Evaluation of Acidic Heteropolysaccharide Structures in *Rhizobium leguminosarum* Biovars Altered in Nodulation Genes and Host Range

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$^1$H-NMR spectroscopy showed that the extracellular heteropolysaccharides (EPS) from derivatives of *Rhizobium leguminosarum* bv. *trifolii* ANU843 altered in pSym nod composition or function (transposon insertions, deletion of pSym, induction by flavone, and introduction of cloned pSym nod regions from ANU843 and *R. l. bv. viciae* 248 on recombinant plasmids into the pSym-cured background of ANU843) differed only in 3-hydroxybutyrate stoichiometry per octaglycosyl unit. This change in EPS was likely to be an indirect effect of altered growth during expression of pSym nod genes in the presence of the flavone. No modifications were found in EPS made by *R. l. bv. phaseoli* 8002 when its resident pSym was deleted or replaced with pSym from *R. l. bv. viciae* 248, or with a derivative of this pSym lacking the host-specific nodulation genes nodFELMNTO. Thus, although certain O-acyl noncarbohydrate substitutions in EPS are affected by pSym nod genes (including the ones that determine host range) in certain backgrounds of *R. leguminosarum*, this change does not occur universally among all strains of *R. leguminosarum*. We conclude that the structure of the acidic EPS does not control host-specific nodulation of white clover, hairy vetch, and beans for the strains of *R. leguminosarum* tested here.

Additional keywords: exopolysaccharide, proton nuclear magnetic resonance, *Rhizobium trifolii*, symbiosis.

Genetic analyses have provided evidence that acidic extracellular heteropolysaccharides (EPS) of most *Rhizobium* species are required for development of the nitrogen-fixing, root-nodule symbiosis with legumes (Chakrovarty et al. 1982; Leigh et al. 1985; Puvanesarajah et al. 1987; Puhler et al. 1988; Diebold and Noel 1989; Gray et al. 1991; Hotter and Scott 1991). Several studies have shown that exogenously added EPS can impact on successful infection of host roots by *R. leguminosarum* bv. *trifolii* (Abe et al. 1984; Skorupska et al. 1985; Djordjevic et al. 1987), *R. melliloti* (Olivares et al. 1984; Puhler et al. 1988), and *R. parasponia* (Djordjevic et al. 1987). Several lines of evidence suggest that the structural features of certain rhizobial EPS may influence the leguminous host range. First, EPS structures differ among several wild-type strains of *R. l. bv. viciae* and *R. l. bv. trifolii* (Kuo and Mort 1986; Philip-Hollingsworth et al. 1989a). Second, a hybrid derivative of wild-type *R. l. bv. viciae* 300, containing cloned host-specific nodulation genes from *R. l. bv. trifolii* ANU843 which broadened its host range (Djordjevic et al. 1986), produced an EPS altered in site and stoichiometry of O-acetyl substituents and stoichiometry of 3-hydroxybutyrate (Philip-Hollingsworth et al. 1989b). Third, a third in effective host range is found in certain rhizobial strains having mutations causing production of a completely different EPS (Chen et al. 1985; Glazebrook and Walker 1989).

Other studies have reported that wild-type rhizobia of different *R. leguminosarum* biovars made similar EPS structures despite different host ranges (Canter-Cremers et al. 1991; O'Neill et al. 1991). In addition, deletion of the symbiotic plasmid (pSym) from certain strains did not change the glycosidic sequences (Carlson et al. 1986) or linkage of noncarbohydrate substitutions in its acidic EPS (Philip-Hollingsworth et al. 1989b; Canter-Cremers et al. 1991; O'Neill et al. 1991). These results have raised questions about the relationship between EPS structure and host range in *R. leguminosarum* biovars.

Because the chromosomal background strongly influences nod expression in certain strains of *R. leguminosarum* (Schlaman et al. 1990), the possibility remains that changes in EPS structure due to nod action may only occur in certain genetic backgrounds of *R. leguminosarum*. To test this hypothesis, several *R. leguminosarum* strains containing various alterations in pSym nod genes that affect host range (Table 1) were grown in defined BIII medium (Dazzo 1982) containing the appropriate antibiotic to retain recombinant plasmids (as was necessary to obtain reproducible results with recombinant strains of *R. l. bv. viciae* 300; Philip-Hollingsworth et al. 1989b), with or without flavone supplement to modulate pSym nod expression (Redmond et al. 1986; Firmin et al. 1986). Because culture age and composition of the medium influence noncarbohydrate substitution in EPS of *R. trifolii* (Abe et al. 1984; Canter-Cremers et al. 1991), careful attention was given to maintain standardized growth conditions in this study, including the preparation of inoculum (Hollingsworth et al. 1984), incubation period, and phase of growth. Measurements of β-galactosidase activity in derivatives of *R. l. bv. trifolii* ANU843 containing lacZ fusions in nodA and nodM showed that expression of pSym nod genes was enhanced.
at least 10-fold by 2-4 μM 4',7-dihydroxyflavone (DHF) under the growth conditions used in this study. The EPS was isolated and depolymerized by a phage-borne glucuronic acid-specific lyase (Hollingsworth et al. 1984). 1H-NMR spectroscopy of the purified oligosaccharidic repeat units was performed at 500 MHz (Hollingsworth et al. 1984; Philip-Hollingsworth et al. 1989a,b). The glycosidic structures of the different EPS oligomers from flavone-grown cultures were also compared by 1H-NMR following base-catalyzed deacylation (Hollingsworth et al. 1988).

In the first study, we performed 1H-NMR spectroscopy of enzymatically produced octaglycosyl repeat units to compare the structure of acidic EPS made by R. l. bv. trifolii ANU843 (see spectra in Hollingsworth et al. 1988) and mutant derivatives when grown for 5 days on BIII plates under conditions that would modulate pSym nod expression. Evaluation of the 1H-NMR spectra of the oligomers indicated that exclusion of this nod-activating flavone during growth, deletion of the resident pSym, or transposon-insertion mutations of selected pSym nod genes (to block some nod gene functions while allowing expression of others) did not result in any detectable change in glycosidic sequence or site of linkage of the different types of noncarbohydrate substitutions (pyruvate, acetate, and 3-hydroxybutyrate) of the EPS. However, quantitation of the proton resonances in the noncarbohydrate regions revealed lower levels of 3-hydroxybutyrate (percentage of reduction in parenthesis) in the EPS oligomers of the parent ANU843 when grown with DHF than 1) without DHF (46%), 2) the pSym-cured ANU845 strain grown with or without DHF (42–47%), and 3) the nod::Tn5 mutants grown with DHF (40–54%, depending on the site of transposon insertion) (Table 2). The calculated standard error of the means, as a measure of variability in stoichiometry within EPS samples from different batch cultures of the same test strain grown identically, ranged from 0.01 to 0.05 moles of 3-hydroxybutyrate per mole of octaglycosyl unit. In addition, the level of 3-hydroxybutyrate substitution in EPS oligomers was lower in the transconjugant strain of ANU845 containing pRt032 (includes the cloned 14-kb HindIII DNA fragment encoding the pSym nod region of R. l. bv. trifolii ANU843) when grown with DHF to induce nod expression rather than without this flavone (Table 2). These results indicate that, under these growth conditions, strains expressing the combined functions of each of the genes (nodABCII, nodD, nodF(R)L, and nodMN) within the 14-kb pSym nod region (as would occur in wild-type ANU843 or recombinant ANU845 pRt032 when grown with DHF) produce an acidic EPS in which approximately one fourth of the repeat units bear a 3-hydroxybutyrate substituent. In contrast, approximately one half of the EPS repeat units bear this substitution in strains or under conditions in which the function(s) of any or all nod genes are altered.

Table 1. Strains of Rhizobium leguminosarum biovarios and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant characteristics</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANU843</td>
<td>Wild-type bv. trifolii</td>
<td>Rolfe et al. 1982</td>
</tr>
<tr>
<td>ANU845</td>
<td>pSym-cured derivative of ANU843</td>
<td>Rolfe et al. 1982</td>
</tr>
<tr>
<td>ANU851</td>
<td>nodD::Tn5 in ANU843 (Kan')</td>
<td>Schofield et al. 1983</td>
</tr>
<tr>
<td>ANU252</td>
<td>nodA::Tn5 in ANU843 (Kan')</td>
<td>Djordjevic et al. 1985</td>
</tr>
<tr>
<td>ANU261</td>
<td>nodD::Tn5 in ANU843 (Kan')</td>
<td>Huang et al. 1988</td>
</tr>
<tr>
<td>ANU262</td>
<td>nodA::Tn5 in ANU843 (Kan')</td>
<td>Huang et al. 1988</td>
</tr>
<tr>
<td>ANU258</td>
<td>nodE::Tn5 in ANU843 (Kan')</td>
<td>Djordjevic et al. 1985</td>
</tr>
<tr>
<td>ANU896</td>
<td>nodM::mudlacZ in ANU843 (Kan')</td>
<td>Weinman et al. 1988</td>
</tr>
<tr>
<td>ANU895</td>
<td>nodN::mudlacZ in ANU845 (Kan')</td>
<td>Weinman et al. 1988</td>
</tr>
<tr>
<td>ANU845pRt032</td>
<td>pRt032 plasmid in ANU845 (Cb')</td>
<td>Schofield et al. 1984</td>
</tr>
<tr>
<td>ANU845pMP225</td>
<td>pMP225 plasmid in ANU845 (Tc')</td>
<td>Spink et al. 1987</td>
</tr>
<tr>
<td>248</td>
<td>Wild-type bv. viciae</td>
<td>Josey et al. 1979</td>
</tr>
<tr>
<td>8002</td>
<td>Wild-type bv. phaseolli</td>
<td>Lamb et al. 1982</td>
</tr>
<tr>
<td>8401</td>
<td>pSym-cured derivative of 8002</td>
<td>Downie et al. 1983</td>
</tr>
<tr>
<td>8401 pRL1J1</td>
<td>pRL1J1 plasmid in 8401</td>
<td>Downie et al. 1983</td>
</tr>
<tr>
<td>A69</td>
<td>derivative of 8401 pRL1J1 deleted in nodFELMNTO</td>
<td>Downie and Surin 1990</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
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<tr>
<td>pRt032</td>
<td>14-kb HindIII pSym nod region of ANU843 containing nodABCII, nodD, nodF(R)L, and nodMN and cloned into pKT240</td>
<td>Schofield et al. 1984</td>
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<tr>
<td>pMP225</td>
<td>pSym nod region of bv. viciae 248 containing nodABCII, nodD, nodFEL, and nodMN cloned into IncP</td>
<td>Spink et al. 1987</td>
</tr>
<tr>
<td>pRL1J1</td>
<td>pSym in bv. viciae 248</td>
<td>Johnston et al. 1978</td>
</tr>
<tr>
<td>pRL1J1A69</td>
<td>derivative of pRL1J1 deleted in nodFELMNTO</td>
<td>Downie and Surin 1990</td>
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</tbody>
</table>

4 Antibiotic concentrations were: kanamycin (Kan) 30 μg/ml, carbenicillin (Cb) 75 μg/ml, tetracycline (Tc) 2 μg/ml.

Table 2. Flavone supplement in the culture medium and molar stoichiometry of pyruvate, acetate, and 3-hydroxybutyrate in the EPS-derived oligosaccharide repeat units from wild-type Rhizobium leguminosarum bv. trifolii ANU843, R. l. bv. viciae 248, R. l. bv. phaseolli 8002, and pSym-nod-modified derivatives

<table>
<thead>
<tr>
<th>Strain</th>
<th>Flavone (μM)</th>
<th>Pyruvate</th>
<th>Acetate</th>
<th>3-Hydroxybutyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>bv. trifolii ANU843 wt</td>
<td>0</td>
<td>1.78</td>
<td>0.95</td>
<td>0.48</td>
</tr>
<tr>
<td>ANU843 wt</td>
<td>4 DHF</td>
<td>1.74</td>
<td>0.91</td>
<td>0.26</td>
</tr>
<tr>
<td>ANU845 (pSym-)</td>
<td>0</td>
<td>1.59</td>
<td>0.86</td>
<td>0.45</td>
</tr>
<tr>
<td>ANU845 (pSym+)</td>
<td>4 DHF</td>
<td>1.65</td>
<td>0.88</td>
<td>0.49</td>
</tr>
<tr>
<td>ANU851 (nodD+)</td>
<td>4 DHF</td>
<td>1.66</td>
<td>0.92</td>
<td>0.48</td>
</tr>
<tr>
<td>ANU252 (nodA-)</td>
<td>4 DHF</td>
<td>1.85</td>
<td>0.88</td>
<td>0.43</td>
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<tr>
<td>ANU261 (nodD)</td>
<td>4 DHF</td>
<td>1.73</td>
<td>0.95</td>
<td>0.50</td>
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<tr>
<td>ANU262 (nodD+)</td>
<td>4 DHF</td>
<td>1.67</td>
<td>0.94</td>
<td>0.56</td>
</tr>
<tr>
<td>ANU258 (nodE+)</td>
<td>4 DHF</td>
<td>1.92</td>
<td>0.89</td>
<td>0.47</td>
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<tr>
<td>ANU896 (nodM-)</td>
<td>4 DHF</td>
<td>1.97</td>
<td>1.03</td>
<td>0.49</td>
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<tr>
<td>ANU895 (nodN-)</td>
<td>4 DHF</td>
<td>1.95</td>
<td>0.99</td>
<td>0.45</td>
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<tr>
<td>ANU845 pRt032</td>
<td>0</td>
<td>1.88</td>
<td>1.00</td>
<td>0.44</td>
</tr>
<tr>
<td>ANU845 pRt032</td>
<td>2 DHF</td>
<td>1.93</td>
<td>1.07</td>
<td>0.28</td>
</tr>
<tr>
<td>ANU845 pMP225</td>
<td>2 NAR</td>
<td>1.92</td>
<td>1.03</td>
<td>0.26</td>
</tr>
<tr>
<td>bv. viciae 248 wt</td>
<td>2 NAR</td>
<td>1.91</td>
<td>1.01</td>
<td>0.38</td>
</tr>
<tr>
<td>bv. phaseolli 8002 wt</td>
<td>2 NAR</td>
<td>1.73</td>
<td>1.60</td>
<td>0.35</td>
</tr>
<tr>
<td>8401 (pSym-)</td>
<td>2 NAR</td>
<td>1.67</td>
<td>1.52</td>
<td>0.37</td>
</tr>
<tr>
<td>8401 pRL1J1</td>
<td>2 NAR</td>
<td>1.70</td>
<td>1.57</td>
<td>0.45</td>
</tr>
<tr>
<td>A69 (AnodFELMNTO)</td>
<td>2 NAR</td>
<td>1.61</td>
<td>1.53</td>
<td>0.33</td>
</tr>
</tbody>
</table>

4 Values are the means of two to four independent EPS preparations. DHF: 4',7-dihydroxyflavone; NAR, naringenin.
The second focus of this work was to examine whether the influence of pSym nod genes on EPS structure, as previously found in recombinant hybrids of R. l. bv. viciae 300 (Philip-Hollingsworth et al. 1989b), occurs in strains of other R. leguminosarum biovars. We compared R. l. bv. trifolii ANU843, R. l. bv. viciae 248, and R. l. bv. phaseoli 8002, since these wild-type strains have different host ranges and derivatives containing heterologous hsn genes have been constructed and were available. Also, since the glycosidic and noncarbohydrate substitutions in the EPS made by these wild-type strains produce proton resonances that are clearly distinguishable by $^1$H-NMR spectroscopy, a change in their acidic EPS structure would be detected by the analytical approach used. All strains in this evaluation were grown as shaken broth cultures in BIII medium with the appropriate flavone to induce nod genes and antibiotic to retain the recombinant plasmid, and the cultures were harvested at the same population density ($9 \times 10^8$ cells per milliliter).

The EPS structures from two recombinant strains of the same pSym-cured background (ANU845) containing the pSym nod region from R. l. bv. trifolii ANU843 or R. l. bv. viciae 248 cloned on plasmids pRT032 and pMP225, respectively, were similar as shown by the $^1$H-NMR spectra of their repeat unit oligosaccharides (Fig. 1). The distribution and relative intensity of the O-acetyl resonances were indistinguishable between the two oligosaccharides, matching the acetylation pattern of the EPS oligomer from the parent R. l. bv. trifolii strain ANU843 (Hollingsworth et al. 1988), and clearly different from those of the R. l. bv. viciae strain 248 (Fig. 1). The same trend was found for the glycosidic sequence of these EPSs based on an $^1$H-NMR study of their de-O-acetylated oligosaccharides (figures not shown). As with transconjugant ANU845 pRT032, the EPS of hybrid transconjugant ANU845 pMP225 had a lower stoichiometry of 3-hydroxybutyrate substitution than the pSym-cured recipient ANU845 (Table 2). In plant nodule tests, these two transconjugants displayed host-specific root nodulation on white clover and hairy vetch, respectively.

Our results, indicating a smaller amount of 3-hydroxybutyrate substitution in EPS of ANU843, ANU845 pRT032, and ANU845 pMP225 than in EPS of the mutant derivatives of ANU843 containing transposon insertions in selected nod genes or the pSym-cured derivative entirely lacking nod genes (ANU845), are detected by two, unambiguous evaluations of the $^1$H-NMR spectra: 1) quantitative comparison to an internal constant present within the octaglycosyl unit itself (the single H-4 proton of the unsaturated hex-4-enopyranosylyluronic acid residue produced by lyase treatment of the native EPS; Hollingsworth et al. 1984); and 2) evaluation of the accompanying changes in pyruvate resonances (8: 1.35–1.50 ppm) that are due to anisotropic effects of the methyl protons of the acetel group from the 3-hydroxybutyrate substituents in close proximity to pyruvate (Hollingsworth et al. 1988). These results are consistent with the change in 3-hydroxybutyrate substitution in EPS of wild-type R. l. bv. trifolii LPR5, its pSym-cured derivative RBL5515, and derivatives of RBL5515 containing pSym from R. l. bv. viciae 248 or R. l. bv. trifolii LPR5 when grown in $^{15}$N minimal medium (Canter-Cremers et al. 1991). This trend was not found when derivatives of LPR5 were grown in $^{15}$N medium (Canter-Cremers et al. 1991) nor with a similar set of strain constructions in another background (R. l. bv. viciae LPR5045) grown in Jensen's medium (O'Neill et al. 1991). However, the methods to measure the stoichiometry of 3-hydroxybutyrate substitution per repeat unit were not described in the report by Canter-Cremers et al. (1991) or based on comparison to an assumed value of 2.0 for pyruvate (O'Neill et al. 1991), which itself is not constant (Abe et al. 1984).

The noncarbohydrate substitutions of EPS oligomers were also compared between a wild-type R. l. bv. phaseoli strain 8002, its pSym-cured derivative strain 8401, a hybrid recombinant containing the Sym plasmid from R. l. bv. viciae strain 248 in the 8401 background (strain 8401 pRLJ1), and a derivative of the latter hybrid strain lacking the nodFELMNT0 hsn genes (strain A69). The portions of the $^1$H-NMR spectra representing the pattern of noncarbohydrate substitution in the EPS oligomers produced by these four strains are shown in Figure 2. These spectra displayed identical features of resonance distribution and relative integration of the pyruvate, acetate, and 3-hydroxybutyrate substitutions (Fig. 2, Table 2). In contrast to the difference in EPS between R. l. bv. trifolii ANU843 and

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**Fig. 1.** Five hundred megahertz $^1$H-NMR spectra of the EPS-derived oligosaccharides obtained from recombinant strains ANU845 pRT032 and ANU845 pMP225, and wild-type *Rhizobium leguminosarum* bv. *viciae* 248. Cultures were grown in the presence of 2 μM nod-activating flavone at 30° C. Inserts on the right of the spectra show an expanded display of the acetate resonances.
its pSym-cured derivative when grown in the presence of nod-inducer flavone, deletion of the Sym plasmid from the wild-type R. l. bv. phaseoli 8002 strain did not change the stoichiometry of 3-hydroxybutyrate substitution in its EPS (Table 2). In addition, the proton resonances of the carbohydrate moieties in both native and de-O-acylated oligosaccharides from acidic EPS of these four strains were indistinguishable (data not shown), indicating no modification of their glycosidic sequence. The \(^1\)H-NMR spectrum corresponding to the glycosidic and acetyl resonance patterns of the EPS oligomer from the wild-type R. l. bv. viciae strain 248 was clearly different from those of the wild-type R. l. bv. phaseoli strain 8002, pSym-cured 8401 strain, and recombinant 8401 pRL1J1 and A69 strains (Fig. 2). The host-specific nodulation phenotypes of these four strains yield expected results based on the requirements of pSym common and host-specific nodulation genes.

There are several important conclusions to draw from this study addressing EPS structure and pSym nod gene expression in R. leguminosarum. First, the reduction in level of 3-hydroxybutyrate in EPS of ANU843 is not linked to white clover host specificity and is likely to be an indirect effect of flavone-induced expression of pSym nod genes. One of several possible explanations is that certain nod-encoded functions may compete or interfere with components of the pathway that incorporates 3-hydroxybutyrate substitutions into acidic EPS. Second, this difference in EPS made by R. l. bv. trifolii ANU843 (and LPR5) does not occur in all R. leguminosarum backgrounds. Third, hybrid transconjugants of ANU845 and 8401 that have changed host range upon introduction of heterologous hsn genes nevertheless retain the acetylation pattern in EPS of the parent strain. These results differ from the reproducible changes in acetylation of EPS made by R. leguminosarum 300 pRt290 hybrid transconjugant grown in the absence and presence of nod-activating flavone (Philip-Hollingsworth et al. 1989b). Thus, although in some R. leguminosarum backgrounds there appear to be changes in EPS that may be influenced by nod gene expression, these structural features do not appear to be determinants that control host specificity in the Rhizobium-legume symbiosis.

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LITERATURE CITED


Fig. 2. Five hundred megahertz \(^1\)H-NMR resonance patterns of the noncarbohydrate substitutions of the EPS-derived oligosaccharides obtained from wild-type R. leguminosarum bv. phaseoli 8002, pSym-cured derivative 8401, and recombinant strains 8401 pRL1J1 and A69. Cultures were grown in the presence of 2 \(\mu\)M nod-activating flavone at 30\(^\circ\) C.


