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

Isabel María López-Lara,1 Guy Orgambide,2 Frank B. Dazzo,2 José Olivares1 and Nicolás Toro1

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**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...
and acidic EPS and in development of root nodule symbiosis; and (iii) a new chromosomal Ipa locus in *Rhizobium* involved in symbiotic development.

**METHODS**

**Bacterial strains and genetic manipulations.** *Rhizobium* sp. GRH2 was originally isolated from root nodules of *A. cyamphylla* (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 TV supplemented with mannitol (YMT). Transposon Tn5 was isolated from root nodules of *A. cyamphylla* (Herrera et al., 1993). Antibiotics were used at the following concentrations: kanamycin sulfate (Km), 180 μg ml⁻¹; tetracycline (Tc), 10 μg 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 EsoRI digested and cloned in plasmid pSUP102. After ligation, DNA was transformed in *E. coli* DH5α (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 Sall 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'-AAAGGTTCCGTTCAAGGC-3'.

**Sequence analysis.** Sequence analysis and homology search were done with the GG2 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 1H-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.

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**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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<tr>
<td><em>Rhizobium</em> sp.</td>
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<tr>
<td>GRH2</td>
<td>Broad-host-range <em>Rhizobium</em> sp. isolated from <em>A. cyamphylla</em> root nodules</td>
<td>Herrera et al. (1985)</td>
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<tr>
<td>GRH2-57</td>
<td>GRH2 derivative obtained by Tn5 mutagenesis, exo-37, EPS⁻</td>
<td>López-Lara et al. (1993)</td>
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<tr>
<td>GRH2-14</td>
<td>GRH2 derivative obtained by Tn5 mutagenesis, exo-14 with altered LPS</td>
<td>This work</td>
</tr>
<tr>
<td>GRH2-50</td>
<td>GRH2 derivative obtained by Tn5 mutagenesis, exo-50, lacks the UDPglucose 4-epimerase</td>
<td>This work</td>
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<tr>
<td><strong>R. meliloti</strong></td>
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<td>Rm2011</td>
<td>Wild-type, Sm⁸</td>
<td>Cassé et al. (1979)</td>
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<td><strong>R. leguminosarum</strong></td>
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<tr>
<td>RS1050</td>
<td>Wild-type, bv. <em>trifolii</em></td>
<td>F. Rodríguez-Quiñones,</td>
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<td>Facultad Farmacia,</td>
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<td>Wild-type, bv. <em>phaselii</em></td>
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<td>122DES</td>
<td>Wild-type, Hup⁺</td>
<td>Emerich et al. (1980)</td>
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<td><strong>A. tumefaciens</strong></td>
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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 Biosquad 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 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 derivative-
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 isps genes containing plasmid pCos4 (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].

Structural evaluation of the acidic heteropolysaccharides from GRH2-14, GRH2-50 and GRH2-50(p501)

Gel filtration analyses indicated that while GRH2-14 produced a similar amount of EPS to the wild-type GRH2, the yield of EPS produced by mutant GRH2-50 was fivefold lower. Based on sugar composition and ¹H-NMR spectral features, the high molecular mass acidic EPS made by the insertion-mutant GRH2-14 was indistinguishable from the wild-type GRH2 acidic EPS, which consists of an octasaccharide-repeat-unit-based polymer with glucose, glucuronic acid and galactose glycosyl components in a 5:2:1 ratio, and acetyl, pyruvyl and 3-hydroxybutyryl substitutions (Lopez-Lara et al., 1993).

In contrast, ¹H-NMR analysis indicated that GRH2-50 EPS was altered in both glycosidic and non-carbohydrate substitutions composition (Fig. 3). The spectrum of this EPS was totally devoid of 3-hydroxybutyryl resonances and displayed a single pyruvyl resonance whose relative integration indicated that GRH2-50 EPS bears a single pyruvate residue per repeat unit, as compared to two residues in GRH2 EPS. Compositional analysis showed that GRH2-50 polysaccharide contains glucose and glucuronic acid in an approximate 3:1 ratio similar to the...
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...
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,*
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 pleiomorphic 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 expanses of the nodule cortex and the central tissue. Nodule cells in this 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 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 LPS and acidic EPS in enabling rhizobia to overcome the host cell barriers in order to achieve nodule invasion in the nitrogen-fixing Rhizobium–Acacia symbiosis.

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 EPS 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 LPS and acidic 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 vulgaris. 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.

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