 1, *A* and *B*). In order to study the influence of expression of nodulation genes on the structure of EPS synthesized by *R. leguminosarum* biovar strains, we first established the basic chemical structure of the EPS isolated from Sym plasmid[3]-cured derivatives of strains LPR5, ANU843,

* This work was supported by the Netherlands Scientific Organization. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Laboratory of Ecotoxicology, R. I. V. M., P. O. Box 1, 3720 BA Bilthoven, The Netherlands.

[1] The abbreviation used is: EPS, exopolysaccharide.

[2] Portions of this paper (including "Materials and Methods," Figs. 2–6, and Tables 1–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

[3] The so called Sym(biosis) plasmids, present in *R. leguminosarum* bacteria, harbor all nodulation and fixation genes necessary to induce nitrogen fixing nodules on the respective host plants. Their average size is 200 kb.

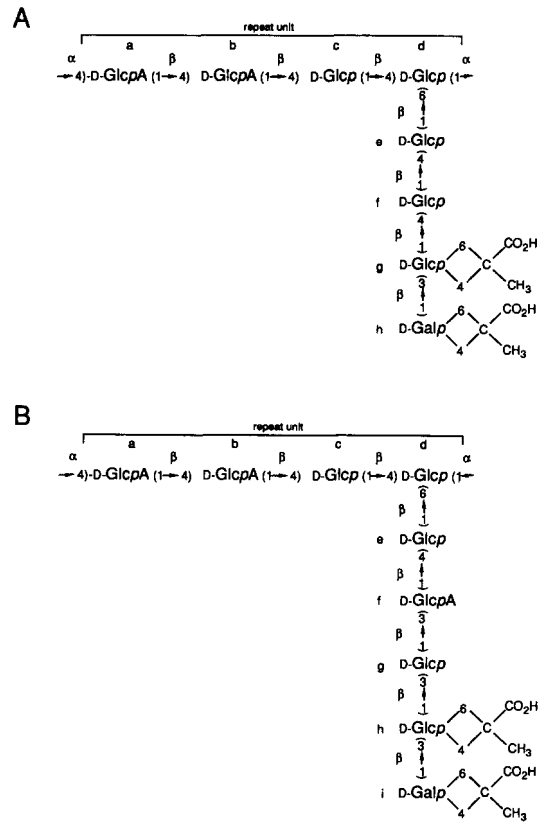

FIG. 1. **Sugar sequence of the repeating units of EPS isolated from *R. leguminosarum* biovar strains.** The non-stoichiometric ester substituents are not shown. *A*, strain LPR5, RBL5515, and ANU845; *B*, strain RBL1387 and 248. Fragments obtained by the action of phage depolymerase have 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid in place of glucuronic acid residue a.

and 248 by both ^1H and ^{13}C NMR spectroscopy.

The EPS of a Sym plasmid-cured derivative of *R. leguminosarum* bv. *trifolii* strain LPR5, namely strain RBL5515, was depolymerized with phage RL38 and purified. After de-esterification of the repeating unit of the EPS thus obtained in the NMR tube by the addition of aliquots of 100 mM NaOD in D_2O, the structure was studied. The ^1H and ^{13}C spectra obtained from the depolymerized and de-esterified EPS isolated from Sym plasmid-cured *R. leguminosarum* biovar strain RBL5515 (Fig. 2*A*) were indistinguishable from those of strain ANU845, indicating an identical sugar sequence.

The ^{13}C spectrum of the de-esterified repeat units of EPS of strains RBL5515 and ANU845 was assigned as follows. Graded acid hydrolysis of purified EPS from strain ANU845 was used to generate oligosaccharide fragments, which were then separated as described previously (Canter Cremers *et al.*, 1991). The ^{13}C NMR spectra of these fragments were assigned by comparison with the reported spectra for similar oligosaccharides (Bock *et al.*, 1984) and by comparing the spectra of larger fragments with those of smaller fragments derived from them (Djordjevic *et al.*, 1986). The set of fragments (Table 2) was sufficient for the assignment of carbons in residues b, c, d, e, and f of the known structure (Hollingsworth *et al.*, 1988; Philip-Hollingsworth *et al.*, 1989b).

Depolymerization changes residue a into 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid. The H-1 and H-4 resonances for this residue occur sufficiently downfield to permit location of H-2 and H-3 by difference decoupling. Using the chemical shifts of these four protons it was possible to measure the chemical shift for C-1, C-2, C-3, and C-4 from the ^{13}C-^1H heteronuclear chemical shift correlation spectrum for the repeat unit. The shifts for C-5 and C-6 were unmistakable. The assignments of residues g and h were obtained by comparing spectra of samples with and without galactose (Canter Cremers *et al.*, 1990), as well as by comparing the effect of the carboxyethylidene substituent on chemical shifts. The assignments of the ^{13}C spectrum are summarized in Table 3.

The spectra of the depolymerized and de-esterified EPS of strain RBL5515 are entirely consistent with the basic chemical structure published for strain LPR5 (McNeil *et al.*, 1986; Fig. 1*A*). We use the latter code given in Fig. 1 as reference for the various sugar residues.

The sugar sequence of the depolymerized and de-esterified EPS isolated from a Sym plasmid-cured derivative of *R. leguminosarum* bv. *viciae* strain 248, namely strain RBL1387, is different (Fig. 1*B*), and the determination of this structure will be reported elsewhere. However, the structure of the backbone of the EPS, namely residues a, b, c, and d (Fig. 1) is identical to that of strain RBL5515 (Fig. 1, *A* and *B*).

Sites of Esterification in the EPS of Strain RBL5515—Information about the sites of esterification in the depolymerized EPS isolated from *R. leguminosarum* strain RBL5515, was obtained by comparing the ^{13}C NMR spectra before and after de-esterification (Fig. 2, *B* and *A*, respectively). Shifts caused by acetylation were distinguished from those due to hydroxybutanoate substitution by examining the octasaccharide repeating units from strains RBL5515 and ANU845, which have different proportions of acetyl and 3-hydroxybutanoyl groups. Chemical shifts for all the protons on residue a were located by homonuclear difference decoupling. In the ^1H spectrum of repeat units from RBL5515 EPS, the triplet due to H-3 of the 3-*O*-acetylglucuronic acid (residue b) which is found slightly upfield from the H-1 resonance of H-1 of residue a (Hollingsworth *et al.*, 1988) is reasonably well separated from other signals (Fig. 3). Difference decoupling, starting with the H-3 resonance, was used to locate H-1, H-2, and H-4 of acetylated residue b. The locations of H-2 and H-4 were in agreement with those reported previously (Hollingsworth *et al.*, 1988) and conformation of the H-1 resonance position was obtained by relayed conherence transfer (Eich *et al.*, 1982) to H-3 following selective excitation of H-1.

The major site of acetylation was the 3-position on the glucuronic residue b, not residue c as previously suggested (Kuo and Mort, 1986). Although spectroscopic differences between glucose and glucuronic acid are not great, there are three independent pieces of evidence for this conclusion.

1) The presence of acetate causes a reduction in the intensity of the C-6 resonance for residue b at 176.2 ppm and the appearance of a new peak at 175.9 ppm (Table 4A; Fig. 4, *A* and *B*).

2) The intensity of the peak at 80.9 ppm is affected by acetylation. This peak is assigned to C-4 of residue b (Table 3) because it was the furthest downfield of all the C-4 resonances and the C-4 resonance for the internal glucuronic acid residue of the hydrolysis fragment β-Glc*p*A-(1→4)-β-Glc*p*A-(1→4)-Glc*p*, is significantly downfield (82.0 ppm) from C-4 for the reducing terminus α-glucose (79.9 ppm) or β-glucose (79.8 ppm). The C-4 resonance for glucuronic acid occurs at 82.0 ppm in the spectra of three other related hydrolysis fragments, but in the octasaccharide, in which residue a has changed from glucuronic acid to 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid, the resonance moves upfield to 80.9 ppm. Since the other C-4 resonances do not move (and are still 1 ppm upfield from the resonance in question), the carbon whose resonance frequency is affected by the change in residue a must be on residue b. Final confirmation of the assignment

was provided by a fortuitous accident during the preparation of depolymerized repeat units from EPS synthesized by mutant strain RBL5515,*exo112*::Tn5. Phage depolymerization of EPS of this strain was performed three times: on two occasions the material obtained was spectroscopically indistinguishable from repeat units of strain RBL5515, but on the third occasion residue a was completely absent. The reason for the loss of this residue a is unknown, but microbial contamination followed by enzymatic degradation must be considered as a possibility. In the ^{13}C spectrum of the modified sample, the peak at 80.9 ppm had disappeared, while a new peak had appeared at 72.9 ppm. The other C-4 resonances were unaffected.

3) Philip-Hollingsworth *et al.* (1989b) reported that the resonance frequency for the H-1 nucleus in 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid residue a was shifted slightly by the presence of acetate. In the present study, it was found that at 30 °C the resonances for all the protons in residue a are affected by acetylation. Samples that contained approximately equal amounts of acetylated and unacetylated material were used, so that measurement of the shifts was not affected by changes in sample conditions. All the shifts were less than 0.05 ppm and were temperature dependent (Fig. 5) and were therefore too small for the acetylation to be on residue a. They are presumably examples of conformational transmission of substitution effects (Dabrowski *et al.*, 1980). A simple hard sphere repulsion model (Rees and Skerret, 1970) would predict no effect of acetate at C-3 of residue b on the extent of rotation around the interresidue bonds connecting residues a and b. We suggest, therefore, that acetate affects hydrogen bonding between the residues (Rees and Skerret, 1970) and that the hydrogen bonding, in turn, affects the average conformation of the whole hexenoic ring.

As discussed above, identification of the site of acetylation depends on correct assignment of the resonances in the unacetylated oligosaccharide. We have nevertheless attempted to assign ^{13}C resonances for the acetylated species. Since the chemical shift for the H-3 proton on acetylated residue b was known, the frequency of the ^{13}C resonance for C-3 of the same residue could be obtained from the two-dimensional correlation spectrum (Fig. 6 and Table 3). The acetylation shift of +1.2 ppm obtained by comparing this frequency with C-3 of the corresponding residue in the unacetylated hydrolysis fragment β-GlcA-(1→4)-β-GlcA-(1→4)-Glc was slightly smaller than the normal range of 1.5 to 4.0 ppm (Bock and Pedersen, 1983), but part of the change can be related to the altered nature of residue a.

We are, however, left with a set of shifts for other carbons that fall well outside the usual range whatever combination of assignment is made. Our preferred assignments for the peaks that are seen only when acetate is present are given in Table 3. The carbon resonance at 81.1 ppm is correlated with a proton resonance that has a similar chemical shift to that for H-4 of residue b in the unacetylated repeat unit (Fig. 6) and is therefore assigned to C-4 of 3-*O*-acetylated residue b, despite the fact that this implies a small positive shift rather than the expected field shift of between 1 and 5 ppm (Bock and Pederson, 1983). The other possible assignments, C-5 and C-2, are not favored because they would mean downfield shifts of 4.6 and 6.1 ppm, respectively, for the carbon signals and the H-2 resonance should be further upfield. Similar arguments were used in arriving at the assignments in Table 3.

The difficulties encountered in assigning the acetylation shifts would have been exactly the same if acetate had been on residue c or any other 1–4-linked glucose residue. A possible explanation for the unusual substituent effects is that the average confirmation of glucuronic acid may be sensitive to changes in the amount of inter- and intraresidue hydrogen bonding. All the ^{13}C resonance frequencies in glucuronic acid are slightly dependent on the degree of ionization of the carboxylate group and the resonance for C-5 varies by as much as 1.8 ppm (Pfeffer *et al.*, 1979).

In agreement with previous studies (Philip-Hollingsworth *et al.*, 1989a) we found methyl proton resonances indicating that there was also 2-*O*-acetate on residue b (Table 4B). The ^{13}C spectral evidence is consistent with this conclusion, but it would not, by itself, be conclusive.

The ^{13}C spectrum also contained evidence for another acetylation site, which could only be on residue d (Table 4D), the reducing terminus of the octasaccharide (Fig. 1). In addition, the ^{1}H spectrum (Fig. 3*A*) contained the methyl resonances previously reported as being specific for acetate on residue d (Philip-Hollingsworth *et al.*, 1989a).

In conclusion, *O*-acetyl groups are found on residue b and d of EPS of *R. leguminosarum* strain RBL5515.

Sites of Esterification in the EPS of Strain ANU845—The ^{1}H (Fig. 3*B*) and ^{13}C NMR spectra (Fig. 2*C*) of the repeating unit of the EPS isolated from *R. leguminosarum* strain ANU845 contained all the peaks assigned to the presence of 2- and 3-*O*-acetates on residue b (Table 4, A and B; Fig. 4*C*). The same was true for the galactose resonances affected by 3-hydroxybutanoyl (Table 4E), but the amounts were somewhat greater than for strain RBL5515.

No evidence was found for acetate on residue d, but there was instead a small amount of acetate at the 2- and 3-positions of residue a, as indicated by the appearance of C-1 peaks at 108.2 and 107.9 ppm (Fig. 2*C*; Table 4C). Furthermore, there were small changes in the resonance frequencies for C-4, C-5, and C-6 of residue a (Table 4C) and for H-1 and H-4 of the same residue.

In conclusion, apart from 3-hydroxybutanoyl groups at residue g, *O*-acetyl groups are found on residues a and b of the repeating unit of EPS isolated from *R. leguminosarum* strain ANU845 (Fig. 1).

Sites of Esterification in the EPS of R. leguminosarum Strain RBL1387—The ^{13}C NMR spectrum of the purified nonasaccharide-repeating unit of the EPS isolated from Sym plasmid-cured *R. leguminosarum* biovar strain RBL1387 (Fig. 2*D*), contained the characteristic resonances indicating the presence of 2- and 3-*O*-acetyl substitution on residue b (Table 4, A and B; Fig. 4*D*). In addition to the methyl proton resonances for the acetates on residue b, the ^{1}H spectrum contained a second pair of acetyl methyl resonances that were not observed in the spectra of material from either of the other strains. As we reported elsewhere (Canter Cremers *et al.*, 1991) these resonances were assigned to acetylations at C-2 and C-4 of residue f (Fig. 1*B*). Apart from the acetylations, EPS of strain RBL1387 also contains 3-hydroxybutanoyl groups, esterified to residue h and i.

Effect of Sym Plasmids on the Esterification Pattern—To test whether the presence of Sym plasmids influenced the esterification pattern of the EPS, we first introduced the *R. leguminosarum* bv. *viciae* Sym plasmid pRL1JI into strains RBL5515, ANU845, and RBL1387. The ^{1}H and ^{13}C NMR spectra showed no change in the sites of 3-hydroxybutanoyl and *O*-acetyl esterification in either strain. In each case there were 2- and 3-*O*-acetyl groups on residue b plus the second acetate indicative of the chromosomal background, on residue a in strain ANU845 pRL1JI, on residue d in strain RBL5515 pRL1JI, and on residue f in strain RBL1387 pRL1JI. Any variation in the amount of the substituents fell within the

range encountered between different batches from the same strain.

Introduction of the Sym plasmid of another cross-inoculation group, namely the *R. leguminosarum* bv. *trifolii* Sym plasmid pSym5, did not influence the site or amount of esterification in either one of these strains.

Also the introduction of either the *R. leguminosarum* bv. *viciae* Sym plasmid pHim or the *R. leguminosarum* bv. *phaseoli* Sym plasmid pSym9 in *R. leguminosarum* strain RBL5515 did not affect the esterification present on its EPS.

In conclusion, the presence of a *R. leguminosarum* Sym plasmid by itself does not influence the site or amount of esterification present in the EPS in any of the *Rhizobium* strains tested.

Influence of Plasmid pRt290 on Esterifications in EPS of R. leguminosarum Strain RBL5515 pRL1JI—Philip-Hollingsworth *et al.* (1989b) reported that the introduction of plasmid pRt290 in *R. leguminosarum* bv. *viciae* strain 300 influenced the site of *O*-acetylation in its EPS, especially when the *nod* genes in this strain were not induced by flavonoids. To check whether this also occurs in the *R. leguminosarum* bv. *viciae* strain we use, we introduced plasmid pRt290, which harbors the *R. leguminosarum* bv. *trifolii* nodulation genes *nodF, E, L, M, N*, in strain RBL5515 pRL1JI and, as a control, in strain RBL5515. The ^{1}H and ^{13}C NMR spectra obtained from the depolymerized EPS isolated from the resulting strains showed no change in site or amount of esterification with *O*-acetyl or 3-hydroxybutanoyl groups compared with that of strain RBL5515 (Fig. 2*B*). Thus, the presence of plasmid pRt290 does not influence the site of *O*-acetylation in *R. leguminosarum* bv. *viciae* strain RBL5515 pRL1JI.

Influence of Induced nod Genes on Esterifications in EPS of R. leguminosarum—To investigate whether expression of *nod* genes influenced the amount or site of esterification, we isolated the EPS from strains RBL5515, RBL5515 pRL1JI, and RBL5515 pSym5 grown in B^- minimal medium at 28 °C for 3 days in the presence or absence of 4 μg/ml naringenin, which is a potent inducer of *nod* gene expression in both *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii* strains (Djordjevic *et al.*, 1987; Zaat *et al.*, 1987). In either case the ^{1}H and ^{13}C NMR spectra obtained from the depolymerized and purified EPS showed no difference in site or amount of esterification.

The *R. leguminosarum* strains RBL5515, RBL5515 PRL1JI, and RBL5515 pSym5 were also grown in the presence of *V. sativa* roots. In respect to site and amount of *O*-acetylation, the ^{1}H and ^{13}C NMR spectra obtained from the depolymerized EPS isolated from these cultures were indistinguishable from that of strain RBL5515 grown in minimal medium (Fig. 2*B*). In EPS isolated from these cultures there was 3-hydroxybutanoyl esterification of O-2 and O-3 of residue g, but the amount was significantly lower than that present in EPS of strain RBL5515 grown in B^- minimal medium. When EPS was isolated from a culture of RBL5515 grown in the same medium as used for the *V. sativa* plants, namely J^{++} medium, the amount of 3-hydroxybutanoyl was comparable to that found in the EPS of strains grown in presence of the *V. sativa* plants. This difference in the amount of 3-hydroxybutanoyl substitution thus seems dependent on the medium used. The presence of flavonoids and other substances secreted by the plant roots seems not to influence the amount or site of esterification in the EPS of the strains tested.

Finally we isolated EPS from a *R. leguminosarum* bv. *viciae* strain in which the *nod* genes are constitutively expressed, namely strain RBL5515 pRL1JI,*nodD2*::Tn5 harboring plasmid pMP604, on which a hybrid *nodD* gene is present. The NodD protein derived from it is able to induce all the promoters in front of the inducible *nod* genes present on the *R. leguminosarum* bv. *viciae* Sym plasmid pRL1JI in the absence of flavonoids (Spaink *et al.*, 1989a). The ^{1}H and ^{13}C spectra obtained from the enzymically depolymerized EPS of this strain were indistinguishable from that of strain RBL5515 (Fig. 2*B*). We therefore conclude that neither the presence nor the induction of *nod* genes influence the site or amount of esterification present in the EPS of *R. leguminosarum* bv. *viciae* strain RBL5515 pRL1JI.

DISCUSSION

In this paper we described the esterification patterns of EPS isolated from three *R. leguminosarum* biovar strains, namely RBL5515, ANU845, and RBL1387.

3-Hydroxybutanoyl Substitutions—In all three strains, 3-hydroxybutanoyl groups are ester linked to the 2- and 3-positions of the terminal galactose residue in the side chain of the EPS (Fig. 1). It is also present on glucose residue h in EPS from strain RBL1387. Like galactose residue i, this residue carries a 4,6-*O*-(1-carboxyethylidene) group.

The amount of 3-hydroxybutanoyl groups present in the EPS depended on the growth medium, which is in agreement with the findings of McNeil *et al.* (1986). When the bacteria were grown under indistinguishable conditions, there were consistently more 3-hydroxybutanoyl groups present on EPS from strain RBL1387 and ANU845 than there was on EPS from strain RBL5515. This may simply be a reflection of the metabolic states for the different strains in the chosen medium. Alternatively, the amount of 3-hydroxybutanoyl esterification may be a consequence of the chromosomal background studied. Neither the presence of Sym plasmids nor the induction of *nod* genes had any effect on the level of hydroxybutanoyl substitution.

In addition, both *R. leguminosarum* bv. *viciae* strain RBL1387 pRL1JI with a high level, as well as strain RBL5515 pRL1JI with a lower level, of hydroxybutanoyl substitution were able to nodulate the same host. It is therefore unlikely that this substituent is involved in determining host specificity within the *R. leguminosarum* biovars.

O-Acetyl Substitutions—In EPS from all strains examined, we found *O*-acetyl groups esterified to residue b (Fig. 1) of the repeating unit. Furthermore, all three *R. leguminosarum* strains RBL5515, ANU845, and RBL1387 showed an additional *O*-acetylation site dependent on the chromosomal background studied.

Our results for strain ANU845, which indicate that it has *O*-acetyl groups esterified to residues a and b (Fig. 1), confirm and extend those of previous workers (Philip-Hollingsworth *et al.*, 1989a). The *O*-acetyl group on residue a of EPS from *R. leguminosarum* biovar strain ANU845 has not previously been reported. Apparently, this acetate does not prevent depolymerization by the phage trans-eliminase, as the phage was able to depolymerize the EPS.

Evidence, although not conclusive, was found for acetylation at both C-2 and C-4 of glucuronic acid residue f in the EPS of *R. leguminosarum* biovar strain RBL1387. Base-catalyzed acetyl migration has been offered (Hollingsworth *et al.*, 1988) as an explanation for the presence of both 2-*O*-acetylation and 3-*O*-acetylation of residue b in repeat units isolated from strain ANU845. Such migration requires the presence of an adjacent free hydroxyl group and cannot, therefore, be the explanation for the acetylation sites on residue f of EPS from strain RBL1387. In analogy, it is possible that the two acetylation sites of residue b in EPS of

R. leguminosarum strain ANU845 and other comparable strains are not due to migration.

Presence of Acetyl Groups on Residue b of the EPS of R. leguminosarum Biovars—The ^{13}C NMR spectra demonstrate unambiguously that one of the sites of acetylation is the same in EPS from all three strains with different chromosomal backgrounds examined. While this is contrary to one of the conclusions drawn in a previous study (Philip-Hollingsworth *et al.*, 1989a), there is no conflict with their and our experimental data. Two of our strains, namely strains ANU845 and RBL5515, are similar to ones examined by Philip-Hollingsworth *et al.* (1989a). They also found that the site of acetylation in EPS from ANU845 was the glucuronic acid residue b, but they reached a different conclusion with respect to EPS from strain LPR5035, which is closely related to our strain RBL5515. Philip-Hollingsworth *et al.* (1989a) found acetylation of residue b in EPS from *R. leguminosarum* bv. *trifolii* strains ANU845, NA-30, and TA1. The last two were also studied by Kuo and Mort (1986), who believed that they had developed a modified methylation analysis procedure that would permit the location of acetate substituents. Kuo and Mort's conclusion that the acetyl groups were on residue c in the EPS of strains NA-30 and TA1 was in direct conflict with the later NMR evidence of Philip-Hollingsworth *et al.* (1989a). Our results for strain ANU845 support the conclusions of Philip-Hollingsworth *et al.* (1989a).

Philip-Hollingsworth *et al.* (1989a) also studied EPS from strains LPR5035 and 128C53, which they found to be spectroscopically identical, but they were unable to locate the characteristic triplet for the H-3 resonance of 3-*O*-acetyl residue b in the ^{1}H spectrum of the repeat units. We, too, found that in a number of cases, the H-3 signal was distinguishable only upon close inspection, but there was no such difficulty with the characteristic ^{13}C resonances. In the absence of other evidence, Philip-Hollingsworth *et al.* (1989a) accepted the finding of Kuo and Mort (1986) that residue c was acetylated in EPS from strain 128C53 and extended it to strain LPR5035, even though they had overturned the same conclusion of Kuo and Mort (1986) concerning EPS from strains NA-30 and TA1, as described above. Our results strongly suggest that the methylation analysis of Kuo and Mort (1986) gave the wrong conclusions for these strains as well, since in EPS of a strain closely related to strain LPR5035, namely strain RBL5515, acetylation occurs to residues b and d.

Influence of nod Genes on the Site of Substitution—Philip-Hollingsworth *et al.* (1989b) reported that the acetylation pattern of the EPS synthesized by *R. leguminosarum* bv. *viciae* strain 300 changed markedly after the introduction of a high copy number plasmid harboring *R. leguminosarum* bv. *trifolii nod* genes *nodF, E, L, M, N*, which are involved in determining host specificity. They, therefore, suggested that the acetylation pattern was involved in determining host specifity.

Neither the introduction of Sym plasmids of various *R. leguminosarum* biovars into the Sym plasmid-cured strains RBL5515, ANU845, or RBL1387, nor the growth of strains RBL5515, RBL5515 pRL1JI, and RBL5515 pSym5 in the presence of either *V. sativa* roots or the flavonoid naringenin had any effect on the esterification pattern of the EPS. In addition, EPS isolated from strain RBL5515 pRL1JI, *nodD2*::Tn5 pMP604, in which the nodulation genes are constitutively expressed, was indistinguishable from that of strain RBL5515. Also no change in the acetylation pattern was observed in the present study, when the multicopy plasmid pRt290 used by Philip-Hollingsworth *et al.* (1989b) was introduced into *R. leguminosarum* bv. *viciae* strain 5515 pRL1JI. The reported changes in acetylation are therefore specific for either *R. leguminosarum* bv. *viciae* strain 300 or for the conditions used.

Role of EPS in Nodulation—Three bacterial strains with different host specificities, namely *R. leguminosarum* bv. *phaseoli* strain RBL5515 pSym9, *R. leguminosarum* bv. *trifolii* strain RBL5515 pSym5, and *R. leguminosarum* bv. *viciae* strain RBL5515 pRL1JI, were found to have EPS with the same *O*-acetylation pattern. Furthermore, three strains which induce nitrogen-fixing nodules on *V. sativa* plants, namely *R. leguminosarum* bv. *viciae* strains RBL5515 pRL1JI, ANU845 pRL1JI, and RBL1387 pRL1JI produce EPS with qualitatively different substitution patterns. Hence, neither the variation in the sugar sequence of the side chain, nor the chromosomal background dependent esterifications sites cause discrimination between these strains. We therefore find it highly unlikely that EPS is a determinant of host specificity. Our conclusions are consistent with those reported by McNeil *et al.* (1986) which were based on EPS isolated from noninduced *Rhizobium* strains only. Experimental data described in the present paper allow us to extend this conclusion to *Rhizobium* strains from which *nod* genes, including those that determine host specifity (Spaink *et al.*, 1989b), were induced.

These conclusions do not preclude the possibility that a common feature of the EPS is required by all *R. leguminosarum* biovars for successful symbiosis. The common features of EPS from strains ANU845, RBL5515, and RBL1387 are 1) the backbone sugar residue sequence, 2) the carboxyethylidinated glucose and galactose residues at the end of the side chain, and 3) a substantial degree of 3-*O*-acetylation of glucuronic acid residue b. The degree of acetylation is known to affect the rheological properties of EPS (Holzwarth and Ogletree, 1979), but whether this can be related to symbiotic efficiency is unclear.

Finally on the basis of our results, we conclude that the acetylation pattern of the EPS of *R. leguminosarum* is determined by the bacterial genome and is not influenced by the expression of *nod* genes, the expression of other genes present on the Sym plasmid, or factors present in the root exudate of *V. sativa* plants.

REFERENCES

Bergey, D. (1983) *Bergey's Manual of Determinative Bacteriology* (Krieg, N. R., Holt, J. G., eds) Vol. 1, 9th ed., Williams and Wilkins, Baltimore

Buchanan-Wollaston, V. (1979) *J. Gen. Microbiol.* **112,** 135–142

Canter Cremers, H. C. J., Spaink, H. P., Wijfjes, A. H. M., Pees, E., Wijffelman, C. A., Okker, R. J. H., and Lugtenberg, B. J. J. (1989) *Plant Mol. Biol.* **13,** 163–174

Canter Cremers, H. C. J., Wijffelman, C. A., Pees, E., Engels, M., Hoogerbruggen, F., Stevens, K., van Dijk, M., and Lugtenberg, B. J. J. (1988) in *Nitrogen Fixation: Hundred Years After*, (Bothe, H., de Bruijn, F. J., and Newton, W. J., eds) p. 484, Gustav Fisher, Stuttgart

Canter Cremers, H. C. J., Batley, M., Redmond, J. W., Eydems, L., Breedveld, M. W., Zevenhuizen, L. P. T. M., Pees, E., Wijffelman, C. A., and Lugtenberg, B. J. J. (1990) *J. Biol. Chem.* **265,** 21122–21127

Canter Cremers, H. C. J., Batley, M., Redmond, J. W., Stevens, K., Breedveld, M. W., Zevenhuizen, L. P. T. M., Lugtenberg, B. J. J., and Wijffelman, C. A. (1991) Carbohydr. Res., in press

Dabrowski, J., Hanfland, P., and Egge, H. (1980) *Biochemistry* **19,** 5632–5658

Djordjevic, M. A., Schofield, P. R., and Rolfe, B. G. (1985) *Mol. Gen. Genet.* **200,** 463–471

Djordjevic, M. A., Rolfe, B. G., Batley, M., and Redmond, J. W., (1987) *EMBO J.* **6,** 1173–1179

Djordjevic, S. P., Batley, M., Redmond, J. R., and Rolfe, B. G. (1986) *Carbohydr. Res.* **148,** 87–99

Djordjevic, S. P., Chen, H., Batley, M., Redmond, J. W., and Rolfe, B. G. (1987) *J. Bacteriol.* **169,** 53–60

Downie, J. A., Knight, C. D., Johnston, A. W. B., and Rossen, L. (1985) *Mol. Gen. Genet.* **198,** 255–262
Eich, G. W., Bodenhausen, G., and Ernst, R. R. (1982) *J. Am. Chem. Soc.* **104,** 3731–3732
Hollingsworth, R. I., Dazzo, F. B., Hallenga, K., and Musselman, B. (1988) *Carbohydr. Res.* **172,** 97–112
Holzwarth, G., and Ogletree, J. (1979) *Carbohydr. Res.* **76,** 277–280
Hooykaas, P. J. J., van Brussel, A. A. N., den Dulk-Ras, H., van Slogteren, G. M. S., and Schilperoort, R. A. (1981) *Nature* **291,** 351–353
Johnston, A. W. B., Beynon, J. L., Buchanon-Wollaston, A. V., Setchell, S. M., Hirsch, P. R., and Beringer, J. E. (1978) *Nature* **276,** 634–636
Kuo, M.-S.K., and Mort, A. J. (1986) *Carbohydr. Res.* **145,** 247–265
Lie, T. A. (1984) *Plant Soil* **82,** 415–425
McNeil, M., Darvill, J., Darvill, A., Albersheim, P., van Veen, R., Hooykaas, P., Schilperoort, R., and Dell, A. (1986) *Carbohydr. Res.* **146,** 307–326
Philip-Hollingsworth, S., Hollingsworth, R. I., and Dazzo, F. B. (1989a) *J. Biol. Chem.* **264,** 1461–1466
Philip-Hollingsworth, S., Hollingsworth, R. I., Dazzo, F. B., Djordjevic, M. A., and Rolfe, B. G. (1989b) *J. Biol. Chem.* **264,** 5710–5714
Priem, W. J. E., and Wijffelman, C. A. (1984) *FEMS Microbiol. Lett.* **25,** 247–251
Robertson, B. K., Åman, P., Darvill, A. G., McNeil, M., and Albersheim, P. (1981) *Plant Physiol.* **67,** 389–400
Rolfe, B. G., Gresshoff, P. M., and Shine, J. (1982) *Plant Sci. Lett.* **19,** 227–284
Skorupska, A., Derylo, M., and Lorkiewicz, Z. (1985) *Arch. Microbiol.* **143,** 307–310
Spaink, H. P., Wijffelman, C. A., Pees, E., Okker, R. J. H., and Lugtenberg, B. J. J. (1987) *Nature* **328,** 337–340
Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Tak, T., GoosendeRoo, L., Pees, E., van Brussel, A. A. N., and Lugtenberg, B. J. J. (1989a) *J. Bacteriol.* **171,** 4045–4053
Spaink, H. P., Weinman, J., Djordjevic, M. A., Wijffelman, C. A., Okker, R. J. H., and Lugtenberg, B. J. J. (1989b) *EMBO J.* **8,** 2811–2818
Sutherland, I. W. (1979) in *Microbiol Polysaccharides and Polysaccharidases* (Berkeley, R. C. W., Gooday, G. W., and Ellwood, D. C., eds) pp. 1–34, Academic Press, New York
van Brussel, A. A. N., Planqué, K., and Quispel, A. (1977) *J. Gen. Microbiol.* **101,** 51–56
van Brussel, A. A. N., Tak, T., Wetselaar, A., Pees, E., and Wijffelman, C. A. (1982) *Plant Sci. Lett.* **27,** 317–325
van Brussel, A. A. N., Zaat, S. A. J., Canter Cremers, H. C. J., Wijffelman, C. A., Pees, E., Tak, T., and Lugtenberg, B. J. J. (1986) *J. Bacteriol.* **165,** 517–522
Vincent, J. M. (1970) *A Manual for the Practical Study of Root-Nodule Bacteria,* International Biological Programme, Handbook No. 15, Blackwell Scientific Publications, Oxford
Wijffelman, C. A., Pees, E., van Brussel, A. A. N., Okker, R. J. H., and Lugtenberg, B. J. J. (1985) *Arch. Microbiol.* **143,** 225–232
Zaat, S. A. J., Wijffelman, C. A., Spaink, H. P., van Brussel, A. A. N., Okker, R. J. H., and Lugtenberg, B. J. J. (1987) *J. Bacteriol.* **169,** 198–204

Supplemental material to

Distribution of O-acetyl Groups in the EPS

Synthesized by Rhizobium leguminosarum Strains Is Not

Determined by the Sym Plasmid

by

Hayo C.J. Canter Cremers, Michael Batley, John W. Redmond,

Ben J.J. Lugtenberg, André H.M. Wijfjes and Carel A. Wijffelman

Materials and Methods

Bacterial strains and plasmids used

All bacterial strains and plasmids used are listed in Table 1. Bacteria were grown in B⁻ (Van Brussel et al, 1977) or J⁺⁺ medium, which is Jensen medium (Vincent, 1970) supplemented with 20% B⁻ medium. If required, antibiotics were added in concentrations as described previously (Canter Cremers et al, 1988). In order to be able to transfer Sym plasmids from one strain to another, we used Sym plasmids marked with transposons in regions that did not affect nodulation ability, namely R.leguminosarum bv viciae Sym plasmid pRL1JI::Tn1831(3) (Priem and Wijffelman, 1984), R.leguminosarum bv trifolii Sym plasmid pSym5::Tn5 (Hooykaas et al., 1981, R.leguminosarum bv phaseoli Sym plasmid pSym9::Tn5 (Hooykaas et al., 1981) and R.leguminosarum bv viciae Sym plasmid pHim::Tn5 (Spaink et al., 1987).

Isolation, purification and characterization of EPS

A supension of Rhizobium bacteria in water was used as an inoculum. In order to make this suspension, bacteria were pelleted from a freshly grown culture in B⁻ minimal medium by centrifugation, resuspended in H_2O, once again pelleted by centrifugation and resuspended in H_2O.In order to isolate EPS, bacteria were grown at 28°C on a rotary shaker in erlenmeyers containing 500 mL of medium for three days after inoculation to A_{620} 0.05 from a bacterial suspension in H_2O. Whenever a comparison is made between induced and non-induced bacteria, the cultures were grown at the same time, using the same rotary shakers and climate rooms to obtain comparable culturing conditions.

The EPS was isolated from the Rhizobium cultures by first pelleting the bacteria by centrifugation for 15 min at 11.000 rpm in a Beckman JA14 rotor. The supernatant was then dialyzed and concentrated in an Amicon hollow fiber apparatus fitted with a HSP100-43 cartridge, which has a 100.000 Da cut off as described previously (Djordjevic et al, 1986). After freeze drying, 0.150 g EPS was dissolved in 20 mL 25mM phosphate buffer pH 7.0, and depolymerized by adding 10^{12} plaque forming units of phage RL38 (Buchanon-Wollaston et al,1979) or phage 4S (Hollingsworth et al, 1984). After 7 days at 28°C, the remaining EPS polymer molecules were pelleted by centrifugation for 10 min at 10.000 rpm after adding two volumes of 96% ethanol to the EPS-phage solution. The ethanol in the supernatant was removed by roto-evaporation at 45°C followed by freeze drying. The material obtained was sequentially purified over Sephadex DEAE A25, Biogel P2 and Dowex 50WX2 columns as described previously (Djordjevic et al, 1986).

From the culture supernatants of the R.leguminosarum strains oligosaccharides, that appeared identical in structure to the repeating units obtained by phage assisted depolymerization of the EPS, could also be isolated according to the method described by Djordjevic et al (1986). This indicates that also the R.leguminosarum bacteria themselves can also synthesize a depolymerase (endo-lyase). The quantities of repeating unit present in the culture supernatants were however so low, about 2 mg per L, that it was more efficient to use phage depolymerized EPS.

Partial hydrolysis of the EPS

A solution of EPS (50 mg/mL) from strain RBL5515 in M trifluoroacetic acid was heated for 1 hour at 100°C for 1 hour. The TFA was removed by freeze drying and the oligosaccharides isolated and purified by sequential ion-exchange and gel-filtration chromatography (Djordjevic et al., 1986). The size of the fragments was estimated by their elution pattern from a Biogel P2 column in comparison to that of model mono-, di-, tri- and tetrasaccharides as described by Djordjevic et al (1986).

Bioassay

The supernatants of cultures of strain RBL5561 pMP604 and other cultures induced with naringenin (Zaat et al, 1987) were checked for biological activity by monitoring the response of plants to the culture supernatants. Therefore the supernatant of relevant cultures was first sterilized for 10 min at 120°C before being placed in tubes with V.sativa plants supported by a metal rack as described by Van Brussel et al (1986). After 4 days, the plants were assayed for root hair deformation and curling with a microscope. This bioassay was performed on the same supernatant from which the EPS was isolated.

NMR analysis

Oligosaccharide samples were dissolved in 99% D_2O and spectra (200mHz for ^1H, 50.3 mHz for ^{13}C) were recorded on a Varian XL-200 spectrometer. The temperature was 20°C, unless otherwise indicated. In the ^1H spectra, the chemical shifts were measured relative to internal trimethylpropanesulphonate at 0 ppm. In the ^{13}C NMR spectra methanol at 50.01 ppm was used as internal standard. The resonance at 72.6 ppm assigned to C2 of residue b in presence of 3-O-acetate at residue b (Table 3A) was only found in EPS of mutant strains that failed to incorporate galactose in their EPS (Canter Cremers et al., 1990). ^1H-^{13}C heteronuclear chemical shift correlation spectra were obtained using the pulse sequence described by Bax and Morris (1981), which permits quadrature detection in both domains. Spectral widths of 3 kHz (2048 points) in the carbon domain and 600 Hz (256 points) in the proton domain were employed. Spectra were displayed n absolute mode and Lorentzian to Gaussian lineshape conversion was performed in each domain.

Isolation of EPS of Rhizobium grown in the presence of V.sativa plants.

For plant assays, V.sativa seeds were surface sterilized by subsequent washing in concentrated H_2SO_4 and hypochlorite as described by Van Brussel et al (1982). After germination at 4°C (Van Brussel et al, 1982), aliquots of about 100 V.sativa seedlings with an average root length of 1 cm were placed on a metal rack in beakers containing 1 L of J⁺⁺ medium each as described previously (Zaat et al, 1987). The beakers were then placed on a magnetic stirrer in our plant growth cabinet, the conditions of which were described previously (Canter Cremers et al, 1989). If required, the medium was inoculated with appropriate Rhizobium bacteria suspensions to an A_{620} 0.05. The

beakers were incubated in the climate chamber for an additional fourteen days, after which the growth medium was harvested and spun for 15 min at 10,000 rpm. The EPS was isolated from the supernatant as described above. In the growth medium of sterile grown V.sativa plants, polysaccharides with an apparent molecular weight of 100,000 Da and greater were not detectable. From the growth medium of V.sativa plants inoculated with a particular Rhizobium strain, about 0.15 g of polysaccharides with an apparent molecular weight of over 100,000 Da could be isolated.

Results

The ^{13}C and ^{1}H NMR spectra of depolymerized and de-esterified EPS of the Sym plasmid free R.leguminosarum strain RBL5515 (Figure 2A) were identical to that of R.leguminosarum strain ANU845, which indicates an identical structure (Figure 1A). The ^{13}C spectrum was assigned with help of oligosaccharide fragments (Table 2), generated by graded acid hydrolysis of the EPS of strain ANU845. The assignments are summarized in Table 3. The sites of esterification with O-acetyl and 3-hydroxybutanoyl groups were determined by 1) comparing the ^{13}C spectra of EPS of strains ANU845 and RBL5515 before and after esterification (Figure 2 and 4); 2) interpretation and comparison of ^{1}H NMR spectra of strains ANU845 and RBL5515 at different temperatures (Figures 3 and 5); 3) interpretation of a ^{1}H-^{13}C correlation spectrum of the depolymerized EPS of strain RBL5515 (Figure 6). The determination of the general structure (Figure 1B) and the sites of esterification in the EPS of another R.leguminosarum strain, namely strain RBL1387, will be reported elsewhere (Canter Cremers et al., 1991). For comparison, the ^{13}C spectrum of its depolymerized EPS is given in Figure 2D.

The sites of esterification with O-acetyl groups in the EPS are: for strain RBL5515 residues b and d (Figure 1A), for strain ANU845 residues b and a and for strain RBL1387 residues b (Figure 1B) and presumably residue f. The EPS of all three strains contains 3-hydroxybutanoyl groups esterified to the terminal galactose residue (Figure 1A,B), whereas strain RBL1387 also contains 3-hydroxybutanoyl groups esterified to residue h.

The site or the amount of esterification with O-acetyl and 3-hydroxybutanoyl groups in the EPS of these strains does not change 1) upon introduction of a Sym plasmid, 2) upon introduction of plasmid pRT290, 3) by incubation of the bacteria harbouring a Sym plasmid in root exudate or in medium containing naringenin or 4) during constitutive expression of the nod genes.

Table 1
Bacterial strains and plasmids

Strain	Relevant characteristics	Source
248	Wild type R.leguminosarum bv viciae isolate	Josey et al, 1979
RBL1387	R.leguminosarum bv viciae strain 248 cured for its Sym plasmid	Priem and Wijffelman, 1984
LPR5	Wild type R. leguminosarum bv. trifolii isolate	McNeil et al., 1986
RBL5515	Strain LPR5 cured of its Sym plasmid, str rif	Priem and Wijffelman, 1984
LPR5045	Strain LPR5 cured of its Sym plasmid, rif	Hooykaas et al, 1981
RBL5561	LPR5045 harbouring R. leguminosarum bv viciae Sym plasmid pRL1JI,nodD2::Tn5, rif	Wijffelman et al., 1985
ANU845	R. leguminosarum bv trifolii strain ANU843 cured of its Sym plasmid, spc	Rolfe et al., 1982
ANU843	R.leguminosarum bv trifolii wild type strain	Rolfe et al., 1982
Plasmids		
pRL1JI	Sym plasmid of R.leguminosarum bv viciae wild type strain 248	Johnston et al, 1978
pSym5	Sym plasmid of R. leguminosarum bv trifolii strain LPR5	Hooykaas et al., 1981
pSym9	Sym plasmid of R. leguminosarum bv phaseoli strain LPR9	Johnston et al, 1978
pHim	Sym plasmid isolated from of R.leguminosarum bv viciae strain Himalaya	Spaink et al., 1989
pRtr843	R.leguminosarum bv trifolii Sym plasmid present in strain ANU843	Rolfe et al., 1982
pMP604	IncP plasmid pMP92 harbouring a hybrid R.leguminosarum bv viciae-R.leguminosarum bv trifolii nodD gene, Tc^r	Spaink et al., 1989
pRt290	DNA fragment harboring R.leguminosarum bv trifolii nod genes nodF,E,L,M,N in Inc Q plasmid	Djordjevic et al., 1986

str streptomycin; rif rifampicin; Tc^r tetracycline

Table 2
Fragments isolated by partial acid hydrolysis of EPS from R.leguminosarum biovar strain ANU845.

I ß-D-Glcp-(1→4)-ß-D-Glcp-(1→4)-D-Glcp
II ß-D-GlcpA-(1→4)-ß-D-Glcp-(1→4)-D-Glcp
III α-D-Glcp-(1→4)-ß-D-GlcpA-(1→4)-ß-D-GlcpA-(1→4)-D-Glcp
IV ß-D-GlcpA-(1→4)-ß-D-GlcpA-(1→4)-ß-D-Glcp-(1→4)-D-Glcp
V ß-D-GlcpA-(1→4)-ß-D-GlcpA-(1→4)-ß-D-Glcp-(1→4)-D-Glcp
ß-D-Glcp-(1→4)-ß-D-Glcp-(1→6)

Table 3
Chemical shifts in ppm for ^{13}C resonances in the nmr spectrum of the de-esterified octasaccharide repeat unit from EPS produced by R.leguminosarum biovar strain RBL5515.

carbon	sugar residue a	b	c	d(α)	d(β)	e	f	g	h
1	100.8	103.3	103.3	93.1	97.1	103.3	103.3	104.0	103.3
2	70.4	75.0	74.0	72.3	74.6	74.0	74.2	74.0	72.7
3	66.6	75.3	75.3	72.3	75.1	75.0	75.3	80.4	66.8
4	108.0	80.9	79.6	79.1	79.1	79.6	79.3	75.9	72.1
5	145.7	76.5	75.9	70.1	-	75.9	76.0	67.5	71.5
6	170.5	176.2	61.0	68.7	68.7	61.0	61.0	65.5	66.0
pyr CH_3								25.6	26.3
pyr C								102.8	102.0
pyr CO								176.5	177.4

Table 4

Effects of esterification in the ^{13}C spectrum of octasaccharides from EPS synthesised by R.leguminosarum biovar strains RBL5515 and ANU845. Chemical shifts in ppm are recorded for ^{13}C peaks that disappear when the material is de-esterified, together with suggested assignments. The substitution shifts are changes in the ^{13}C resonance position relative to the de-esterified molecule. The ^{1}H chemical shifts for the larger peaks were obtained from the heteronuclear correlation spectrum.

A) shifts in ppm caused by 3-O-acetate on residue b present in the repeating unit of EPS of R.leguminosarum strains RBL5515 or ANU845

sugar residue	position	^{13}C chemical shift	^{13}C substitution shift	1H chemical shift
a	2	70.6	+0.2	3.89
a	3	67.3*	+0.1	4.19
b	1	104.7	+1.4	4.51
b	2	72.6	-2.4	3.54
b	3	76.5	+1.2	5.12
b	4	81.1	+0.2	3.85
b	5	78.1	+1.6	3.89
b	6	175.9	-0.3	
c	4	77.6	-2.0	3.81

B) shifts in ppm caused by 2-O-acetate on residue b present in the repeating unit of EPS of R.leguminosarum biovar strains RBL5515 or ANU845

sugar residue	position	^{13}C chemical shift	^{13}C substitution shift	^{1}H chemical shift
b	1	100.8*	-2.5	4.87
b	2	73.6	-1.4	
b	3	73.3	-2.0	
c	4	79.1	-0.5	3.75

C) shifts in ppm caused by 3-O-acetate and 2-O-acetate on residue a present in the repeating unit of EPS of R.leguminosarum biovar strains ANU845

sugar residue	position	3-O-acetate ^{13}C chemical shift	3-O-acetate ^{13}C substit. shift	2-O-acetate ^{13}C chemical shift	2-O-acetate ^{13}C substit. shift
a	1	99.0	-1.8	98.4	-2.4
a	2	70.9	+0.5	72.0	+1.6
a	4	108.2	+0.1	107.9	-0.1
a	5	145.9	+0.2		
a	6	170.4	-0.1		

D) shifts in ppm caused by 3-O-acetate and 2-O-acetate on residue d present in the repeating unit of EPS of R.leguminosarum biovar strain RBL5515

sugar residue	position	3-O-acetate ^{13}C chemical shift	3-O-acetate ^{13}C substit. shift	2-O-acetate ^{13}C chemical shift	2-O-acetate ^{13}C substit. shift
d(α)	1	93.0	-0.1	90.6	-2.5
d(β)	1	96.9	-0.2	95.3	-1.8

E) shifts in ppm caused by 3-O-hydroxybutanoyl and 2-O-hydroxybutanoyl on residue h present in the repeating unit of EPS of R.leguminosarum biovar strains RBL5515 and ANU845.

sugar residue	position	3-O-hydroxybut. ^{13}C chemical shift	3-O-hydroxybut. ^{13}C substit. shift	2-O-hydroxybut. ^{13}C chemical shift	2-O-hydroxybut. ^{13}C substit. shift
g	3	80.5	+0.1	81.6	+1.2
h	1	102.4	-0.9		
h	3	69.0	+2.2	67.2	+0.4
h	5	70.9	-0.6	72.0	+0.5
h	pyr C	101.9	-0.1		
h	pyr CH_3	26.2	-0.2		

* The shift is observed at 50°C but not at 20°C.

* Obscured by the a1 peak, this resonance is seen in the heteronuclear correlation spectrum.

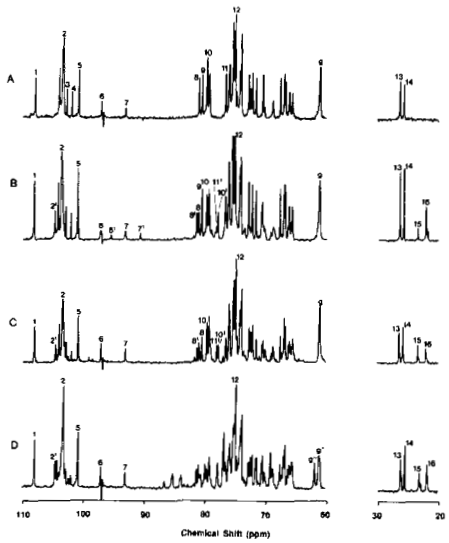

Figure 2

^{13}C nmr spectra of repeating units isolated from the depolymerized EPS of R.leguminosarum biovar strains.

A) R.leguminosarum strain RBL5515 after de-esterification

B) R.leguminosarum strain RBL5515

C) R.leguminosarum strain ANU845

D) R.leguminosarum strain RBL1387.

1: C4 of 4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid (residue a); 2: C1 of glucuronic acid residue b; 2' C1 of 2-O-acetyl glucuronic acid residue b; 3: C1 of the carboxyethylidene residue of 4,6-O-(1-carboxyethylidene)glucose (residue g); 4: C1 of the carboxyethylidene residue of 4,6-O-(1-carboxyethylidene)galactose (residue h); 5: C1 of 4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid residue a; 6: C1 of β-glucose residue d and, in spectrum B, C1 of 3-O-acetyl- β-glucose residue d; 6', C1 of 2-O-acetyl β-glucose residue d; 7: C1 of α-glucose residue d and, in spectrum B, C1 of 3-O-acetyl-α-glucose residue d; 7', C1 of 2-O-acetyl α-glucose residue a; 8: C4 of glucuronic acid residue b; 8' C4 of 3-O-acetyl glucuronic acid residue b; 9: C3 of 4,6-O-(1-carboxyethylidene)glucose residue g; 10: C4 of glucose residue c; 10', C4 of glucose residue c adjacent to 3-O-acetyl glucuronic acid residue b; 11: C5 of glucuronic acid residue b; 11', C5 of 3-O-acetyl glucuronic acid residue b; 12: C3 of glucuronic acid residue b; 12', C3 of 3-O-acetyl glucuronic acid residue b, is situated at the same place as resonance 11 in panel A; 13: Methyl of 4,6-O-(1-carboxyethylidene)galactose residue h; 14: Methyl of 4,6-O-(1-carboxyethylidene)glucose residue g; 15: Hydroxybutanoyl methyl; 16: Acetate methyls; g: (Spectrum A,B,C) C6 of glucose residues c,e and f; Spectrum D: g', C6 of glucose residues c and e; g", C6 of glucose residue g.

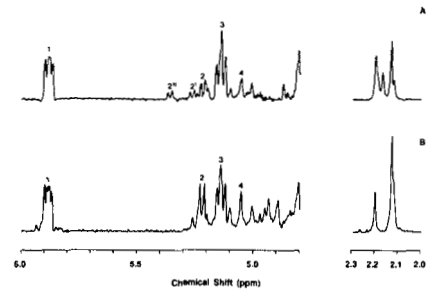

Figure 3

^{1}H spectrum at 30°C of the repeating unit of EPS isolated from R.leguminosarum biovar strains A) RBL5515 and B) ANU845.

1: resonance of H4 of 4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid.

2: resonance of H1 of α-glucose residue d; 2',2" resonances of H1 of 2-O-acetyl α-glucose and 3-O-acetyl α-glucose (residue d).

3: resonance of H1 of 4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid.

4: resonance of H3 of 3-O-acetyl glucuronic acid residue b.

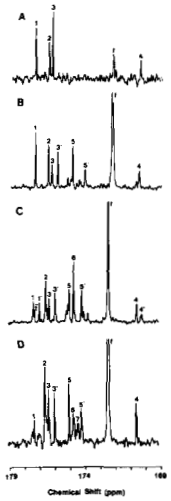

Figure 4

Carbonyl carbon resonances in the ^{13}C nmr spectrum of the repeating unit of EPS from R.leguminosarum biovar strains.

A) R.leguminosarum strain RBL5515 after deesterification

B) R.leguminosarum strain RBL5515

C) R.leguminosarum strain ANU845

D) R.leguminosarum strain RBL1387

The proposed assignments are:

1: carboxylate of 4,6(1-carboxyethylidene)galactose; 1',1" : carboxylates of 2- and 3-O-hydroxybutanoyl galactose; 2: carboxylate of 4,6(1-carboxyethylidene)glucose; 3: C6 of glucuronic acid residue b; 3' C6 of 3-O-acetyl glucuronic acid residue b; 4: C6 of 4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid; 4' C6 of 3-O-acetyl-4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid; 5: acetyl of 3-O-acetyl glucuronic acid; 5' acetyl of 2-O-acetyl glucuronic acid; 6: hydroxybutanoyl of 3-O-hydroxybutanoylgalactose; 7: hydroxybutanoyl of 3-O-hydroxybutanoylglucose; f: formate.

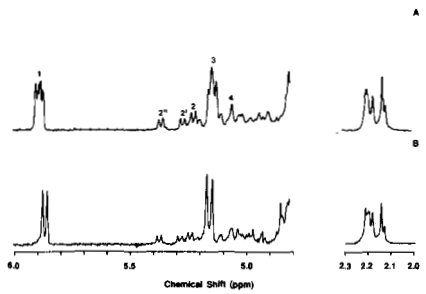

Figure 5

Effect of temperature on the ^{1}H spectrum of the repeating unit of R.leguminosarum biovar strain RBL5515 at 30°C (A) and 70°C (B). The samples contains approximately equal amounts of substituted and unsubstituted b residues. The indicated resonances are explained in the legend to Figure 3.

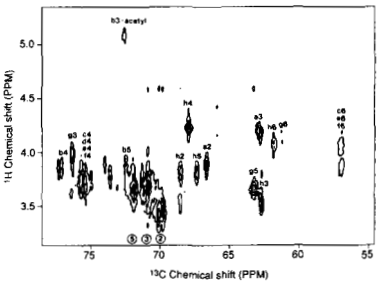

Figure 6

Part of the ^{1}H-^{13}C correlation spectrum at 50°C obtained from the repeating unit from EPS of strain RBL5515. Resonance assignments are designated using the letter code given in Figure 1, followed by the position of the nuclei in the pyranose ring. The circled numbers indicate the general region in which resonances for the carbons in glucose residues c,d,e and f are expected. The resonance labelled b3-acetyl is that for the C3 and H3 nuclei in 3-O-acetyl glucuronic acid (residue b).