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# 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 heterpolysaccharides (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. meliloti* (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 EPSs 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 change 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).

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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 $\beta$-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). $^1$H-NMR spectroscopy of the purified oligosaccharidic repeat units was performed at 500 MHz (Hollingsworth *et al.* 1984; Philip-Hollingsworth *et al.* 1989ab). The glycosidic structures of the different EPS oligomers from flavone-grown cultures were also compared by $^1$H-NMR following base-catalyzed deacylation (Hollingsworth *et al.* 1988).

In the first study, we performed $^1$H-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 $^1$H-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*::Tn*5* 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 *Hin*dIII 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 (*nodABCIJ, nodD, nodFE(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* biovars and plasmids used in this study

| | Relevant characteristics[a] | Source/Reference |
|---|---|---|
| Strains | | |
| ANU843 | Wild-type bv. *trifolii* | Rolfe *et al.* 1982 |
| ANU845 | pSym-cured derivative of ANU843 | Rolfe *et al.* 1982 |
| ANU851 | *nodD*::Tn*5* in ANU843 (Kan$^r$) | Schofield *et al.* 1983 |
| ANU252 | *nodA*::Tn*5* in ANU843 (Kan$^r$) | Djordjevic *et al.* 1985 |
| ANU261 | *nodI*::Tn*5* in ANU843 (Kan$^r$) | Huang *et al.* 1988 |
| ANU262 | *nodJ*::Tn*5* in ANU843 (Kan$^r$) | Huang *et al.* 1988 |
| ANU258 | *nodE*::Tn*5* in ANU843 (Kan$^r$) | Djordjevic *et al.* 1985 |
| ANU896 | *nodM*::mud*lacZ* in ANU843 (Kan$^r$) | Weinman *et al.* 1988 |
| ANU895 | *nodN*::mud*lacZ* in ANU843 (Kan$^r$) | Weinman *et al.* 1988 |
| ANU845pRt032 | pRt032 plasmid in ANU845 (Cb$^r$) | Schofield *et al.* 1984 |
| ANU845pMP225 | pMP225 plasmid in ANU845 (Tc$^r$) | Spaink *et al.* 1987 |
| 248 | Wild-type bv. *viciae* | Josey *et al.* 1979 |
| 8002 | Wild-type bv. *phaseoli* | Lamb *et al.* 1982 |
| 8401 | pSym-cured derivative of 8002 | Downie *et al.* 1983 |
| 8401 pRL1JI | pRL1JI plasmid in 8401 | Downie *et al.* 1983 |
| A69 | derivative of 8401 pRL1J1 deleted in *nodFELMNTO* | Downie and Surin 1990 |
| Plasmids | | |
| pRt032 | 14-kb *Hin*dIII pSym *nod* region of ANU843 containing *nodABCIJ*, *nodD*, *nodFE(R)L*, and *nodMN* and cloned into pKT240 | Schofield *et al.* 1984 |
| pMP225 | pSym *nod* region of bv. *viciae* 248 containing *nodABCIJ*, *nodD*, *nodFEL*, and *nodMNTO* cloned into IncP | Spaink *et al.* 1987 |
| pRL1J1 | pSym in bv. *viciae* 248 | Johnston *et al.* 1978 |
| pRL1J1A69 | derivative of pRL1JI deleted in *nodFELMNTO* | Downie and Surin 1990 |

[a] 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. *phaseoli* 8002, and pSym- *nod*-modified derivatives[a]

| | | Molar stoichiometry per octaglycosyl unit | | |
|---|---|---|---|---|
| Strain | Flavone (μM) | Pyruvate | Acetate | 3-Hydroxybutyrate |
| bv. *trifolii* ANU843 wt | 0 | 1.78 | 0.95 | 0.48 |
| ANU843 wt | 4 DHF | 1.74 | 0.91 | 0.26 |
| ANU845 (pSym-) | 0 | 1.59 | 0.86 | 0.45 |
| ANU845 (pSym-) | 4 DHF | 1.65 | 0.88 | 0.49 |
| ANU851 (*nodD*-) | 4 DHF | 1.66 | 0.92 | 0.48 |
| ANU252 (*nodA*-) | 4 DHF | 1.85 | 0.88 | 0.43 |
| ANU261 (*nodI*-) | 4 DHF | 1.73 | 0.95 | 0.50 |
| ANU262 (*nodJ*-) | 4 DHF | 1.67 | 0.94 | 0.56 |
| ANU258 (*nodE*-) | 4 DHF | 1.92 | 0.89 | 0.47 |
| ANU896 (*nodM*-) | 4 DHF | 1.97 | 1.03 | 0.49 |
| ANU895 (*nodN*-) | 4 DHF | 1.95 | 0.99 | 0.45 |
| ANU845 pRt032 | 0 | 1.88 | 1.00 | 0.44 |
| ANU845 pRt032 | 2 DHF | 1.93 | 1.07 | 0.28 |
| ANU845 pMP225 | 2 NAR | 1.92 | 1.03 | 0.26 |
| bv. *viciae* 248 wt | 2 NAR | 1.91 | 1.01 | 0.38 |
| bv. *phaseoli* 8002 wt | 2 NAR | 1.73 | 1.60 | 0.35 |
| 8401 (pSym-) | 2 NAR | 1.67 | 1.52 | 0.37 |
| 8401 pRL1JI | 2 NAR | 1.70 | 1.57 | 0.45 |
| A69 (Δ*nodFELMNTO*) | 2 NAR | 1.61 | 1.53 | 0.33 |

[a] 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*-acylated 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 nodulation tests, these two transconjugants displayed host-specific root nodulation on white clover and hairy vetch, respectively.

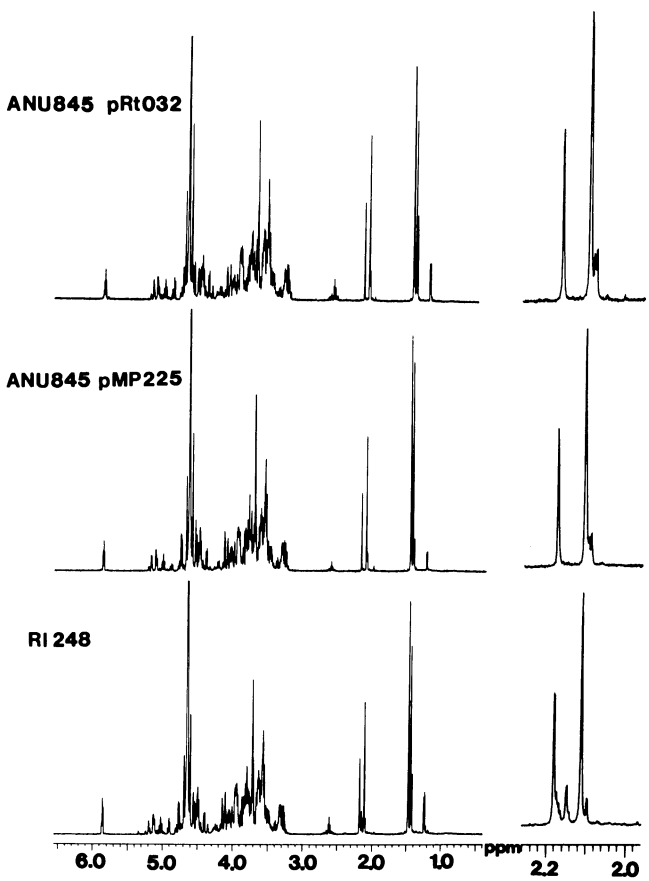


**Fig. 1.** Five hundred megahertz $^1$H-NMR spectra of the EPS-derived oligosaccharides obtained from recombinant strains ANU845 pRt032 and ANU845 pMP225, and wild-type *Rhizobium leguminosarum* bv. *viciae* 248. Cultures were grown in the presence of 2 $\mu$M *nod*-activating flavone at 30° C. Inserts on the right of the spectra show an expanded display of the acetate resonances.

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-eno-pyranosyluronic acid residue produced by lyase treatment of the native EPS; Hollingsworth *et al.* 1984); and 2) evaluation of the accompanying changes in pyruvate resonances ($\delta$: 1.35–1.50 ppm) that are due to anisotropic effects of the methyl protons of the acetal 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 $\beta^-$ minimal medium (Canter-Cremers *et al.* 1991). This trend was not found when derivatives of LPR5 were grown in $J^{++}$ medium (Canter-Cremers *et al.* 1991) nor with a similar set of strain constructions in another background (*R. l.* bv. *viciae* LPR5045) grown in Jensens 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 pRL1JI), and a derivative of the latter hybrid strain lacking the *nodFELMNTO 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

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 pRL1JI 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.

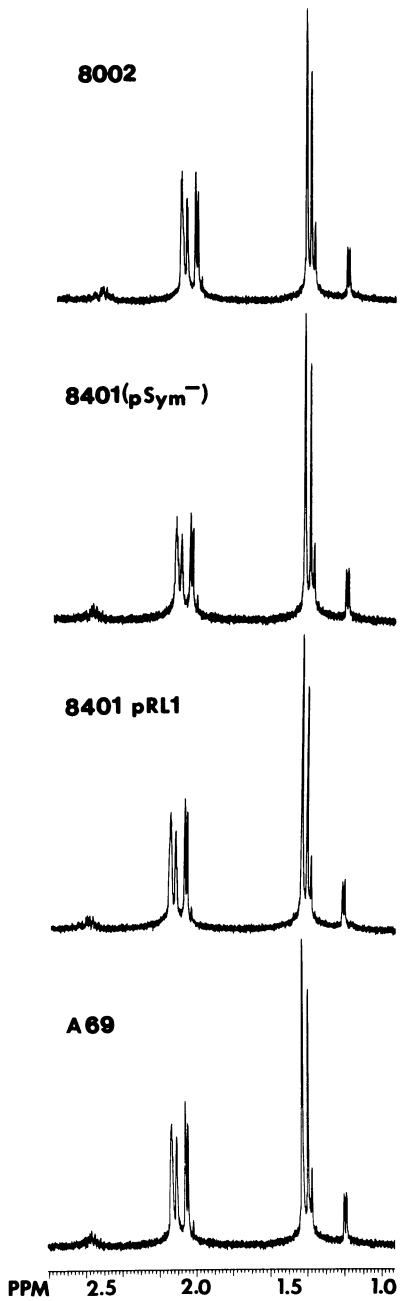


**Fig. 2.** Five hundred megahertz $^1$H-NMR resonance patterns of the noncarbohydrate substitutions of the EPS-derived oligosaccharides obtained from wild-type *R. leguminosarum* bv. *phaseoli* 8002, pSym-cured derivative 8401, and recombinant strains 8401 pRL1JI and A69. Cultures were grown in the presence of 2 $\mu$M *nod*-activating flavone at 30° C.

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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