Surface polysaccharide mutants of *Rhizobium* sp. (*Acacia*) strain GRH2: major requirement of lipopolysaccharide for successful invasion of *Acacia* nodules and host range determination

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Two transposon Tn5-induced mutants of wild-type broad-host-range *Rhizobium* sp. GRH2 were isolated and found to harbour different alterations in surface polysaccharides. These mutants, designated GRH2-14 and GRH2-50, induced a few, empty nodules on *Acacia* and lost the ability to nodulate most host herbaceous legumes. Whereas mutant GRH2-14 produces an acidic exopolysaccharide (EPS) similar to the wild-type, the acidic EPS of mutant GRH2-50 lacks galactose and the pyruvyl and 3-hydroxybutryl substituents attached to this sugar moiety. In addition, both mutants GRH2-50 and GRH2-14 were altered in smooth lipopolysaccharides (LPS). DNA sequence analyses of the corresponding Tn5 insertions revealed that strain GRH2-50 was mutated in a DNA locus homologous to *galE*, and in vitro enzyme assays indicated that the UDPglucose 4-epimerase (*GalE*) activity was missing in this mutant strain. DNA hybridization studies showed that the GRH2-50 mutant DNA has homologous sequences within the different biovars of *Rhizobium leguminosarum*. However, no DNA homology to GRH2-14 altered DNA was found in those rhizobial strains, indicating that it represents a new chromosomal *lps* locus in *Rhizobium* sp. (*Acacia*) involved in symbiotic development.

**Keywords**: *Rhizobium* sp. GRH2, legume tree, polysaccharide, Tn5

INTRODUCTION

*Acacia* is the largest and most diverse genus of legume trees, containing approximately 1500 species, and serves as a model legume for studies of symbiotic nitrogen fixation under conditions of desiccation stress. We are investigating the role(s) of *Rhizobium* surface glycoconjugates in the development of nitrogen-fixing root-nodule symbiosis with *Acacia*. For these studies, we are using wild-type *Rhizobium* sp. GRH2 which was originally isolated from root nodules of *Acacia cyanophylla* in Chile (Herrera et al., 1985). A unique aspect of this rhizobial strain is its wide host range, which includes legume trees (e.g. *Acacia, Prosopis*) and a diversity of herbaceous legumes such as *Trifolium, Lotus, Phaseolus, Vicia* and *Siratro* (Herrera et al., 1985; López-Lara et al., 1993). Although many reports suggest an involvement of *Rhizobium* acidic heteropolysaccharides (exopolysaccharides; EPS) and lipopolysaccharides (LPS) in symbiotic infection of herbaceous legume roots (Abe et al., 1984; Canter Cremers et al., 1990; Carlson, 1982; Dazzo et al., 1992; Herrera et al., 1985; Lamb et al., 1982; Maier & Brill, 1978; Müller et al., 1988; Noel et al., 1986; Reuber et al., 1991; Zhan et al., 1992), little is known about their importance in root infection of legume trees. We have recently shown that the excreted acidic heteropolysaccharide produced by wild-type GRH2 and *R. leguminosarum* bv. *trifolii* ANU843 are very similar in structure, and the analysis of an *Exo*−mutant derivative of GRH2 indicates that this surface glycoconjugate is important for invasion of host cells in *Acacia* nodules (López-Lara et al., 1993). In this study, we isolated and characterized two different surface polysaccharide mutants of GRH2 in order to examine the role of smooth LPS in symbiotic development. This study of new GRH2 mutants identifies: (i) a major requirement of smooth LPS for successful invasion of *Acacia* nodules and in determination of host range; (ii) a dual role of *exoB* encoding UDPglucose 4-epimerase involved in production of both smooth LPS

**Abbreviation**: EPS, exopolysaccharide.
and acidic EPS and in development of root nodule symbiosis; and (iii) a new chromosomal $lp$ locus in *Rhizobium* involved in symbiotic development.

**METHODS**

**Bacterial strains and genetic manipulations.** *Rhizobium* sp. GRH2 was originally isolated from root nodules of *A. cyanophylla* (Herrera et al., 1985). Other bacterial strains used in this work are listed in Table 1. Bacteria were routinely grown in minimal medium (MM; Robertsen et al., 1981), TY (Beringer, 1974) or TY supplemented with mannitol (YMT). Transposon Tn5: $mob$ random mutagenesis was done by mating *Escherichia coli* S17-1 (pSUP5011) with *Rhizobium* sp. GRH2. Kanamycin (Km)-resistant transconjugants were selected on MM agar (López-Lara et al., 1993). Antibiotics were used at the following concentrations: kanamycin sulfate (Km), 180 $\mu$g ml$^{-1}$; tetracycline (Tc), 10 $\mu$g ml$^{-1}$. Plasmid isolation, random primer DNA labelling, DNA hybridization, and Southern blotting were performed according to standard procedures (Maniatis et al., 1982).

**Cloning of the GRH2-14 and GRH2-50 Tn5 insertions.** The total DNA of mutants GRH2-14 and GRH2-50 was EcoRI digested and cloned in plasmid pSUP102. After ligation, DNA was transformed in *E. coli* DH5a (Bethesda Research Laboratories). Km-resistant colonies were selected and analysed for plasmid content. Plasmid clones with the Tn5 insertion were designated pIS14 and pIS50.

**DNA sequencing.** The left junctions of Tn5 insertions 14 and 50 were cloned as SalI fragments in pUC18 from plasmids pIS14 and pIS50, respectively. The sequence data were obtained by the chain-termination method (Sanger et al., 1977) using the ISS0L specific primer 5'-AAAGTTCCGTCGACGACG-3'.

**Sequence analysis.** Sequence analysis and homology search were done with the GCG software package (Devereux et al., 1984). The amino acid sequences deduced from the nucleotide sequences were compared with the actual version in the Swissprot and PIR Protein Data Banks using the program FASTA (Pearson & Lipman, 1988).

**LPS analyses by SDS-PAGE.** Cell cultures (1.5 ml) were pelleted, suspended in SDS sample buffer (Laemmli, 1970), denatured at 100 °C for 3 min, and treated with proteinase K. After discontinuous SDS-PAGE, gels were stained by periodic acid/silver for carbohydrates (Hitchcock & Brown, 1983).

**Isolation and fractionation of the EPS components.** The native EPS was isolated and fractionated as previously described (López-Lara et al., 1993). Alternatively, EPS was obtained from bacteria grown on solid medium. Cells were suspended in 10 mM sodium phosphate buffer (5–7 ml per plate), and pelleted by centrifugation. EPS was isolated by precipitation of the culture supernatant with 2 vols cold ethanol. The precipitated EPS was redissolved in water and then dialysed against water for 48 h at 4 °C.

**Compositional and spectroscopic analyses of the EPS.** Sugar composition, glycosidic linkage determination and $^1$H-NMR analyses of the native EPS were carried out as previously described (López-Lara et al., 1993).

**Nodulation assays.** Plants were grown on nitrogen-free medium (Rigaud & Puppo, 1975) under microbiologically controlled conditions and inoculated as described by Olivares et al. (1980). Nodulation of tree legumes and herbaceous legumes was followed for 6 months and 1 month, respectively.

**Table 1. Strains used or constructed in this work**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td><em>Rhizobium</em> sp.</td>
<td>Broad-host-range <em>Rhizobium</em> sp. isolated from <em>A. cyanophylla</em> root nodules</td>
<td>Herrera et al. (1985)</td>
</tr>
<tr>
<td>GRH2</td>
<td><em>R. meliloti</em> Wild-type, Sm$^R$</td>
<td>Cassé et al. (1979)</td>
</tr>
<tr>
<td>GRH2-14</td>
<td><em>VF39</em> Wild-type, bv. <em>trifolii</em></td>
<td>F. Rodríguez-Quitones, Facultad Farmacia, Universidad de Sevilla, Spain</td>
</tr>
<tr>
<td>GRH2-14</td>
<td><em>B. japonicum</em> Wild-type, Hup$^+$</td>
<td>Emerich et al. (1980)</td>
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</table>
Enzyme assay for UDPglucose 4-epimerase. Bacteria were grown on MM medium containing glucose or galactose as sole carbon source. Cells were disrupted by sonication and debris removed by centrifugation. Cell-free extracts were used to measure the UDPglucose 4-epimerase activity according to Fukasawa et al. (1980).

Plant microscopy. Root nodules were processed as previously described (Lopez-Lara et al., 1993). Two micrometre sections were stained with alkaline toluidine blue solution and examined by light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy using a Philips CM-10 electron microscope. Computer-assisted image analysis of nodule histology was performed using Bioquand System IV software as described previously (Dazzo & Petersen, 1989).

RESULTS

Isolation and characterization of Rhizobium sp. GRH2 surface polysaccharide mutants

Rhizobium sp. GRH2 was mutagenized with Tn5::mob, and Km' transconjugants were selected on MM agar with mannitol as carbon source. By screening 10000 random Tn5 insertion mutants, colonies with a non-mucoid, supermucoid or a rough morphology were selected visually (Lopez-Lara et al., 1993). Two of the mutant strains with a rough colony morphology were designated GRH2-14 and GRH2-50. A Southern blot of EcoRI-digested total DNA of GRH2-14 and GRH2-50 mutant strains, probed with Tn5, showed a single insertion located in 2.4 and 3.2 kb EcoRI fragments, respectively (data not shown). In addition, Southern blotting an electrophoretic gel of Eckhart lysates from these mutants using Tn5 probe indicated that the Tn5 insertions were located on the chromosome, not on any of the five large plasmids (Toro et al., 1984). To determine whether the Tn5 insertions were responsible for the altered colony morphology exhibited by the mutants, the insertions were cloned as EcoRI fragments (Fig. 1a) in the pSUP102 vector and transferred back to the wild-type strain GRH2. Transconjugants showed the same colony morphology to that of GRH2-14 and GRH2-50 mutant strains indicating that the Tn5 insertion was responsible for the rough colony phenotype. These results were further confirmed by hybridization of EcoRI-digested total DNA from

Fig. 1. (a) Restriction fragments containing the Tn5 insertion of GRH2-14 and GRH2-50 mutant strains. (b) Restriction map of GRH2-50 complementing plasmid p501. R, EcoRI; B, BamHI; H, HindIII; S, SalI.

Fig. 2. LPS profiles on an SDS-polycrylamide gel. Periodic acid/silver-stained SDS-polyacrylamide gel (12%) of proteinase-K-treated SDS extracts of Rhizobium sp. GRH2 (lane 1), EPS GRH2-57 (lane 2), GRH2-50 (lane 3), GRH2-50 complemented with plasmid p501 (lane 4) and GRH2-14 (lane 5).

GRH2 and mutants using the mutant cloned restriction fragments as DNA probes.

The rough colony phenotype exhibited by GRH2-14 and GRH2-50 strains suggested that they were lps mutants. Proteinase-K-digested cell extracts of wild-type and mutant strains were separated by SDS-PAGE and stained by the periodic acid/silver procedure. As shown in Fig. 2, the relative staining intensity of the slow-migrating carbohydrate band of 'smooth' LPS present in the wild-type was considerably reduced in the mutants. Apparently, the GRH2-14 and GRH2-50 mutants have an alteration in the smooth LPS, due likely to a loss in O-antigen content. Reintroduction of the corresponding Tn5 insertions into the wild-type GRH2 also reproduced the altered LPS profile.

Complementation of Rhizobium sp. GRH2-14 and GRH2-50 mutants

We attempted to restore the wild-type phenotype of GRH2-14 and GRH2-50 mutants by carrying out a conjugative mating with a wild-type GRH2 cosmid (pLAFR1) clone bank (Lopez-Lara et al., 1993). Mucoid colonies, occurring at a frequency of 0.1%, were obtained only in the case of GRH2-50 and no complementation was achieved for the GRH2-14 mutant strain. Cosmids isolated from the mucoid colonies of GRH2-50 deriva-
tives were identical and designated p501. A restriction map of cosmid p501 is shown in Fig. 1(b). Upon reintroduction of cosmid p501 into mutant GRH2-50, the wild-type mucoid phenotype was restored, which indicated that this phenotype was encoded by plasmid p501. Hybridization of p501 with the EcoRI Tn5-mutant fragment isolated from GRH2-50 further confirmed that the DNA inserted in p501 carries the wild-type DNA that was mutated by the Tn5 insertion. In addition, plasmid p501 also restored the wild-type LPS profile (Fig. 2, lane 4) of mutant GRH2-50 but not of mutant strain GRH2-14.

Identification of DNA homologous to GRH2-14 and GRH2-50 mutant loci in Rhizobium

Genomic DNA from various Rhizobium species was examined for homology to the mutant loci of GRH2-14 and GRH2-50 by Southern blot hybridization. DNA fragments homologous to the GRH2-50 DNA probe (Fig. 1a) were found in R. leguminosarum bv. viciae, bv. trifolii and bv. phaseoli but no homology was detected with Rhizobium meliloti, Bradyrhizobium japonicum or Agrobacterium tumefaciens DNA. On the contrary, no DNA fragments homologous to the GRH2-14 DNA probe (Fig. 1a) were found in the four fast-growing rhizobia analysed nor in B. japonicum or A. tumefaciens (data not shown). In addition, no homology was detected with the R. leguminosarum strain VF39 (Priefer, 1989).

Sequencing of Tn5 insertions

In order to better understand the phenotypes of GRH2-14 and GRH2-50 mutations, we determined the DNA sequence of the corresponding left Tn5-DNA junctions. The partial amino acid sequences deduced were compared with sequences in the Swissprot and PIR Data Banks. No homologous sequences were found to the predicted product encoded by the mutant locus of GRH2-14. On the other hand, a significant homology (up to 43%) to GalE protein (UDPglucose 4-epimerase) from different organisms was found for the putative protein encoded by the GRH2-50 mutant locus. This result suggested that strain GRH2-50 was an exoB mutant.

Analysis of the UDPglucose 4-epimerase activity in wild-type GRH2 and derivative strains

To further confirm that strain GRH2-50 was an exoB mutant, cell-free extracts from wild-type GRH2 and the mutant GRH2-50 were assayed for the presence of UDPglucose 4-epimerase activity. Data indicated that this enzyme activity was present in the wild-type [99 nmol substrate used min⁻¹ (mg protein)⁻¹] but absent in the GRH2-50 mutant strain. Similar values were obtained for cells grown with glucose or galactose as the sole carbon source indicating a constitutive expression. In the cell-free extract prepared from GRH2-50 complemented with cosmid p501, the enzymic activity was recovered [98 nmol substrate used min⁻¹ (mg protein)⁻¹].
wild-type EPS, but no trace of a galactosyl component was detected.

$^1$H-NMR spectroscopy and compositional analyses indicated that the introduction of plasmid p501 into the GRH2-50 background resulted in restoration of the production of the wild-type EPS. The $^1$H-NMR spectrum of GRH2-50 (p501) EPS was superimposable with that of the GRH2 EPS for both glycosidic and non-carbohydrate substitutions resonances (Fig. 3). Assuming a stoichiometry of two pyruvyl residues per GRH2 EPS repeat unit (López-Lara et al., 1993), the pyruvate/acetate/3-hydroxybutyrate ratio was 2:00:1:62:0:84 for the EPS of the complemented mutant, as compared to 2:00:1:56:0:78 in the wild-type EPS. In addition, both the EPS sugar ratios were similar (glucose/glucuronic acid/galactose approximately 5:2:1), showing the restoration of the galactose moiety as a component of the EPS of the complemented strain.

The structures of GRH2-50 and GRH2-50 (p501) EPS were further examined by GC/MS analysis of the

Fig. 4. Longitudinal sections of an effective root nodule of A. cyanophylla infected with wild-type *Rhizobium* sp. (*Acacia*) strain GRH2 examined by light microscopy (a) and transmission electron microscopy (b). Note the numerous infected host cells interspersed within the large central zone in (a), and a portion of an infected host cell with numerous symbiosomes containing pleomorphic bacteroids surrounded by peribacteroid membranes in (b). Scale bars: (a) 100 µm; (b) 1 µm. CW, Cell wall; B, bacteroid; PM, peribacteroid membrane.
glycosidic linkage composition. The analysis of GRH2-50 (p501) EPS showed the predominance of 4-linked glucose, and the occurrence of 4-linked glucuronic acid, 4,6-linked glucose, 4,6-linked galactose and 3,4,6-linked glucose. This pattern of partially methylated alditol acetates was similar to the one obtained from the GRH2 wild-type EPS (López-Lara et al., 1993). In the GRH2-50 EPS, 4-linked glucose was also predominant; 4-linked glucuronic acid and 4,6-linked glucose were also present but no other derivative was found. This result reinforced the above NMR and GC/MS data indicating that the GRH2-50 mutation results in an altered EPS lacking the galactose component, as well as one pyruvyl moiety and 3-hydroxybutyryl substitutions, whereas the introduction of the p501 plasmid into the GRH2-50 background restores the production of the wild-type EPS.

**Symbiotic phenotype of GRH2-14 and GRH2-50 mutant strains**

Wild-type strain GRH2 is able to elicit reddish, elongated root nodules within 20 d of inoculation on *A. cyanophylla,*
Rhizobium sp. LPS mutants and symbiotic behaviour

Fig. 6. Longitudinal sections of an ineffective root nodule of A. cyanophylla induced by lps mutant GRH2-50, examined by light microscopy (a and b) and transmission electron microscopy (c and d). Scale bars: (a) 100 μm; (b) 20 μm; (c) 2 μm; (d) 2 μm. NP, Nodule parenchyma; CT, central tissue; V, vacuole; GM, electron-dense granular material.

Acacia melanoxylon and Prosopis chilensis plants. Inoculation of these tree-legumes with GRH2-14 and GRH2-50 mutant strains resulted in alteration of their root system leading to shorter and thicker roots without the formation of reddish, elongated nodules. Occasionally, inoculated roots of tree legumes developed a few small white nodules after 1 month of incubation with either of these two mutant strains. Reintroduction of the corresponding Tn5 insertions into the wild-type GRH2 also reproduced the altered symbiotic phenotype. Combined light and transmission electron microscopy of median longitudinal sections through 1-month-old A. cyanophylla nodules indicated that many host cells in the central tissue were invaded by the wild-type strain GRH2, which developed into pleomorphic endosymbiotic bacteroids within symbiosomes (Fig. 4a, b). In contrast, Acacia nodules were not invaded by either GRH2-14 or GRH2-50 mutant strains (Figs 5a–c and 6a–c). Other distinguishing mor-
phological features of median sections through ‘empty’ nodules induced by GRH2-14 and GRH2-50 mutant strains were a smaller proportional area of the central tissue (7% and 15%, respectively, compared to 59% for nodules induced by wild-type GRH2) and a more expansive nodule parenchyma between the peripheral nodule cortex and the central tissue. Nodule cells in this layer surrounding the central tissue stained intensely with toluidine blue (Figs 4a, 5a and 6a) and contained large vacuoles in which was deposited electron-dense granular material that was not bacteria (Figs 5d and 6d). Neither GRH2-14 nor GRH2-50 mutant strains were able to induce nodules on the herbaceous legumes which are nodulated by the GRH2 wild-type strain, such as *Lotus corniculatus*, *Vicia hirsuta*, *Trifolium subterraneum*, *Trifolium repens* and *Trifolium incarnatum*. However, like wild-type GRH2, mutants GRH2-14 and GRH2-50 elicited nodules on *Phaseolus vulgaris*.

**DISCUSSION**

Broad-host-range rhizobia are very useful for investigating the molecular mechanisms of symbiotic interactions between bacteria and plants. For this study, we have used wild-type *Rhizobium* sp. strain GRH2, which was isolated from root nodules of *A. cyanophylla* and has a broad host range which includes legume trees and herbaceous legumes (Herrera *et al.*, 1985). Here we report the characterization of two mutant strains of GRH2 which are defective in production of smooth LPS. One of them, strain GRH2-50, is an *exoB* mutant which lacks UDPglucose 4-epimerase activity. A consequence of this mutation is the inability to produce UDPgalactose and thus it is defective in production of glycoconjugates containing galactosyl components. In a previous study (López-Lara *et al.*, 1993), we found that the acidic EPS of wild-type GRH2 is closely related to the EPS of wild-type *R. leguminosarum* bv. *trifolii* ANU843 (Hollingsworth *et al.*, 1988). The simultaneous lack of galactose, one pyruvyl residue, and the 3-hydroxybutyryl substituents in the EPS of the GRH2-50 *exoB* mutant is best explained by proposing that the EPS of wild-type GRH2, like wild-type ANU843, has a galactose residue at the terminus of the branch in each oligosaccharide repeat unit which is substituted with one pyruvate residue and is also the site of esterification of 3-hydroxybutyrate substitutions. The inability of the GRH2-50 *exoB* mutant to produce smooth EPS implies that UDPgalactose is also required for synthesis of this cell surface polysaccharide. Preliminary studies of GRH2 LPS suggest that the sugar composition is different from that of strain ANU843, and that the LPS of mutant GRH2-50 lacks galactose and contains galacturonic acid instead (S. Hollingsworth, personal communication).

Attempts to complement the mutant phenotypes exhibited by GRH2-14 and GRH2-50 with a wild-type cosmid bank were only successful with the latter mutant strain. Southern blot hybridization and complementation studies showed that both GRH2-50 and GRH2-14 mutant strains carry a single Tn5 insertion. Reintroduction into the wild-type strain GRH2 of the corresponding Tn5 insertions reproduced the colony morphology, LPS and symbiotic mutant phenotypes. Thus, the lack of complementation of strain GRH2-14 suggests that: (i) the wild-type DNA was not well represented in the genomic bank used, (ii) the mutation could be dominant, or (iii) several copies of the wild-type DNA region could be lethal. Whereas the GRH2-50 mutant locus is well conserved among *R. leguminosarum* strains, GRH2-14 mutant DNA did not show homology in these rhizobial strains nor with pCos4. In addition, sequence analysis of the left junction of the Tn5 insertion 14 did not reveal homologous sequences in the Swissprot and PIR Protein Data Banks. Therefore, the GRH2-14 mutant locus may represent a newly described *ips* gene for *Acacia* rhizobia.

In a previous study we reported an EPS mutant derivative of GRH2 (mutant GRH2-57) which was partially defective in nodule invasion in *Acacia*. It still nodulated this host but exhibited a fivefold reduction in nodule occupancy (López-Lara *et al.*, 1993). The LPS mutant strains described here (GRH2-14 and GRH2-50) have even more drastic defects in their symbiotic phenotypes on this tree legume host. These mutant strains are only able to induce a few small white nodules in *Acacia*, and these nodules are devoid of bacteria. Therefore, whereas a mutation which blocks acidic EPS biosynthesis results in a partial loss in ability to fully invade *Acacia* nodules, a mutation which blocks biosynthesis of smooth LPS completely prevents bacterial invasion of nodule cells on this preferred tree legume host. This demonstrates the critical importance of EPS in enabling rhizobia to overcome the host cell barriers in order to achieve nodule invasion in the nitrogen-fixing *Rhizobium–Acacia* symbiosis.

Nodulation tests with a diversity of legumes have revealed that these polysaccharide mutants of *Acacia* rhizobia are also altered in host range. In a previous study (López-Lara *et al.*, 1993), the EPS mutant GRH2-57 lost the ability to nodulate clovers and vetch, but retained the ability to nodulate *Phaseolus* and *Lotus*. Therefore, the importance of EPS in nodulation and host range on herbaceous legume hosts is correlated with the type (determinant or indeterminant) of nodule ontogeny. In contrast, the LPS mutant strains GRH2-14 and GRH2-50 were able to nodulate *Phaseolus* but not *Lotus*. These results suggest that the symbiotic defect exhibited by these two LPS mutants on herbaceous legume hosts was not dependent on the nodule ontogeny. Thus, our results highlight the contributing role(s) of the surface polysaccharides (LPS and acidic EPS) in determining the host range of *Acacia* rhizobia.

**ACKNOWLEDGEMENTS**

This work was supported by Comisión Asesora de Investigación Científica y Técnica grants BIO90-0747 and BIO93-0677 (to J.O. and N.T.), National Institute of Health Grant no. GM-34331-06 and the Michigan State University Biotechnology Research Center (to F.B.D.). I.L.L. was supported by a M.E.C. fellowship. We thank H. S. Pankratz and J. Palma for technical assistance.
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