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Genetic and Chemical Characterization of a Mutant That Disrupts Synthesis of the Lipopolysaccharide Core Tetrasaccharide in *Rhizobium leguminosarum*

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A 2-kb region that complements the Tn*5*-derived lipopolysaccharide (LPS) rough mutant *Rhizobium leguminosarum* RU301 was sequenced. Two open reading frames (ORFs) were identified. The first ORF (*lpcA*) is homologous to a family of bacterial sugar transferases involved in LPS core tetrasaccharide biosynthesis. ORF2 (*lpcB*), in which Tn*5* transposed, has no significant homology to any DNA in the GenBank-EMBL databases. Chemical characterization of LPS produced by strain RU301 demonstrated that the 3-deoxy-D-manno-2-octulosonic acid (Kdo) residue which normally attaches the core tetrasaccharide to the O chain was missing, suggesting that *lpcB* may encode a CMP-Kdo:LPS Kdo transferase.

The lipopolysaccharides (LPSs) of gram-negative bacteria are a major chemical constituent of the outer membrane and are implicated as having a role in infection of the legume symbiont by rhizobia (3, 10, 11). In determinate bean nodules, LPS rough mutants of *Rhizobium leguminosarum* bv. *phaseoli* accumulate in the infection thread without forming bacteroids, and nitrogen fixation does not occur (11). Development may proceed further in indeterminate nodules, and low rates of nitrogen fixation can sometimes be detected (3, 5). Two forms of LPS exist: LPS I, consisting of lipid A, core tri- and tetrasaccharides, and the O-chain polysaccharide, and LPS II, which lacks the O-chain polysaccharide (10, 16). *R. leguminosarum* bv. *viciae* RU301 has been isolated and characterized as a Tn*5* mutant that contains only LPS II (12). Complementing clones were obtained from both *R. leguminosarum* bv. *phaseoli* and *R. leguminosarum* bv. *viciae* gene libraries, and a common clustering of LPS and *dct* genes was demonstrated (12).

Noel (10) defined five regions involved in LPS biosynthesis in *Rhizobium etli* CE3: the α-, β-, and γ-*lps* regions, *exoBC*, and a poorly defined region which contains the *lps-166* mutation. Strain RU301 is not complemented by cosmids corresponding to the α-, β-, or γ-*lps* or the *exoB* region (12). A 3.4-kb *Eco*RI-*Hin*dIII fragment from the cosmid pIJ1848 (9), which is derived from *R. leguminosarum* bv. *phaseoli*, complemented strain RU301 (Fig. 1) and was used as a probe to determine if there was any homology to cosmid DNA encoding the α-, β-, or γ-*lps* region. Hybridization analysis supported the view that the complementing region constituted a region separate from those previously described (12). The 3.4-kb fragment also complements the mutant strain VF-39-86 (14), which has been characterized as having a truncated core tetrasaccharide component containing the disaccharide mannose (1-5) 3-deoxy-D-manno-2-octulosonic acid (Kdo) (16). A smaller *Pst*I-*Eco*RI subclone, pRU74 (2.4 kb), complemented strain RU301 but not VF-39-86, indicating that at least two genes involved in LPS core tetrasaccharide synthesis may be present in this previously undefined region (12).

Subclones derived from pRU74 were cycle sequenced [Exo

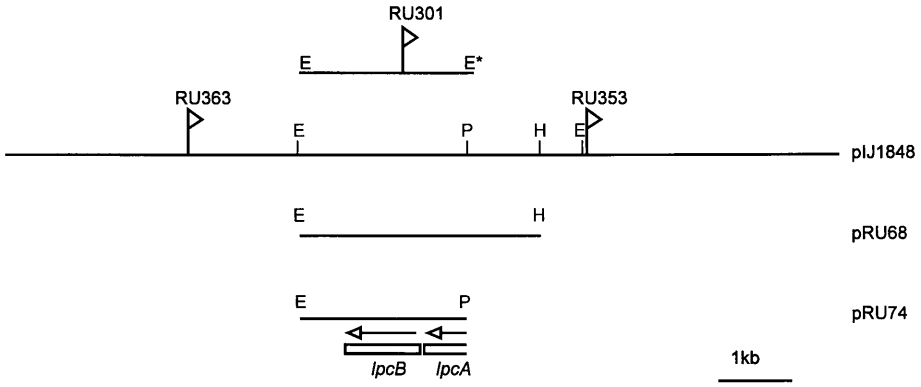

FIG. 1. Map of the LPS region of *R. leguminosarum* bv. *phaseoli*. pIJ1848 complements the LPS mutant strains RU301 and VF-39-86. The position of Tn*5* in *R. leguminosarum* bv. *viciae* RU301 is shown above. Subclone pRU68 complements both mutants, while pRU74 complements only strain RU301. ORFs are indicated as open boxes. Tn*5-lacZ* mutant strains RU353 and RU363 define the limits of the region. Restriction sites are abbreviated as follows: E, *Eco*RI; H, *Hin*dIII; and P, *Pst*I. The asterisk refers to a restriction site present in strain 3841 DNA but not in pIJ1848.

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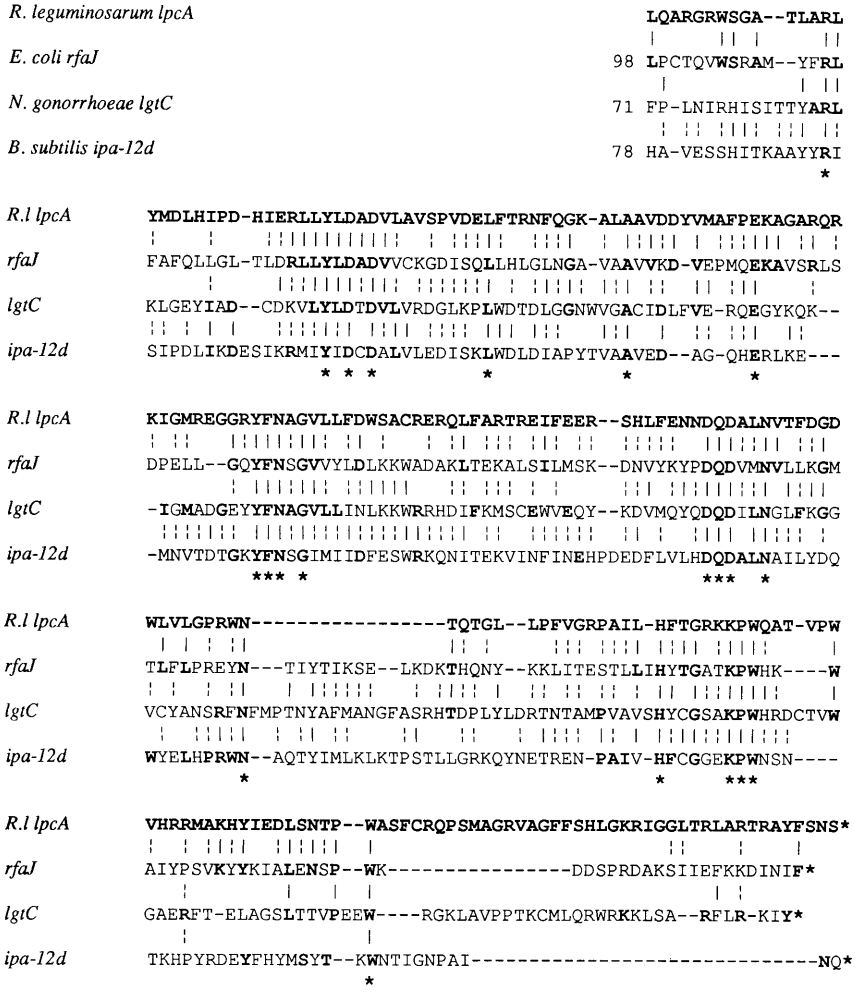

FIG. 2. Alignment of *lpcA* (ORF1) with sugar transferases from the EMBL database, based on a CLUSTALV alignment of the four peptides and subsequently corrected for pairwise TFASTA alignments (using the GCG package [5a]). Residues in boldface show identity with *lpcA*. Residues conserved in all peptide sequences are indicated by asterisks. Conserved substitutions are indicated by broken vertical segments, and identical residues are indicated by solid vertical segments. *E. coli rfaJ* encodes a putative LPS 1,2-glucosyltransferase (13). *N. gonorrhoeae lgtC* encodes a lipo-oligosaccharide 1,4-galactosyltransferase (7). *B. subtilis ipa-12d* has a predicted peptide sequence similar to those of the LPS 1,3-galactosyltransferase and LPS 1,2-glucosyltransferase of *E. coli* and *S. typhimurium* (6).

(−)Pfu DNA Cyclist; Stratagene) in accordance with the manufacturer's recommendations, and two open reading frames (ORFs) were identified. ORF1 constitutes the 3′ region of an incomplete ORF translated from the *Pst*I end of pRU74 (Fig. 1). This ORF has homology to the carboxy termini of members of a semiconserved family of glucosyl and galactosyl transferases involved in synthesis of the LPS core tetrasaccharide in gram-negative bacteria, including *rfaIJ* from *Escherichia coli* and *Salmonella typhimurium*. Homology was also observed with *lgtC* from *Neisseria gonorrhoeae* and *ipa-12d* from *Bacillus subtilis* (Fig. 2). This gene may be the one mutated in strain VF-39-86 (16). Genetic evidence to support this possibility includes the observation that the larger, 3.4-kb *Eco*RI-*Hin*dIII clone pRU68 complements strain VF-39-86 while pRU74 does not (12) (Fig. 1). Chemical analysis indicates that in strain VF-39-86, the core tetrasaccharide is truncated at the site of addition of galactose to α-Man*p*-(1→5)-Kdo*p* (where *p* indicates pyranose) (see below and reference 16), indicating that the gene may be a UDPgalactose:LPS α-1,6-galactosyltransferase. We suggest that ORF1 be given the designation *lpcA* (for lipopolysaccharide core synthesis).

ORF2 is 867 bp long and codes for a putative 289-amino-acid protein with a relative molecular weight of 32,000. TFASTA and BLAST searches did not identify any significant homology between ORF2 and any DNA or protein in the available databases. However, Tn*5* was located within ORF2 in strain RU301, and the LPS produced by this mutant was analyzed.

Mild acid hydrolysis of the LPSs from *R. leguminosarum* strains (this paper and references 1, 2, 8, and 15) and from *R. etli* (1, 2) produced the following core oligosaccharides, as determined by high-performance anion-exchange liquid chromatography (HPAEC) with a Carbopac PA1 column from Dionex (2): (i) monomeric Kdo (designated OS1), (ii) monomeric GalA (OS2), (iii) α-Gal*p*-(1→6)-[α-GalA*p*-(1→4)]-α-Man*p*-(1→5)-Kdo*p* (OS3), (iv) the 4,7- or 4,8-anhydro-Kdo version of the above tetramer (OS4), and (v) α-GalA*p*-(1→4)-[α-GalA*p*-(1→5)]-Kdo*p* (OS5).

A polyacrylamide gel electrophoresis profile of LPS produced by strain RU301 indicated the complete absence of the O chain (12). The HPAEC profiles of the core oligosaccharides released by mild acid hydrolysis of the LPSs from strain RU301 and the parent strain, 3841, are shown in Fig. 3. The profile of the parent LPS is identical to that reported (2) for

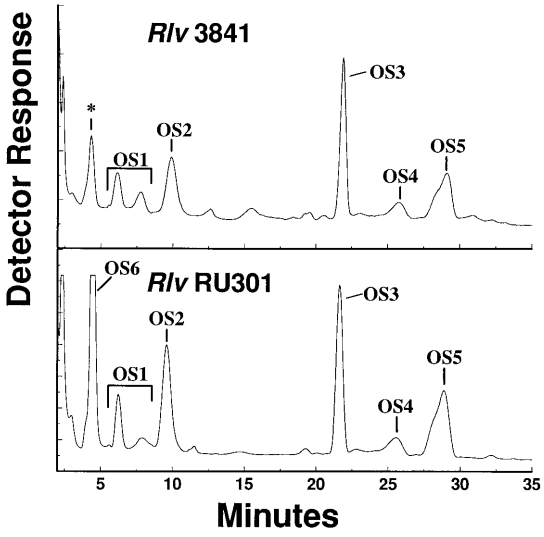

FIG. 3. HPAEC elution profiles of core oligosaccharides released by mild acid hydrolysis of *R. leguminosarum* bv. *viciae* (*Rlv*) 3841 and RU301 LPSs. The peak marked with an asterisk in the strain 3841 profile is not the same as OS6 from strain RU301 LPS, since its retention time is slightly but significantly less than that of OS6. The identities of the remaining peaks are as indicated in the text.

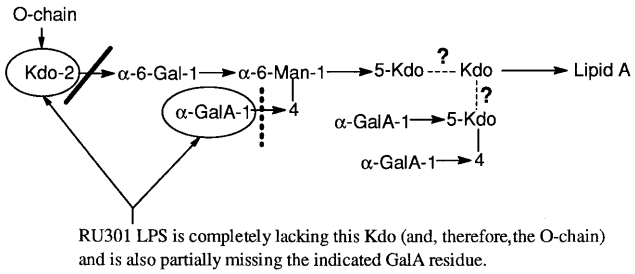

FIG. 4. The structural arrangement of the core region of *R. leguminosarum* and *R. etli* CE3 LPS as previously described (2). The defects in strain RU301 LPS are as indicated.

the LPS from *R. etli* CE3 and shows the presence of all of the above-described oligosaccharides. The profile of strain RU301 oligosaccharides shows the above-mentioned components and an additional oligosaccharide designated OS6. OS6 has the same retention time as a previously reported oligosaccharide from a mutant of *R. etli* CE3, strain CE358 (2). That oligosaccharide, α-Gal*p*-(1→6)-α-Man*p*-(1→5)-Kdo*p*, has also been found in strain CE109 (2). OS6 from strain RU301, which has Kdo at its reducing end, was isolated, and analysis of its neutral sugars suggested that it contained a 1:1 Gal:Man ratio, which is consistent with the structure of the oligosaccharide from strain CE358 reported above. Thus, strain RU301 produced both the normal core tetrasaccharide (OS3) and a version which lacked the GalA residue (OS6). In addition, methylation analysis of the LPS from strain RU301 before and after mild acid hydrolysis indicated that the Kdo residue which is normally attached to C-6 of the core galactosyl residue (2) was missing. Only terminally linked galactose was detected in the LPS both before and after mild acid hydrolysis. Galactose linked to C-6 was not detected prior to mild acid hydrolysis. Since current evidence indicates that it is this Kdo residue which joins the O chain to the core region, its absence in strain RU301 LPS may explain the complete lack of the O chain in this LPS. Furthermore, the amount of galacturonic acid attached to mannose was reduced, indicating that the above-mentioned Kdo residue may normally be attached to galactose prior to the transfer of the galacturonosyl residue. The defects in the core region of strain RU301 LPS are shown in Fig. 4. In summary, these data indicate that ORF2 may encode a CMP-Kdo transferase responsible for the transfer of Kdo to the core tetrasaccharide galactosyl residue. However, the available evidence is insufficient to warrant assigning a defined enzyme activity to this gene product, and we suggest that it be designated *lpcB*.

Several mutants of *R. etli* CE3 produce altered core oligosaccharides that are similar but not identical to those from strain RU301. In strain CE358, the tetrasaccharide (OS3) is replaced by the α-Gal*p*-(1→6)-α-Man*p*-(1→5)-Kdo*p* trisaccharide (OS6) (2). Analysis of strain CE109 LPS shows that like in strain RU301, the core region consists of OS6 and the normal tetrasaccharide (OS3) (1, 2). However, the LPSs from both strains CE358 and CE109 contain Kdo at C-6 of the core galactosyl residue (2). Analysis of strain CE350 illustrates that its core oligosaccharides give an HPAEC profile (2) identical to those of its own parent strain, CE3, and the parent of RU301, strain 3841. However, strain CE350 partially, but not completely, lacks the Kdo attached to C-6 of the core galactosyl residue and does produce some forms of LPS with truncated O chains (2). All of these mutations in strain CE3 are in the α-*lps* region (2, 4). All of the other α-*lps* region mutants contain a core region identical to that found in the CE3 parent strain (2). Thus, these *R. etli* mutants all have LPS phenotypes different from that of strain RU301. This may explain why the α-*lps* region from strain CE3 does not complement the mutation in strain RU301.

The sequence downstream of *lpcB* does not have any homology to genes involved in LPS core synthesis. Furthermore, the region is flanked by transposon mutant strains RU363 and RU353 (Fig. 1), neither of which apparently affects LPS core biosynthesis (12). Data presented here allow identification of two genes involved in the sequential addition of galactose and Kdo to the core tetrasaccharide of *R. leguminosarum*. Since these genes do not correspond to any previously identified in *Rhizobium* species, we suggest they constitute the δ-*lps* region in accordance with the nomenclature of Noel (10).

Nucleotide sequence accession number. The sequences of the two ORFs derived from pRU74 were submitted to EMBL and given accession number X94963.

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