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Expression of *Rhizobium leguminosarum* CFN42 Genes for Lipopolysaccharide in Strains Derived from Different *R. leguminosarum* Soil Isolates

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Two mutant derivatives of *Rhizobium leguminosarum* ANU843 defective in lipopolysaccharide (LPS) were isolated. The LPSs of both mutants lacked O antigen and some sugar residues of the LPS core oligosaccharides. Genetic regions previously cloned from another *Rhizobium leguminosarum* wild-type isolate, strain CFN42, were used to complement these mutants. One mutant was complemented to give LPS that was apparently identical to the LPS of strain ANU843 in antigenicity, electrophoretic mobility, and sugar composition. The other mutant was complemented by a second CFN42 *lps* genetic region. In this case the resulting LPS contained O-antigen sugars characteristic of donor strain CFN42 and reacted weakly with antiserum against CFN42 cells, but did not react detectably with antiserum against ANU843 cells. Therefore, one of the CFN42 *lps* genetic regions specifies a function that is conserved between the two *R. leguminosarum* wild-type isolates, whereas the other region, at least in part, specifies a strain-specific LPS structure. Transfer of these two genetic regions into wild-type strains derived from *R. leguminosarum* ANU843 and 128C53 gave results consistent with this conclusion. The mutants derived from strain ANU843 elicited incompletely developed clover nodules that exhibited low bacterial populations and very low nitrogenase activity. Both mutants elicited normally developed, nitrogen-fixing clover nodules when they carried CFN42 *lps* DNA that permitted synthesis of O-antigen-containing LPS, regardless of whether the O antigen was the one originally made by strain ANU843.

Leguminous plants and bacteria of the genera *Rhizobium* and *Bradyrhizobium* enter into a symbiosis characterized by nitrogen-fixing root nodules. Early steps in development of the symbiosis involve mutual recognition of plant and bacteria, induction of cortical cell division, and (in most cases) invasion of root hairs by way of infection threads. As gram-negative eubacteria, rhizobia have an outer membrane containing lipopolysaccharide (LPS). LPS is composed of two regions: lipid A, which anchors the LPS molecule in the outer leaflet of the outer membrane, and a polysaccharide portion, which projects into the environment. Being a major cell surface molecule, LPS is likely to play a role in early symbiotic events.

The polysaccharide of *Rhizobium leguminosarum* LPS consists of a variable portion and a portion whose composition is conserved among wild-type isolates that have been studied previously (4, 6, 7). The conserved portion is released from the LPS by mild acid hydrolysis as two oligosaccharides that together are composed of galactose, mannose, galacturonic acid, and at the reducing ends, 3-deoxy-D-*manno*-octulosonic acid in a 1:1:3:2 molar ratio (4, 5, 14a). The variable portion is found in a longer polysaccharide that is released by mild acid hydrolysis. Its composition varies from strain to strain (4, 26). The variable portion carries the dominant antigenic determinants of wild-type strains and is referred to as the O antigen, in keeping with the terminology devised for enteric bacteria (4, 6, 16).

LPSs from different *R. leguminosarum* strains result in distinct banding patterns when displayed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The bands can be classified into two groups according to their mobilities: LPS I and LPS II. LPS I occurs as one to several bands with a wide range of electrophoretic mobilities; all bands migrate more slowly than LPS II. Both LPS I and LPS II contain the sugar residues of the conserved oligosaccharides, but only LPS I bands carry O antigen (6). In mutants that lack LPS I, LPS II (often in modified form) occurs in much greater concentrations (6, 8, 19).

Five genetic regions that are required for LPS synthesis have been identified in *R. leguminosarum* biovar *phaseoli* CFN42 by complementation of Lps mutants with cloned wild-type *lps* DNA (8, 11). Mutations within these regions result in LPS that lacks the O antigen or that has greatly decreased amounts of it. Mutants with such LPS defects elicit incomplete bean nodule development in which infection threads abort (8, 19).

In this study, two Lps mutants from *R. leguminosarum* biovar *trifolii* ANU843 were isolated. Both mutants lacked O antigen and elicited incompletely developed nodules on clover. Complementation with cloned DNA of strain CFN42 restored normal ANU843 LPS and proficient nodulation to one of the mutants. The second mutant was restored to Lps^+ by a different region of the CFN42 genome, and in this case, the LPS of the resulting strain contained CFN42 O-antigen sugars and some CFN42 antigenic determinants. Even though it carried CFN42 O-antigen structural features, this transconjugant strain also elicited nitrogen-fixing nodules on clover. Therefore, considerable latitude in LPS O-antigen structure is permitted in the symbiosis with clover, as predicted from the documented variability in LPS compositions of *R. leguminosarum* strains with similar host ranges (4) and the interconversion of host ranges by exchanging Sym (symbiosis) plasmids or certain *nod* genes between *R.*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
R. leguminosarum biovar *phaseoli* CFN42	Wild-type Nod$^+$ Fix$^+$	18
R. leguminosarum biovar *trifolii*		
ANU843	Wild-type Nod$^+$ Fix$^+$	R. W. Carlson
TB4	Nal^r Str^r derivative of ANU843 Nod$^+$ Fix$^+$	This work
TB104	*lps-104* LpsI$^-$ Ndv$^-$ Nal^r Str^r Km^r	This work
TB112	*lps-112* LpsI$^-$ Ndv$^-$ Nal^r Str^r Km^r	This work
R. leguminosarum biovar *viciae*		
128C53	Wild-type Ery^r Nod$^+$ Fix$^+$	R. W. Carlson
LB2	Sp^r derivative of 128C53 Nod$^+$ Fix$^+$	This work
Plasmids[b]		
pCOS126	27.0 kb of CFN42 DNA cloned in pLAFR1	8
pDEL27	Derivative of pCOS126 carrying only 7.5 kb of CFN42 DNA	8
pCOS109.11	30.1 kb of CFN42 DNA cloned in pLAFR1	8
pRK2013	RK2 derivative, Km^r Tra$^+$	12
pSUP2021	Vector for Tn*5* mutagenesis	22

[a] The indicated nodulation phenotypes are for the following host plants that were examined: *R. leguminosarum* biovar *phaseoli*, *Phaseolus vulgaris*; *R. leguminosarum* biovar *trifolii*, *T. hybridum*; *R. leguminosarum* biovar *viciae*, *P. sativum*. Abbreviations: Nal, nalidixic acid; Str, streptomycin; Km, kanamycin; Sp, spectinomycin; Ery, erythromycin; Nod$^+$ Fix$^+$, wild-type nodule development and nitrogen fixation; Ndv$^-$, nodules developed incompletely.
[b] Plasmids not listed here are shown in Fig. 4.

leguminosarum strains (3, 13). Results of this study also help to define the roles of these two *lps* genetic regions in LPS biosynthesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are listed in Table 1, along with their relevant characteristics. Rhizobial strains were grown at 30°C in rich media (TY) (2) unless otherwise noted. Yeast-mannitol (AMA) and minimal (Y) media have been described previously (23, 24). *Escherichia coli* strains were grown at 37°C in LB medium (17). Solid media contained 15 g of Bacto-Agar (Difco Laboratories, Detroit, Mich.) per liter. Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were incorporated into the media at the following concentrations: 30 μg of nalidixic acid per ml, 30 μg of kanamycin per ml, 200 μg of streptomycin per ml, 200 μg of spectinomycin per ml, and 5 μg (*R. leguminosarum*) or 15 μg (*E. coli*) of tetracycline per ml.

Mutagenesis. Mutants of *R. leguminosarum* TB4 were generated by Tn*5* mutagenesis by using the suicide vector pSUP2021 (22). Portions of fully grown cultures of strain TB4 (0.6 ml) and *E. coli* SM17-1 (22) containing pSUP2021 (0.4 ml) were spread onto the surface of a TY agar plate and incubated overnight at 30°C. This mixed lawn of bacteria was suspended in 3.0 ml of 0.1 M $MgSO_4$ and plated onto TY agar containing streptomycin, nalidixic acid, and kanamycin to screen for LPS mutants on the basis of colony morphology. Colonies with a dry or rough appearance were subjected to further characterization of LPS by SDS-PAGE.

SDS-PAGE. Bacterial cultures were grown to the late log phase in liquid TY. Harvested cells were resuspended in SDS sample buffer and heated at 100°C for 5 min, as described previously (8). Before being applied to a polyacrylamide gel, some samples were then incubated with freshly prepared proteinase K (Sigma) at a 0.5-μg/μl final concentration for 1 h at 37°C. Following discontinuous SDS-PAGE, the resolving gels (15% polyacrylamide) were stained by using a silver staining kit (Bio-Rad Laboratories, Richmond, Calif.) by a previously described procedure that includes a periodate oxidation step (8). Bands corresponding to LPS I and LPS II were identified by comparing them with SDS-polyacrylamide gels of purified LPS of strains CFN42 and ANU843 (6, 7).

Immunoblots. Following SDS-PAGE, gels were electroblotted onto nitrocellulose and incubated with the appropriate antiserum (or monoclonal antibody). The nitrocellulose was then incubated with a secondary antibody, goat anti-rabbit immunoglobulin G (IgG) (polyclonal sera) or rabbit anti-rat IgG (monoclonal sera), conjugated to horseradish peroxidase. Bands were visualized by a color reaction by using 4-chloro-1-naphthol as the peroxidase substrate. Monoclonal antibodies were kindly provided by N. J. Brewin and J. P. Knox of the John Innes Institute, Norwich, England. Polyclonal rabbit antisera were raised against washed rhizobial cells of strain CFN42 or ANU843 (6).

Bacterial matings. Intergeneric crosses were performed by using a triparental mating system (12) in which pRK2013 was used as the helper plasmid. *E. coli* strains carrying the recombinant plasmids with rhizobial DNA or pRK2013 were grown at 37°C in LB liquid containing either tetracycline (to maintain pRK290 and pLAFR1 carrying rhizobial DNA) or kanamycin (for maintaining pRK2013). The *R. leguminosarum* recipient strains were grown at 30°C in TY liquid. Portions of fully grown cultures of the recipient strain (0.5 ml) and each donor strain (0.2 ml each) were spread onto the surface of a TY agar plate and incubated at 30°C overnight. This mixture was suspended in 3.0 ml of 0.1 M $MgSO_4$, and appropriate dilutions were plated onto TY agar containing nalidixic acid and tetracycline.

Plant tests. Nodulation assays of strains derived from ANU843 were performed on *Trifolium hybridum* cv. Alsike. Seeds were surface sterilized with 50% commercial bleach for 15 min and then washed extensively with sterile water. Seeds were germinated at 30°C on moistened filter paper. The 2- to 3-day-old seedlings were planted on RBN (24) agar slants in tubes (18 by 150 mm) and inoculated with 0.25 ml of a fully grown culture. Five plants per inoculum were used in

each experiment. To assay for nitrogenase activity, the tubes were plugged with rubber serum caps, and 0.5 cm^3 of acetylene was injected. Tubes were incubated at room temperature for 1.5 h, at which time a 0.25-cm^3 sample was taken and analyzed by gas chromatography.

Strains derived from 128C53 were tested for their nodulation proficiency on *Pisum sativum* cv. Laxton Progress. Seeds were surface sterilized as described above. Germination was on moistened filter paper at 30°C for 1 day and then at room temperature in the dark for an additional 2 days. Three-day-old seedlings were planted in modified Leonard jars (1) and inoculated with 0.5 ml of a fully grown culture.

Bacteria were isolated from nodules 5 to 6 weeks after inoculation. Nodules were excised from the roots, surface sterilized with 10% commercial bleach for 10 min, and then washed three times with sterile water. Individual nodules were crushed in 1.0 ml of PDB dilution buffer (14) containing 0.25 M mannitol, 0.25 M sorbitol, 2 mM $CaCl_2$, and 2 mM KH_2PO_4 (pH 5.8 to 6.0) for osmotic protection. Appropriate dilutions of this buffer were plated onto BMM minimal agar plates containing 0.2 M mannitol to obtain viable cell counts.

Light microscopy. Nodules were excised from plants 4 to 6 weeks after inoculation, fixed in 2% glutaraldehyde, and embedded in Spurr resin as described previously (23). Thick sections were stained with toluidine blue.

Compositions. Purified LPS was acid hydrolyzed, and alditol acetate derivatives were analyzed by gas chromatography on a capillary column (Supelco SP2330). Uronic acids were identified by reduction of the carboxyl groups before acetylation, or their trimethylsilyl methyl glyosides were prepared and analyzed by gas chromatography by using a 30-m capillary column (DB-1; J&W Scientific, Folsom, Calif.) (25). 3-Deoxy-D-*manno*-octulosonic acid and uronic acids were quantitated by colorimetric assays, as described previously (6, 25).

RESULTS

Isolation and characterization of Lps mutants of strain ANU843. Two Lps mutants, TB104 and TB112, were isolated following Tn*5* mutagenesis of TB4, a Str^r Nal^r derivative of *R. leguminosarum* biovar *trifolii* ANU843. Both mutants were detected initially because of their dry, rough colony morphology on TY agar. Strain TB112 exhibited a slower rate of growth in liquid TY, AMA, or Y medium than did the wild type. Full growth was reached in 2 days for TB112 versus 1 day for TB4. Strain TB104, in TY or Y liquid, displayed a more drastic reduction in growth rate, requiring 3 days to reach full growth. There was no difference in the rate of growth of TB104 in AMA compared with that of TB112. Both mutants grew equally well on TY and AMA agar, although they grew slightly slower than the wild-type strain. Strains TB104 and TB112 required 4 days for the colonies to reach a particular size compared with 3 days for TB4. Characteristic of *R. leguminosarum* Lps mutants (19), strains TB104 and TB112 agglutinated during growth in liquid TY. SDS-PAGE revealed that the mutants lacked LPS I (Fig. 1A and 2A, lanes 3), which was previously shown to be the O-antigen-containing LPS (7).

Complementation by *lps* DNA from strain CFN42. Mutants TB104 and TB112 were tested for complementation by two *lps* regions cloned from the genome of *R. leguminosarum* biovar *phaseoli* CFN42 (8). One of these regions was found in the overlapping DNA inserts of plasmids pCOS109.11 and pCOS109.10. The second region was carried in plasmid pDEL27. These plasmids were transferred to the Lps mutants of ANU843.

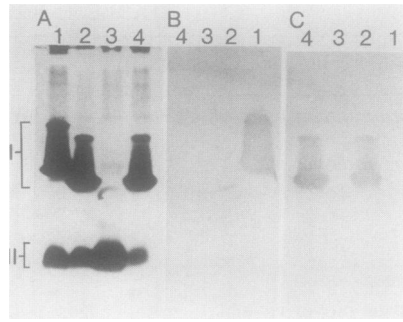

FIG. 1. Complementation of TB104 as determined by SDS-PAGE and immunological reactivity. Proteinase K-treated cell extracts were prepared from strains CFN42 (lanes 1), TB4 (lanes 2), TB104 (lanes 3), and TB104(pDEL27) (lanes 4). (A) Periodate silver-stained gel. The positions of LPS I and LPS II bands are indicated in the left margin. (B and C) Immunoblots of gels duplicating the one shown in panel A reacted with rabbit polyclonal antiserum against CFN42 (B) or ANU843 (C).

One Lps mutant, TB104, was complemented to Lps^+ by plasmid pDEL27. The LPS banding pattern of the transconjugant TB104(pDEL27) was identical to that of the parental strain TB4 on SDS-PAGE (Fig. 1A, lanes 2 and 4). The LPS I of the transconjugant was cross-reactive with antisera directed against wild-type *R. leguminosarum* biovar *trifolii* ANU843 (anti-ANU843), but not with antisera (anti-CFN42) against wild-type *R. leguminosarum* biovar *phaseoli* CFN42 from which the DNA was cloned (Fig. 1B and C, lanes 4). Therefore, it appears that the LPS I specific to the recipient genetic background is restored by this DNA.

The other mutant, TB112, was complemented to Lps^+ by plasmid pCOS109.11. However, the banding pattern of LPS from transconjugant TB112(pCOS109.11) on SDS-PAGE

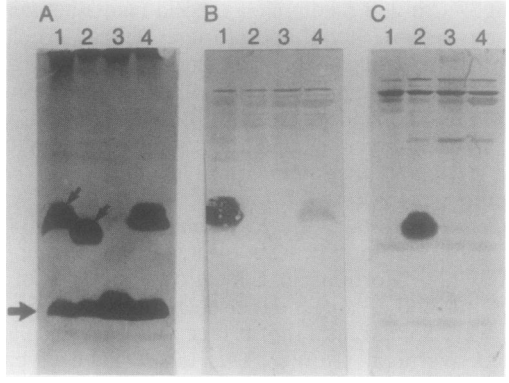

FIG. 2. Complementation of TB112, as determined by SDS-PAGE, and immunological reactivity. SDS extracts of harvested cells were prepared from the following strains: CFN42 (lanes 1), ANU843 (lanes 2), TB112 (lanes 3), and TB112(pCOS109.11) (lanes 4). (A) Periodate silver-stained gel. The LPS I bands of CFN42 and ANU843 are indicated by the arrows in lanes 1 and 2. LPS I was more tightly resolved than it was in Fig. 1. The position of LPS II is indicated by the large arrow on the left side of the panel. (B and C) Immunoblots of gels duplicating the one shown in panel A reacted with rabbit polyclonal antiserum against CFN42 (B) or ANU843 (C). The LPS I of TB112(pCOS109.11) was detected in lane 4 of panel B but not in lane 4 of panel C. Note that protein bands were also observed because, unlike in Fig. 1, the samples were not treated with proteinase K. The antisera were raised by injecting rabbits with bacterial cells rather than purified LPS.

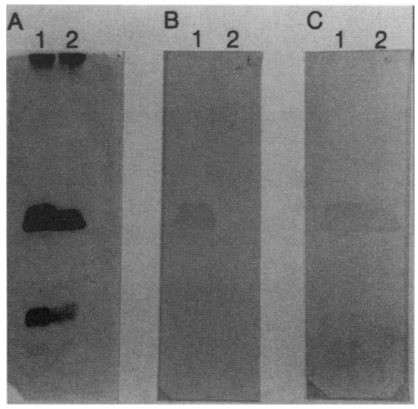

FIG. 3. Reaction of TB112(pCOS109.11) LPS with monoclonal antibodies that recognize LPS I of strain CFN42. SDS-PAGE and immunoblotting were performed as described in the legend to Fig. 2 by using the following strains: CFN42 (lanes 1) and TB112(pCOS 109.11) (lanes 2). (A) Periodate silver-stained gel. (B) Immunoblot of a gel duplicating the one shown in panel A reacted with monoclonal antibody preparation JIM28. Monoclonal antibodies JIM27 and JIM29 gave similar reactions. (C) Immunoblot of a gel duplicating the one shown in panel A reacted with monoclonal antibody JIM26.

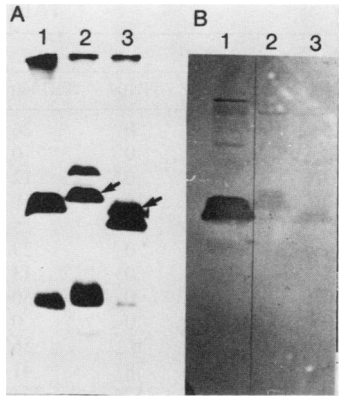

FIG. 5. Expression of pCOS109.11 in *R. leguminosarum* biovar *trifolii* TB4 and *R. leguminosarum* biovar *viciae* LB2. SDS-PAGE and immunoblotting were performed as described in the legend to Fig. 2 by using the following strains: CFN42 (lanes 1), LB2 (pCOS 109.11) (lanes 2), and TB4(pCOS109.11) (lanes 3). (A) Periodate silver-stained gel. Arrows indicate the LPS I molecule that was unique to LB2(pCOS109.11) (lane 2) and TB4(pCOS109.11) (lane 3). (B) Immunoblot of a gel duplicating the one shown in panel A reacted with antiserum against CFN42.

was not the same as that of Lps$^+$ strain TB4, from which mutant TB112 was derived; rather, it was similar to that of strain CFN42, the source of the cloned DNA (Fig. 2A). The LPS I of TB112(pCOS109.11) did not cross-react with anti-ANU843 antisera but reacted slightly with anti-CFN42 antisera (Fig. 2B and C, lanes 4). Four monoclonal antibodies that recognized four distinct structures within LPS I of CFN42 were tested against LPS of transconjugant TB112 (pCOS109.11) by immunoblotting. Only monoclonal antibody JIM26 reacted with the LPS I of TB112(pCOS109.11) (Fig. 3).

When the overlapping plasmid, pCOS109.10, was transferred to mutant TB112, no LPS I was produced. This suggests that at least the two *Eco*RI fragments on the left end of the CFN42 DNA of pCOS109.11 (Fig. 4) are needed, since they were not present in pCOS109.10. To determine whether other DNA fragments of pCOS109.11 were required, various plasmids carrying rhizobial DNA fragments from pCOS 109.11 were transferred to TB112. Derivative plasmids pE109, pE347, pDEL2, pDEL11, pDEL15, and pDEL2-2 (Fig. 4) (J. Cava, Ph.D. dissertation, Marquette University, Milwaukee, Wis., 1988; J. R. Cava, H. Tao, and K. D. Noel, Mol. Gen. Genet., in press) were tested; but only pDEL2 was able to complement TB112 to Lps I$^+$. The LPS I made in TB112(pDEL2) transconjugants was identical in SDS-PAGE mobility and antigenicity to the LPS I of TB112 (pCOS 109.11) transconjugants (data not shown).

Expression of cloned CFN42 *lps* genes in other wild-type *R. leguminosarum* strains. The plasmids pCOS126, pCOS 109.10, and pCOS109.11 were transferred to strain TB4 (*R. leguminosarum* biovar *trifolii*) and strain LB2 (*R. leguminosarum* biovar *viciae*). Plasmid pCOS126 carried the 7.5-kilobase (kb) *Eco*RI fragment of pDEL27 and 20 kb of contiguous DNA from strain CFN42. It had no effect on the LPS of either strain, as detected by SDS-PAGE. On the other hand, when strain TB4 or LB2 contained pCOS109.11, two LPS I species were produced (Fig. 5, lanes 2 and 3). One of these was the normal LPS I of the recipient. The other LPS I was weakly recognized by anti-CFN42 but not by antisera against the LPS of the recipient strains. In TB4 (pCOS109.11) the SDS-PAGE mobility of this second LPS band was the same as that of the LPS I of transconjugant TB112(pCOS109.11), whereas the LPS I unique to transcon-

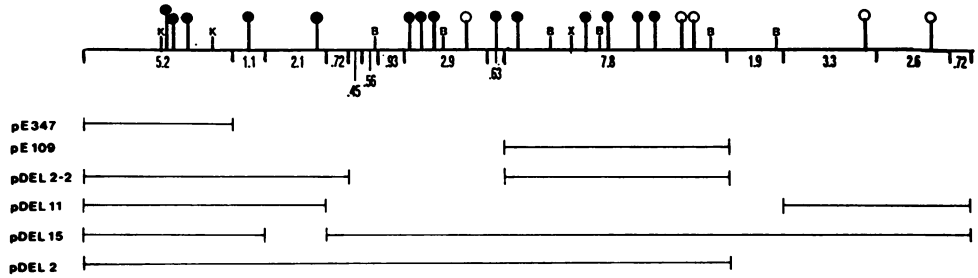

FIG. 4. Restriction map of the rhizobial DNA of pCOS109.11 and its derivative plasmids (Cava, Ph.D. dissertation). The plasmids derived from pCOS109.11 are indicated by the bars below the restriction map. The plasmids designated with the prefix pE are fragments of pCOS 109.11 subcloned into pRK290 (12). Those indicated with the prefix pDEL are deletion derivatives of pCOS109.11 carrying the rhizobial DNA indicated by the bars. Sites at which Tn*5* insertions affect LPS synthesis in CFN42 are indicated by solid circles above the restriction map, whereas open circles represent sites at which insertions have no effect on LPS (Cava et al., in press). Distances (in kilobase pairs) between *Eco*RI sites are shown just below the line that represents the rhizobial DNA of pCOS109.11. Other restriction sites are indicated just above this line. Abbreviations: B, *Bam*HI; K, *Kpn*I; X, *Xho*I.

TABLE 2. Sugar composition of purified LPS

Component[a]	Content (μg of sugar per mg of LPS) in:								
	CFN42	ANU843[b]	TB104	TB104(pCOS126)[b]	TB112	TB112(pCOS109.11)	TB4(pCOS109.11)	LB2(pCOS109.11)	128C53
20M6DH[c]	25	37 (10)	0	26 (10)	0	30	45	10	0
30M6DH[c]	48	0 (0)	0	0 (0)	0	58	23	21	0
Fucose	63	19 (5.3)	0	12 (4.7)	0	48	33	64	160
Mannose	40	24 (6.6)	19	16 (6.2)	17	32	42	57	120
Galactose	23	22 (6.1)	tr[d]	15 (5.8)	tr	22	37	19	22
3NM36DH	0	41 (11)	0	29 (11)	0	0	40	0	0
2A26DH	15	18 (5.0)	0	13 (5.0)	0	8.4	25	20	0
Heptose	0	73 (20)	0	50 (19)	0	0	76	0	0
Rhamnose	0	0 (0)	0	0 (0)	0	0	0	35	130
Uronate	118[e]	87[e] (24)	tr	56[e] (22)	tr	70[e]	92[e]	76[e]	97[f]
KDO	52	40 (11)	38	41 (16)	10	34	34	53	27
Total	384	361	57	258	27	302	447	355	556

[a] Abbreviations: 2OM6DH, 2-*O*-methyl-6-deoxyhexose; 3OM6DH, 3-*O*-methyl-6-deoxyhexose; 3NM36DH, 3-*N*-methyl-3,6-dideoxyhexose; 2A26DH, 2-amino-2,6-dideoxyhexose; KDO, 3-deoxy-D-*manno*-2-octulosonic acid.

[b] Percentages (in parentheses) are of total detected carbohydrate.

[c] These are *O*-methylrhamnoses in strain CFN42 and in the transconjugants in which they appeared (R. W. Carlson, unpublished data).

[d] tr, Trace amounts.

[e] The uronic acid values are for the sum of glucuronic and galacturonic acids, both of which are present in the indicated LPSs.

[f] The LPS of 128C53 contained only galacturonic acid.

jugant LB2(pCOS109.11) migrated more slowly. When pCOS109.10 was introduced into strain TB4 or LB2, only the normal LPS I of the recipient was synthesized. These results and the complementation of mutant TB112 demonstrate that pCOS109.11 encodes enough information to direct O-antigen synthesis, but the resulting LPS I somehow differs antigenically from CFN42 LPS I.

LPS sugar compositions. The LPS sugar compositions of various strains are given in Table 2. Mutants TB104 and TB112 lacked the O-antigen sugars of ANU843: a 2-*O*-methyl-6-deoxyhexose, fucose, a 3-*N*-methyl-3,6-dideoxyhexose, a 2-amino-2,6-dideoxyhexose, and heptose. In addition, the LPS of both mutants contained drastically reduced amounts of galactose and galacturonic acid, both of which are sugars of the conserved oligosaccharides. The LPS sugar composition of transconjugant TB104(pCOS126) was similar to that of ANU843, the grandparent of strain TB104. Although gas chromatographic analysis gave a lower value for total sugars in the former preparation, the relative content of each glycosyl residue within the total sugars was almost identical in the two preparations.

Composition analysis of LPS from TB112(pCOS109.11) indicated that a 3-*O*-methyl-6-deoxyhexose unique to CFN42 LPS was present. Moreover, a 3-*N*-methyl-3,6-dideoxyhexose and heptose, the two sugars unique to ANU843 LPS, were missing. When pCOS109.11 was transferred to wild-type strains TB4 (*R. leguminosarum* biovar *trifolii*) and LB2 (*R. leguminosarum* biovar *viciae*), two LPS I molecules were synthesized. Composition analysis of total LPS revealed that these transconjugants contained *O*-antigen sugars unique to CFN42, in addition to the sugars of the recipient strain TB4 or LB2 (Table 2). For example, transconjugant LB2(pCOS109.11) contained four CFN42 O-antigen sugars (a 2-*O*-methyl-6-deoxyhexose, a 3-*O*-methyl-6-deoxyhexose, glucuronic acid, and a 2-amino-2,6-dideoxyhexose) plus those sugars that are normally found in strain LB2.

Plant tests. Like Lps mutants of *R. leguminosarum* biovar *phaseoli* CFN42 (19), mutants TB104 and TB112 elicited nodules that emerged 2 to 3 days later than normal and that never completely developed. Four weeks after inoculation, mutant-inoculated roots displayed only 2% of the nitrogenase activity observed in roots inoculated with wild-type strain TB4. The mutant-induced nodules remained small and white thereafter, and their weak nitrogenase activity did not increase, whereas the large, red nodules induced by strain TB4 continued to increase in activity. The mutant-inoculated plants had stunted and chlorotic shoots, indicative of nitrogen deficiency. The nodules induced by strains TB104 and TB112 were observed microscopically 6 weeks after the inoculation of plants. The number of infected plant cells in mutant-induced nodules was much less than that in nodules elicited by the wild type. Furthermore, bacterial proliferation in the infected cells was less extensive than it was in nodules induced by the wild type (Fig. 6). A total of 10- to 100-fold fewer viable bacteria per nodule were isolated from the mutant-induced nodules compared with those from wild-type-induced nodules.

The complemented mutants carrying CFN42 *lps* DNA were tested for nodulation of clover. Transconjugants TB104(pCOS126) and TB112(pCOS109.11) elicited large, red nodules that displayed wild-type levels of nitrogenase activity. In light micrographs, nodules induced by TB104 (pCOS126) and TB112(pCOS109.11) were indistinguishable from nodules induced by wild-type strain TB4 (Fig. 6). The nodules were well developed and fully infected. Transconjugants TB4(pCOS109.11) and LB2(pCOS109.11) were tested for nodulation proficiency on their respective host plants, clover and pea. Both nodulated as well as present strains TB4 and LB2 did.

DISCUSSION

It appears that *lps* DNA cloned in pDEL27 specifies functions which are conserved between *R. leguminosarum* CFN42 and ANU843. One mutant derived from strain ANU843 was restored to the LPS characteristic of ANU843 by plasmid pDEL27, even though the cloned DNA was from strain CFN42. In addition, the presence of this DNA in wild-type strains ANU843 and 128C53 [TB4(pCOS126) and LB2(pCOS126)] did not affect LPS content, consistent with the idea that this CFN42 *lps* region encodes functions that duplicate those that are already present. One explanation for the Lps phenotype of TB104(pDEL27) is that the *lps* DNA of pDEL27 encodes an enzyme(s) that is involved in the

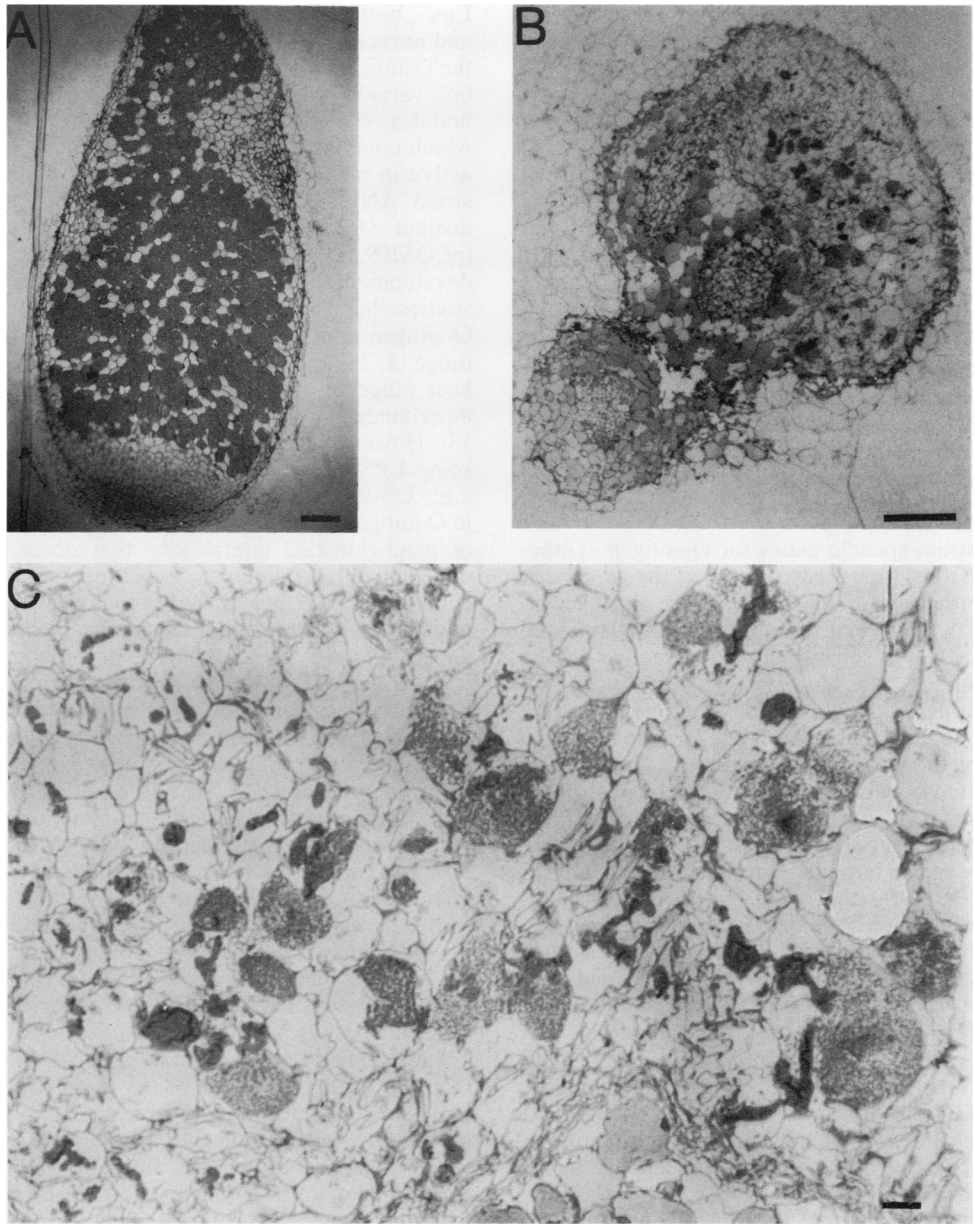

FIG. 6. Light microscopy of clover nodules. (A) Thick section of a nodule induced by transconjugant TB104(pCOS126) 4 weeks after inoculation. Bar, 100 μm. (B) Thick section of a nodule induced by TB112 6 weeks after inoculation. Bar, 100 μm. (C) A region of the nodule section shown in panel B was enlarged to show more clearly the infected cells. Bar, 10 μm.

synthesis of the conserved portion (core) of the *R. leguminosarum* LPS polysaccharide. Restoration of a normal core to TB104 would then allow for attachment of the ANU843 O antigen encoded by DNA elsewhere in the genome. This possibility is supported by analysis of the composition of mutant TB104 LPS. Not only were O-antigen sugars lacking, but only trace amounts of the core sugars galactose and galacturonic acid were detected. Therefore, it seems that TB104 is missing a function required for core synthesis and that pDEL27 DNA restores this function.

On the other hand, some of the *lps* genes cloned in pCOS 109.11 specified strain-specific functions that were required for synthesis of the O antigen of strain CFN42. This conclusion was supported by analysis of mutant TB112 when it carried this DNA. Mutant TB112 was derived from strain ANU843. However, the LPS of transconjugant TB112 (pCOS109.11) did not react with antiserum prepared against ANU843 cells, and it did not contain either of the LPS sugars unique to ANU843. Instead, the LPS of this transconjugant contained all of the sugars previously reported (6) to be present in the LPS of strain CFN42, including a 3-*O*-methyl-6-deoxyhexose unique to CFN42. Likewise, the presence of pCOS109.11 DNA in wild-type strains TB4 (from wild-type isolate ANU843) and LB2 (from wild-type isolate 128C53) conferred on them an additional LPS I that reacted antigenically like the LPS I of transconjugant TB112(pCOS109.11). From a comparison of the LPS compositions of strain LB2 and transconjugant LB2(pCOS109.11), it appears that pCOS 109.11 DNA carries at least the genes needed to incorporate four O-antigen sugars of strain CFN42 into LPS molecules.

Although the unique LPS I specified by pCOS109.11 DNA in ANU843 and 128C53 genomic backgrounds resembled CFN42 LPS I, immunoblot analysis showed that it is not identical to LPS I of CFN42. The reaction with polyclonal antiserum was weak, and only one of four monoclonal antibodies recognized the LPS I of transconjugant TB112 (pCOS109.11). Recent analysis (R. W. Carlson, unpublished data) has revealed the presence of tri-*O*-methylfucose in CFN42 LPS and its absence from the LPS of transconjugant strains TB112(pCOS109.11), TB4(pCOS109.11), and LB2 (pCOS109.11). Perhaps this residue is part of one or more major structural determinants for the binding of CFN42-specific antibodies. Apparently, strain-specific DNA outside the *lps* DNA cloned in pCOS109.11 is required to synthesize and attach this residue. It may be pertinent that the only monoclonal antibody that reacted with LPS of transconjugant TB112(pCOS109.11) also reacts with LPS III, a minor SDS-PAGE band exhibited by certain mutants derived from strain CFN42 (8; B. A. Brink, E. L. Kannenberg, and N. J. Brewin, unpublished data).

In addition to strain-specific genes for O-antigen synthesis, plasmid pCOS109.11 must carry some of the genes that are needed for synthesis of the conserved core oligosaccharides. One mutant derivative of strain CFN42 complemented to Lps^+ by pCOS109.11 appears to be defective in the conserved LPS oligosaccharides (R. W. Carlson, R. L. Hollingsworth, and K. D. Noel, Carbohydr. Res., in press). Composition data suggest that strain TB112 is also defective in the synthesis of the core oligosaccharides. Therefore, if the mutation affected only one gene, it should be possible to complement strain TB112 so that wild-type ANU843 LPS is restored by transferring a portion of pCOS109.11 DNA that contains this core gene but not O-antigen genes. Of six derivatives of pCOS109.11 that were tested, only pDEL2, containing approximately 22.4 kb of DNA, was able to confer LPS I synthesis. This LPS I appeared to be identical to the LPS I synthesized when the entire cosmid was present; it was not ANU843 LPS I. Indeed, if TB112 carries a mutation that affects only a core gene, it is paradoxical that pCOS109.11 and pDEL2 do not confer upon TB112 both ANU843 LPS I and the other LPS I, as occurs in the wild-type strain TB4 carrying pCOS109.11. A possible explanation is that the mutation of TB112 is a polar mutation or a deletion, eliminating genes for both core and ANU843 O-antigen synthesis.

Genetic regions specifying the LPS core (*rfa*) or O antigen (*rfb*) have been transferred between enteric bacteria (16). The results are analogous to those of this report, in that *rfa* genes from one species restored to a Rfa^- mutant of another species its native LPS, whereas transferred *rfb* genes conferred the O antigen of the donor. Also in accordance with the results of this report, a *Salmonella* strain carrying two rfb^+ regions from different strains expresses antigenic characters of both O antigens. Of particular relevance are transfers of *rfb* DNA from *Shigella flexneri* into *E. coli* K-12 (which lacks O antigen). The transconjugants express the basic structure of the *S. flexneri* O polysaccharide but lack the serotype specificity of the *S. flexneri* donor, because of the absence of glucosylations and O acetylations directed by *S. flexneri* prophage genes that map elsewhere.

Mutants TB104 and TB112 elicited poorly developed nodules with much lower than normal bacterial populations. The reduction in nitrogen-fixing activity was probably due solely to poor nodule development and lower bacterial population; it was not necessary to invoke an effect of Lps^- on the activity of individual bacteroids. When restored to Lps^+, both strains formed nodules with normal development and normal levels of nitrogen-fixing activity. It appears that the O antigen is required for successful nodulation of clover, but variation in O-antigen structure is tolerated. Clover nodules elicited by transconjugant TB112(pCOS109.11), which contains a unique LPS, were as fully developed and active in nitrogen fixation as nodules elicited by wild-type strain ANU843 were. In addition, the presence of two distinct O antigens, as in TB4(pCOS109.11) and LB2 (pCOS109.11), did not appear to inhibit clover or pea nodule development. These results are consistent with chemical studies that have shown that there is great variability in O-antigen compositions among strains with the same host range (4, 26) and genetic studies which have shown that the host range of an *R. leguminosarum* strain is changed by inheritance of a different Sym plasmid or *nod* gene cluster (3, 13). However, it remains an open question whether there is some LPS structural element that depends on host range, e.g., *hsn*-specified modifications. Moreover, subtle changes in O-antigen structure may affect the efficiency or frequency of plant-rhizobial interactions that occur early in nodule development. Preliminary experiments suggest that transconjugant TB112(pCOS109.11) elicits certain early events of nodulation, including nodule meristem induction, at a reduced frequency (Frank Dazzo, personal communication).

The roles of LPS in the rhizobium-legume symbiosis are not clear, since Lps mutants of various strains have different nodulation phenotypes. A *Bradyrhizobium japonicum* 110 Lps^- derivative fails to elicit any visible nodule development on soybeans (21). On the other hand, LPS O antigen may not be required at all in symbiosis by *Rhizobium galegae* and *Rhizobium meliloti* SU47 (9, 15). Lps mutants of *R. leguminosarum* CFN42 induce nodules on beans in which infection threads are initiated but usually abort within the root hair cell (8, 19). The interiors of these nodules are devoid of bacteria, and nitrogenase activity is not detected at all. The Lps mutants described in this report induced clover root nodules which were infected sufficiently to give very weak nitrogenase activity. *R. leguminosarum* Lps mutants behave similarly on *Vicia hirsuta* (9, 20). Therefore, it seems that the extent of infection by Lps^- *R. leguminosarum* varies in a host-dependent manner. One explanation, invoking LPS as a signal molecule, is that the requirement for the signal becomes critical at different times during nodule development in different hosts. Another possibility is that LPS may protect the bacteria from host toxins or otherwise suppress host defense mechanisms. In that case, the extent of infection by the Lps mutants might depend on the severity of the host defenses or the timing, if the defenses are induced by the presence of the bacteria.

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LITERATURE CITED

1. **Appelbaum, E. R., D. V. Thompson, K. Idler, and N. Chartrain.** 1988. *Rhizobium japonicum* USDA 191 has two *nodD* genes that differ in primary structure and function. J. Bacteriol. **170**:12–20.
2. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84**:188–198.
3. **Beynon, J. L., J. E. Beringer, and A. W. B. Johnston.** 1980. Plasmids and host-range in *Rhizobium leguminosarum* and *Rhizobium phaseoli*. J. Gen. Microbiol. **120**:421–429.
4. **Carlson, R. W.** 1984. Heterogeneity of *Rhizobium* lipopolysaccharides. J. Bacteriol. **158**:1012–1017.
5. **Carlson, R. W., R. L. Hollingsworth, and F. B. Dazzo.** 1988. A core oligosaccharide component from the lipopolysaccharide of *Rhizobium trifolii* ANU843. Carbohydr. Res. **176**:127–135.
6. **Carlson, R. W., S. Kalembasa, D. Turowski, P. Pachori, and K. D. Noel.** 1987. Characterization of the lipopolysaccharide from a *Rhizobium phaseoli* mutant that is defective in infection thread development. J. Bacteriol. **169**:4923–4928.
7. **Carlson, R. W., R. Shatters, J.-L. Duh, E. Turnbull, B. Hanley, B. G. Rolfe, and M. A. Djordjevic.** 1987. The isolation and partial characterization of the lipopolysaccharides from several *Rhizobium trifolii* mutants affected in root hair infection. Plant Physiol. **84**:421–427.
8. **Cava, J. R., P. M. Elias, D. A. Turowski, and K. D. Noel.** 1989. *Rhizobium leguminosarum* CFN42 genetic regions encoding lipopolysaccharide structures essential for complete nodule development on bean plants. J. Bacteriol. **171**:8–15.
9. **Clover, R. H., J. Kieber, and E. R. Signer.** 1989. Lipopolysaccharide mutants of *Rhizobium meliloti* are not defective in symbiosis. J. Bacteriol. **171**:3961–3967.
10. **deMaagd, R. A., A. S. Rao, I. H. M. Mulders, L. G. Roo, M. C. M. van Loosdrecht, C. A. Wijffelman, and B. J. J. Lugtenberg.** 1989. Isolation and characterization of mutants of *Rhizobium leguminosarum* bv. *viciae* 248 with altered lipopolysaccharides: possible role of surface charge or hydrophobicity in bacterial release from the infection thread. J. Bacteriol. **171**:1143–1150.
11. **Diebold, R., and K. D. Noel.** 1989. *Rhizobium leguminosarum* exopolysaccharide mutants: biochemical and genetic analyses and symbiotic behavior on three hosts. J. Bacteriol. **171**:4821–4830.
12. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**:7347–7351.
13. **Djordjevic, M. A., R. W. Innes, C. A. Wijffelman, P. R. Schofield, and B. G. Rolfe.** 1986. Nodulation of specific legumes is controlled by several distinct loci in *Rhizobium trifolii*. Plant Mol. Biol. **6**:389–401.
14. **Gresshoff, P. M., and B. G. Rolfe.** 1978. Viability of *Rhizobium* bacteroids isolated from soybean nodule protoplasts. Planta **142**:329–333.

14a. **Hollingsworth, R. I., R. W. Carlson, F. Garcia, and D. A. Gage.** 1989. A new core tetrasaccharide component from the lipopolysaccharide of *Rhizobium trifolii* ANU843. J. Biol. Chem. **264**: 9294–9299.

15. **Lipsanen, P., and K. Lindstrom.** 1989. Lipopolysaccharide and protein patterns of *Rhizobium* sp. (*Galega*). FEMS Microbiol. Lett. **58**:323–328.
16. **Makela, P. H., and B. A. D. Stocker.** 1984. Genetics of lipopolysaccharide, p. 59–137. *In* E. T. Rietschel (ed.), Handbook of endotoxin, vol. 1: chemistry of endotoxin. Elsevier Science Publishing, Inc., New York.
17. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 433. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Noel, K. D., A. Sanchez, L. Fernandez, J. Leemans, and M. A. Cevallos.** 1984. *Rhizobium phaseoli* symbiotic mutants with transposon Tn*5* insertions. J. Bacteriol. **158**:148–155.
19. **Noel, K. D., K. A. VandenBosch, and B. Kulpaca.** 1986. Mutations in *Rhizobium phaseoli* that lead to arrested development of infection threads. J. Bacteriol. **168**:1392–1401.
20. **Priefer, U. B., S. Priesler, C. Schmidt, D. Kapp, and A. Puhler.** 1988. *Rhizobium leguminosarum* cell surface mutants affected in their symbiotic ability, p. 51–52. *In* R. Palacios and D. P. S. Verma (ed.), Molecular genetics of plant-microbe interactions. American Phytopathological Society, St. Paul, Minn.
21. **Puvanesarajah, V., F. M. Schell, D. Gerhold, and G. Stacey.** 1987. Cell surface polysaccharides from *Bradyrhizobium japonicum* and a nonnodulating mutant. J. Bacteriol. **169**:137–141.
22. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1**:784–791.
23. **VandenBosch, K. A., K. D. Noel, Y. Kaneko, and E. H. Newcomb.** 1985. Nodule initiation elicited by noninfective mutants of *Rhizobium phaseoli*. J. Bacteriol. **162**:950–959.
24. **Wacek, T., and W. J. Brill.** 1976. Simple, rapid assay for screening nitrogen fixing ability in soybean. Crop Sci. **16**: 519–522.
25. **York, W. S., A. G. Darvill, M. McNeil, T. T. Stevenson, and P. Albersheim.** 1985. Isolation and characterization of plant cell walls and cell-wall components. Methods Enzymol. **118**:3–40.
26. **Zevenhuizen, L. P. T. M., I. Scholten-Koerselman, and M. A. Posthumus.** 1980. Lipopolysaccharides of *Rhizobium*. Arch. Microbiol. **125**:1–8.