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Eight symbiotic mutants defective in lipopolysaccharide (LPS) synthesis were isolated from *Rhizobium leguminosarum* biovar *phaseoli* CFN42. These eight strains elicited small white nodules lacking infected cells when inoculated onto bean plants. The mutants had undetectable or greatly diminished amounts of the complete LPS (LPS I), whereas amounts of an LPS lacking the O antigen (LPS II) greatly increased. Apparent LPS bands that migrated between LPS I and LPS II on sodium dodecyl sulfate-polyacrylamide gels were detected in extracts of some of the mutants. The mutant strains were complemented to wild-type LPS I content and antigenicity by DNA from a cosmid library of the wild-type genome. Most of the mutations were clustered in two genetic regions; one mutation was located in a third region. Strains complemented by DNA from two of these regions produced healthy nitrogen-fixing nodules. Strains complemented to wild-type LPS content by the other genetic region induced nodules that exhibited little or no nitrogenase activity, although nodule development was obviously enhanced by the presence of this DNA. The results support the idea that complete LPS structures, in normal amounts, are necessary for infection thread development in bean plants.

Lipopolysaccharide (LPS) is found on the outer membrane of gram-negative bacteria. It consists of lipid A, which anchors it to the outer membrane, and a polysaccharide portion that extends into the cell surroundings. Besides being necessary for the structural integrity of the outer membrane, LPS molecules are likely to be involved in important interactions between the cell and its environment. LPS is proposed to protect bacterial pathogens against animal host defenses (20, 30, 35, 38). It may protect against plant defenses also. Mutants of the plant pathogen *Erwinia chrysanthemi* having severe LPS alterations exhibit avirulence which may have resulted from bacterial susceptibility to plant defenses (37). Strains of *Pseudomonas solanacearum*, another plant pathogen, are avirulent when both LPS and exopolysaccharide are altered (13).

Bacteria of the genera *Rhizobium* and *Bradyrhizobium* form a nitrogen-fixing symbiosis with leguminous plants. The bacteria infect the plant roots and reside in nodules, whose development is induced by the bacteria. Various roles for rhizobial LPS in the symbiosis have been proposed.

It has been suggested that LPS polysaccharides interact with the lectins of legumes (42) as a host-specific recognition step (17). Hrabak et al. (14) reported that growth-phase-dependent changes occurred in the LPS composition of one strain of *Rhizobium leguminosarum* and that these changes may be required for recognition of the LPS by white clover. Other studies, however, have reported contradictory or mixed results concerning LPS and host-specific lectin binding (6, 16, 32). Moreover, strains that nodulate the same host can have very different LPS compositions (7). Although in at least some systems legume lectins probably do interact with LPS, such interactions are not necessarily limited to attachment to the root surface or to host specificity.

A few rhizobial Lps mutants have been described. A nonnodulating mutant of *Bradyrhizobium japonicum* has been shown to lack a substantial portion of the LPS polysaccharide (34). Another mutant strain of *B. japonicum* nodulates very slowly (40) and is reported to lack portions of the LPS polysaccharide (21). ExoB⁻ and ExoC⁻ mutants of *Rhizobium meliloti* SU47 are altered in LPS as well as exopolysaccharide (19).

The LPS polysaccharide of *R. leguminosarum* appears to consist of an inner portion, relatively conserved among related strains, and an outer O-antigen portion, whose structure varies in a strain-dependent manner (7, 8). Recently, two Lps mutants of *R. leguminosarum* bv. *phaseoli* CFN42 have been described (29). The LPS of each mutant is missing the O antigen (9, 29). These mutants induce bean root nodules in which infection is initiated. However, the infection threads cease to develop, usually within the infected root hair, and as a consequence no plant cells in the nodule become infected. In each mutant, the LPS defect and the abortive infection phenotype appear to be due to the same mutation (29). The phenotype is consistent with LPS being required to protect against host defenses, among other possibilities. It is not consistent with a role for O antigen in a host-specific event required for nodule induction.

In this report, we describe the isolation, properties, and genetic analysis of other Lps mutants of strain CFN42. The mutants exhibited the symbiotic defect of the two mutants described previously (9, 29). The majority of *lps* mutations were clustered in two genetic regions. Complementation of each mutation with cloned DNA restored apparently complete LPS synthesis. Concomitantly, ability to infect roots was improved. This work represents the initial genetic analysis of LPS in this strain of *R. leguminosarum*. It gives further support to an essential role of LPS O antigen during rhizobial infection of bean plants.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains used are listed in Table 1. *Rhizobium* strains were grown on TY medium (41) at 30°C. *Escherichia coli* strains were cultured at 37°C in LB medium (26). Solid media contained 15 g of

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics[a]	Reference
Strain		
R. leguminosarum[b]		
CE3	*str-1*	28
CE8	*ery-1*	27a
CE109	*lps-109*::Tn*5 str-1* Lps⁻ Ndv⁻	29
CE113	*lps-113 str-1* Tn*5* Lps⁻ Ndv⁻	29
CE121	*lps-121*::Tn*5 str-1* Lps⁻ Ndv⁻	This work
CE125	*lps-125 str-1* Lps⁻ Ndv⁻	This work
CE126	*lps-126 str-1* Lps⁻ Ndv⁻	This work
CE148	*lps-148 str-1*, Tn*5* Lps⁻ Ndv⁻	This work
CE166	*lps-166*::Tn*5 str-1* Lps⁻ Ndv⁻	This work
CE168	*lps-168*::Tn*5 str-1* Lps⁻ Ndv⁻	This work
CE183	*lps-183*::Tn*5 str-1* Lps⁻ Ndv⁻	This work
CE309	*lps-309*::Tn*5 str-1* Lps⁻ Ndv⁻	This work
E. coli		
HB101	*recA* Str^r Eyr^r	23
1843	pJB3; Tc^r	2
Plasmid		
pSUP202	pBR325 derivative	39
pSUP2021	pSUP202::Tn*5*	39
pRK209	Broad host range; Tc^r Tra⁻	10
pLAFR1	Cosmid derivative of pRK290	12
pRK2013	Tra⁺ Km^r	10
pCOS109.11	30.1-kbp DNA insert in pLAFR1	This work
pCOS126	27.0-kbp DNA insert in pLAFR1	This work
pE109	7.8-kbp *Eco*RI fragment in pRK290	This work
pE168	7.5-kbp *Eco*RI fragment in pRK290	This work
pDEL23	Deletion derivative of pCOS109.11	This work

[a] *str-1* and *ery-1* confer resistance to streptomycin and erythromycin, respectively. Lps⁻ implies defective LPS rather than absence of LPS; Ndv⁻, induced nodules are defective in development (and lack nitrogenase activity).

[b] All *R. leguminosarum* strains are derivatives of the field isolate CFN42, which nodulates bean plants (28). Strains CE125 and CE126 were isolated after nitrosoguanidine mutagenesis. The others were isolated after Tn*5* mutagenesis. In mutants CE113 and CE148, the *lps* mutations are not due to Tn*5* insertion.

agar (Difco Laboratories, Detroit, Mich.) per liter. Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were incorporated into the growth media at the following concentrations (in micrograms per milliliter): kanamycin, 30; erythromycin, 10; streptomycin, 200; nalidixic acid, 30; and tetracycline, 5 for *Rhizobium* cultures and 15 for *E. coli* cultures.

Isolation of mutants. Strain CE3 or CE8 was mutagenized with nitrosoguanidine (27a) or transposon Tn*5* by means of the suicide vector pSUP2021 (39). Resulting bacterial clones were screened on bean plants grown in agar vials for symbiotic nitrogenase activity (28). A total of 11 of the 1,200 survivors from nitrosoguanidine treatment and 39 of the 4,060 kanamycin-resistant clones after Tn*5* mutagenesis were symbiotically defective. Independently generated strains confirmed to be symbiotic mutants were then screened by sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE) for altered LPS banding patterns.

Colony morphology was the basis for a second isolation procedure. After mutagenesis, colonies were screened for their appearance on TY agar. Those having a dry or rough appearance were screened further for LPS characteristics on SDS-PAGE. This screening procedure works only on a medium, such as TY, that suppresses exopolysaccharide

production. Lps mutants isolated by either procedure grew at normal rates in minimal liquid or on agarose media.

Gel electrophoresis. Cell cultures were harvested after full growth in TY broth by centrifugation of 1-ml samples at 13,000 × *g* for 1 min. Pelleted cells were washed once in 1 ml of 10 mM Tris–5 mM MgCl₂–10 mM 2-mercaptoethanol (pH 7.5) and then suspended in 130 μl of SDS sample buffer (18) (0.0625 M Tris [pH 6.8], 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.001% bromophenol blue). Dialyzed phenol-water extracts or column fractions were diluted directly into SDS buffer. After discontinuous SDS- (18) or deoxycholate (DOC)-PAGE (25), gel slabs were stained with a silver staining kit (Bio-Rad Laboratories, Richmond, Calif.) according to the instructions of the manufacturer with the following modification: after being fixed in 40% methanol–10% acetic acid, gels were treated with 0.7% sodium metaperiodate, 0.65% isopropanol, and 0.26% glacial acetic acid for 5 min, followed by a 30-min wash in glass-distilled water. After this treatment, the procedure suggested by the manufacturer was continued. SDS-plus-DOC-gel electrophoresis was modified from a DOC-PAGE procedure (25). Samples were prepared in SDS sample buffer (as described above) in place of DOC sample buffer (25). The stacking gel was composed of 3% acrylamide, and the resolving gel was 15% acrylamide. Samples were applied without preelectrophoresis (25) of the gel. After electrophoresis, the gel was shaken for 2 days in several changes of 40% methanol–10% acetic acid until the gel was free of a milky white precipitate. Staining then was performed as above. LPS III and LPS IV were observed after SDS, DOC, or SDS-plus-DOC electrophoresis, but the resolution of the bands was best by the SDS-plus-DOC method.

Immunoblots. Polyclonal rabbit antisera were developed against washed rhizobial cells, and gels were electroblotted onto nitrocellulose and stained by indirect enzyme-linked immunosorbence as described previously (9).

Light microscopy. Nodules were harvested from plants 8 and 14 days after inoculation. Specimens were fixed, embedded in Spurr resin, and stained with toluidine blue, using the procedures outlined previously (41).

Bacterial crosses. Intergeneric triparental matings (10) to effect complementation were performed by mixing 0.2 ml of the *E. coli* donor with 0.2 ml of the helper strain [HB101 (pRK2013)] and 0.5 ml of the *R. leguminosarum* recipient strain. All cultures had been grown to full density. The mixture was spread on a TY agar plate and incubated overnight at 30°C. Mating plates were suspended in 3 ml of 0.1 M MgSO₄, and a dilution series was plated onto TY agar containing nalidixic acid (recipient selection) and tetracycline (plasmid selection). *R. leguminosarum-R. leguminosarum* matings for determining chromosomal linkage were performed in essentially the same manner except that 0.5 ml of both donor and recipient were used. Recipients carrying Tn*5* were selected on TY agar containing kanamycin and erythromycin.

DNA isolation and manipulation. Construction of the cosmid library of the genome of strain CFN42 has been described (27a). The library was maintained in a mixture of *E. coli* HB101 cells cultured in LB medium with tetracycline and stored in 16% glycerol at −70°C. Cosmids identified by complementation of particular rhizobial mutants were returned to *E. coli* HB101 by transformation and maintained in this strain for further analysis. Plasmid DNA was isolated by an alkaline lysis procedure (23). Total *Rhizobium* DNA was isolated and purified by the method of Meade et al. (24). *Eco*RI fragments containing the Tn*5* insertions from the
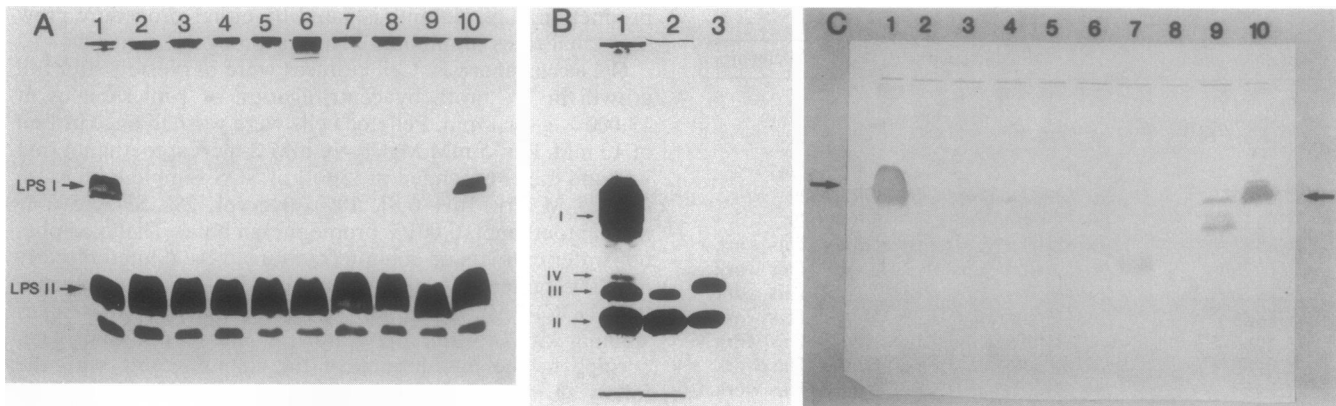
FIG. 1. Electrophoretic and immunological characterization of mutants. (A) SDS-PAGE of total SDS extracts of *R. leguminosarum* strains. Lanes: 1, CE3; 2, CE113; 3, CE126; 4, CE148; 5, CE168; 6, CE109; 7, CE121; 8, CE125; 9, CE309; 10, CE166. Characteristics of CE183 (not shown) were identical to those of CE125. Bands running below LPS II did not partition with LPS during phenol-water extraction. (B) SDS-plus-DOC-PAGE of cell extracts. Lanes: 1, CE3; 2, CE109; 3, CE121. Lanes were not adjacent during the run but were from the same slab. Suspected forms of LPS are indicated by arrows. Lanes carried 100 (lane 1), 18 (lane 2), and 35 (lane 3) μg of material from the dialyzed aqueous phases of phenol-water extracts. (C) Immunoblot, incubated with antiserum against CE3 cells, of a duplicate gel corresponding to the one shown in panel A. Arrows indicate the positions of LPS I. In each panel, electrophoresis was from top to bottom. The contents of 4 × 10⁸ cells were applied to each lane of panels A and C.

transposon-induced mutants were cloned into pSUP202 (39) by ligation of EcoRI-digested total *Rhizobium* and vector DNAs. *E. coli* HB101, after cold CaCl₂ treatment, was transformed with the ligation mixture by heat shock (23). Transformants were selected on LB agar with ampicillin and kanamycin. EcoRI fragments from the cosmid clones were subcloned into pRK290 after isolation of the desired fragments from low-temperature agarose-gel slices by phenol-chloroform extraction. All ligations were carried out with a 1:1 insert-to-vector ratio at 4°C, using T4 DNA ligase (Pharmacia Fine Chemicals, Piscataway, N.J.). DNA probes for colony and Southern hybridizations (23) were labeled with [³²P]dCTP (Dupont, NEN Research Products, Boston, Mass.), using a nick translation kit purchased from Amersham Corp., Arlington Heights, Ill. EcoRI restriction maps of pCOS109.11 and pCOS126 were determined by an in vitro deletion method (4).

LPS purification. A 50-liter culture of bacteria grown at 30°C in TY broth to early stationary phase was harvested. The cells were washed, frozen, and extracted with hot phenol-water (9). The material from the aqueous phase was dialyzed, freeze-dried, dissolved in water, and treated with RNase A (9). Samples with 100 to 200 mg of material were applied to a Sepharose 4B column (2.6 by 50 cm) in buffer composed of 0.30 M triethylamine and 0.10 M EDTA. Fractions were assayed for hexose (29) and 3-deoxy-D-manno-2-octulosonic acid (KDO) content (29). KDO-containing fractions were pooled, dialyzed against several changes of deionized water for 5 days, and freeze-dried.

DOC-Sephadex column chromatography of the phenol-water extract of mutant CE121 was a modification of the procedure described by Peterson and McGroarty (31). A Sephadex G-150-50 (Sigma) column (1.5 by 120 cm) was used with a column buffer consisting of 0.16 M NaCl, 0.2% DOC, 8 mM Tris, 0.016% NaN₃, and 0.1 M EDTA (pH 8.0). Phenol-water-extracted material from CE121 did not dissolve unless this higher concentration of EDTA was used. A 30-mg portion of the extract was dissolved in 2.0 ml of this buffer and applied to the column. KDO-containing fractions were analyzed by SDS-PAGE.

LPS sugar composition. Purified LPS was acid hydrolyzed, and alditol acetate derivatives (1) of monosaccharide com-

ponents were analyzed by gas chromatography on a Supelco SP2330 column, using a 5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, Calif.). The oven temperature was programmed to increase 2°C/min from 150 to 240°C. Uronic acids were identified by reduction of the carboxyl groups before acetylation was performed (11).

Plant tests. *Phaseolus vulgaris* L. cv. Negro jamapa was grown in pouches inoculated with *R. leguminosarum* bv. *phaseoli* as previously described (28). After plants were grown for 2 weeks, nodulation was scored. Nitrogenase activity was assayed by acetylene reduction. Nodules were isolated, surface sterilized in 1/10-strength commercial hypochlorite bleach, rinsed extensively with sterile water, and crushed. Liberated bacteria were counted and characterized by being spread on TY agar and replica plated onto selective media.

RESULTS

Characteristics of LPS from the wild-type strain. The purification, immunochemical properties, and chemical composition of LPS from wild-type strain CE3 have been reported previously (9, 29). On SDS-PAGE, purified LPS yields two prominent bands, LPS I and LPS II (9, 29). The same bands predominated when unfractionated detergent extracts of cells were analyzed by SDS- or DOC-PAGE and stained by periodate-silver as described in Materials and Methods (Fig. 1A, lane 1). A third band (band A), which migrated faster than did LPS II, was also detected (Fig. 1A, lane 1). This band may represent lipid A, since purified lipid A from strain CE3 (9) applied to SDS-PAGE gave a band with the same mobility. Two minor bands (III and IV) were revealed when unfractionated detergent extracts and the aqueous phases of phenol-water extracts (29) were loaded for SDS-plus-DOC-PAGE in high amounts (Fig. 1B, lane 1). Presence in the phenol-water extract, mobility in detergent-PAGE, and periodate-silver staining indicated that bands III and IV probably were LPS also.

Immunochemical analysis and examination of polysaccharide fragments after partial acid hydrolysis have shown that the O antigen of strain CE3 is in LPS I whereas LPS II lacks O antigen (9). For instance, when SDS-PAGE blots of

unfractionated cell extracts of CE3 were incubated with polyclonal antiserum directed against this strain, LPS I was the predominant band recognized (Fig. 1C, lane 1). LPS II was detected only slightly and only at concentration higher than those represented in Fig. 1C.

Isolation of mutants and characterization of LPS. Most of the Lps mutants were obtained from a screen for symbiotically defective mutants after Tn5 or nitrosoguanidine mutagenesis. Of the resulting 50 symbiotically defective strains, 7 produced abnormal LPS. Mutant CE309 was isolated instead by screening directly for rough colonies on TY agar plates after Tn5 mutagenesis. This mutant turned out to be symbiotically defective also. Colonies of the other mutants on TY agar also were rough in appearance. All mutants agglutinated when grown in TY broth.

LPS of the mutants was examined by SDS- or DOC-PAGE of unfractionated cell extracts, stained with periodate-silver, or blotted onto nitrocellulose and probed with the antiserum discussed above that recognizes LPS I but not LPS II. Two mutants, CE109 and CE113, described previously (9, 29), were included for comparison. For simplicity, LPS from a mutant which comigrated with a wild-type LPS form was given the same designation. This nomenclature is not meant to imply that an LPS II, for example, from a mutant is chemically identical to wild-type LPS II. In all of the mutants, LPS I was less abundant (or undetectable) and LPS II was more abundant than in the wild type (Fig. 1).

To confirm the validity of analyzing mutant LPS by means of SDS- or DOC-PAGE of unfractionated extracts, LPS of mutants CE113, CE166, and CE309 was purified by gel filtration of phenol-water extracts as previously described for strains CE109 and wild-type CE3 (9, 29). Even from these rough mutants the LPS appeared in the aqueous phase of the phenol-water extract, although it often was necessary to extract the phenol phase with water two or more times to obtain a good yield. The purified LPS from each mutant exhibited the same bands on SDS- or DOC-PAGE as those shown in Fig. 1 except that the band at the top of the gel and the band migrating faster than LPS II (band A) were missing.

LPS I was detected in three mutants. Two strains (CE148 and CE309) produced very small quantities of LPS I detected by immunoblotting (Fig. 1C, lanes 4 and 9) but not by silver staining. Mutant CE166 synthesized LPS I in quantities readily detectable by silver staining (Fig. 1A, lane 10). On an immunoblot, this LPS was recognized by the antiserum to a degree qualitatively equivalent to the recognition of the wild-type LPS I (Fig. 1C, lane 10). Purified LPS from strains CE166 and wild-type CE3 was analyzed for sugar composition by gas chromatography (Table 2). As predicted by SDS-PAGE, the amount of O antigen in LPS of mutant CE166 was at most half that in wild-type LPS and, given the likelihood of incomplete aqueous extraction of LPS II, probably less than that. The O-antigenic sugars (9) were found in approximately the wild-type ratios (Table 2).

LPS I was not detected in any of the remaining mutants under the conditions described for Fig. 1. From mutants CE113, CE125, CE126, CE168, and CE183, only the abundant LPS II band(s) was seen (Fig. 1). Strain CE121 uniquely exhibited an SDS-plus-DOC-PAGE band that migrated similarly to but was much more pronounced than wild-type band IV (Fig. 1B, lane 3). Mutant CE109 uniquely yielded a rather prominent SDS-plus-DOC-PAGE band comigrating with wild-type band III (Fig. 1B, lane 2). The antiserum that recognizes LPS I reacted with the LPS IV band of CE121 but not with the LPS III band of CE109 (Fig. 1C, lanes 6 and 7). The LPS of strain CE121 was fractionated by DOC-gel

TABLE 2. Sugar composition of purified LPS from strains CE3 and CE166

LPS sugar(s)	% of total sugar in LPS[a]		Ratio of O-antigen sugars[b]	
	CE3	CE166	CE3	CE166
O-methyl-6-deoxyhexoses	21	11	35	39
Fucose	18	10	30	35
Mannose	12	15		
Galactose	7.3	14		
Galacturonic acid	21	43		
Glucose	Trace	Trace		
Glucuronic acid	17	5.5	28	20
2-Amino-2,6-dideoxyhexose	4.1	1.6[c]	7	6

[a] Only sugars analyzed by gas chromotography are shown. Values are relative to the total integrated peaks of these sugars. KDO by colorimetric assay was 2.0 and 2.5% of LPS mass from strains CE3 and CE166, respectively. Ribose from contaminating RNA is not shown.

[b] Data for percent total sugar were recalculated by comparing sugars found only in the O-antigen-containing polysaccharide released from wild-type LPS by mild acid hydrolysis (9).

[c] Content was low and subject to great variability.

filtration (Fig. 2A). The LPS IV and LPS II from this strain were not completely separated, but in three KDO-containing fractions LPS IV was the only band detected by SDS-PAGE (Fig. 2B, lanes 7 to 9). LPS IV of this strain was therefore inferred to contain KDO, the signature sugar of LPS.

Mutant CE309, already mentioned as producing LPS I in very low amounts, was unique in two respects. First, it produced an LPS II with greater SDS-PAGE mobility than that of the wild-type LPS II (Fig. 1A, lane 9). Second, SDS-PAGE of CE309 extracts gave bands, migrating somewhat faster than LPS I, that were revealed by the antiserum but not detected by silver staining (Fig. 1C, lane 9).

Absence of LPS I on periodate-silver-stained SDS- or DOC-PAGE of extracts of all mutants except CE166 revealed the presence of dimly staining bands that migrated somewhat more slowly than did LPS I. These bands were found in the aqueous phase of phenol-water extracts and were unaltered after protease treatment (not shown). On the other hand, they were not detected in immunoblots using antisera that would have recognized an equivalent amount of wild-type LPS I. Therefore, although these bands may represent LPS, the PAGE mobility and lack of immunochemical detection indicate that they are distinct from LPS I.

Mapping of mutations by molecular cloning and complementation. To isolate cloned DNA that covered the lps mutations, two strategies were used. In the first strategy, the EcoRI DNA fragment carrying the mutation of strain CE109 was used as a probe in colony hybridization to isolate two clones with overlapping DNA from a cosmid library of the wild-type genome. The second strategy took advantage of the altered colony morphology of the Lps mutants. The cosmid library was transferred to a mutant by triparental mating, and complementing cosmids were isolated from the rare smooth colonies that appeared. Cosmid pCOS126, which complemented strain CE126, and five cosmids carrying overlapping DNA inserts which complemented mutant CE309 were isolated in this way.

The cloned DNA identified by these approaches was used in complementation tests with the Lps mutant strains. Complementation was revealed by lack of agglutination during growth in TY broth, restoration of LPS I and approximately the normal LPS I/LPS II ratio on SDS-PAGE (Fig. 3).
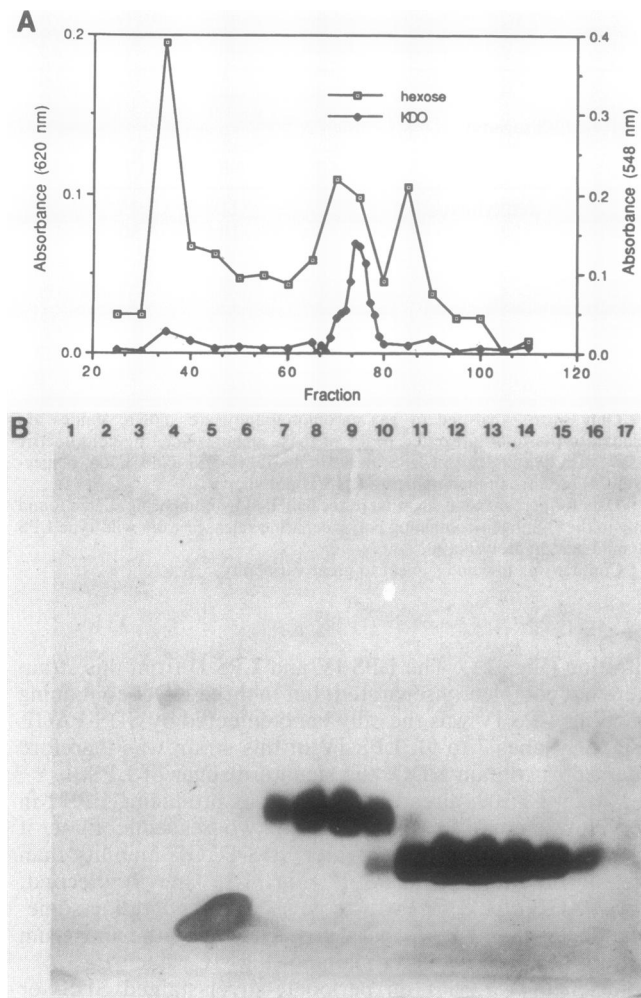
FIG. 2. (A) DOC-gel filtration of the phenol-water extract of strain CE121, performed as described in Materials and Methods. Absorbance was measured at 620 and 548 nm in the assays of hexose (anthrone) and KDO, respectively. (B) SDS-PAGE of gel filtration fractions. Lanes 1 to 17 correspond to fractions 63 to 79 of panel A.
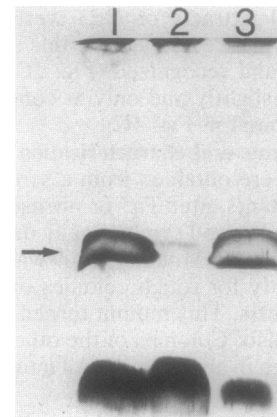

FIG. 3. Example of the restoration of LPS I (arrow) by complementing DNA as revealed by SDS-PAGE. Extracts of cells of the following strains were applied: CE3 (lane 1), CE121 (lane 2), CE121(pCOS109.11) (lane 3).

kbp insert of pDEL23 (derived from pCOS109.11; Fig. 5) complemented all four of these mutants (CE109, CE121, CE125, and CE183) to Lps$^+$. A 7.5-kbp EcoRI fragment subcloned from pCOS126 (Fig. 5) was sufficient to complement strains CE113, CE126, CE148, and CE168 to Lps$^+$.

Complementation of mutant CE166. Probing with the cloned EcoRI fragment carrying the Tn5 insertion of CE166 indicated that this fragment was not located in any of the cosmids described above. However, the presence of pCOS109.11 increased the LPS I content in strain CE166 (Fig. 6). Since the ratio between LPS I and LPS II on SDS-PAGE consistently was not as large as in the wild-type strain CE3, pCOS109.11 may have suppressed the CE166 mutation rather than carrying the DNA for true complementation. Another indication of the effect of pCOS109.11 was that transconjugant CE166(pCOS109.11) had obviously greater nodulating ability than did strain CE166 (see below).

Strains CE113 and CE148 also carried Tn5 insertions that were not located in the DNA that complemented them. By using R-plasmid (pJB3)-mediated conjugation, the Tn5 insertions of strains CE113 and CE148 were shown to be unlinked to the lps mutations. In no case was the transfer of Tn5 accompanied by the lps mutation. However, in pJB3-mediated transfer from CE166, the Tn5 insertion cotransferred 20

Strains CE109, CE121, CE125, and CE183 were all complemented to Lps$^+$ by cosmid pCOS109.11. Cosmid pCOS126 complemented the LPS defect in strains CE113, CE126, CE148, and CE168. Only mutant CE309 was complemented by the five overlapping cosmids isolated by complementing this mutant. These five cosmids restored the wild-type mobility of LPS II as well as LPS I to strain CE309. Antisera raised against wild-type cells reacted with the LPS I of each complemented mutant and the LPS I of the wild-type strain to the same degree (according to qualitative intensity of staining on immunoblots; Fig. 4).

EcoRI restriction maps of the rhizobial DNA of pCOS 109.11 and pCOS126 were constructed (Fig. 5). The lps mutations were mapped to specific fragments of these maps as follows. The EcoRI fragment carrying the lps::Tn5 mutation of strain CE109 hybridized to the 7.8-kilobase-pair (kbp) EcoRI fragment of pCOS109.11 (Fig. 5). A plasmid carrying only this wild-type fragment (pE109) complemented mutants CE109 and CE125 (Fig. 4, lane 4). Likewise, the EcoRI fragments carrying the transposon insertions of strains CE121 and CE183 hybridized to the 2.9- and 0.63-kbp EcoRI fragments, respectively, of pCOS109.11 (Fig. 5). The 13.3-
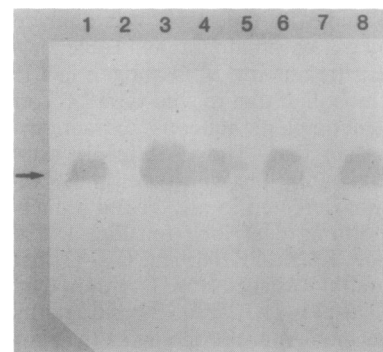

FIG. 4. LPS I of various complemented strains compared with CE3 LPS I by immunoblot. SDS extracts of pelleted cells were separated by SDS-PAGE, and the blot of the gel was stained by using antiserum against CE3 cells. Lanes: 1, CE3; 2, CE109; 3, CE109(pCOS109.11); 4, CE109(pE109); 5, CE113; 6, CE113 (pCOS126); 7, CE126; 8, CE126(pCOS126).
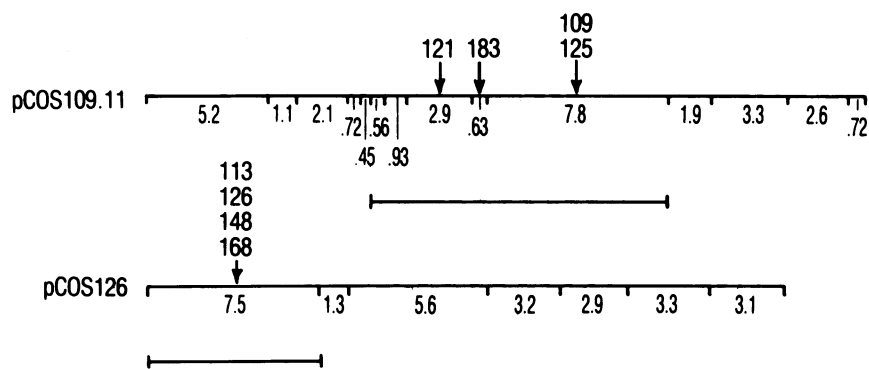
FIG. 5. *Eco*RI restriction maps of the rhizobial DNA of cosmids pCOS109.11 and pCOS126. Arrows indicate within which *Eco*RI restriction fragments the *lps* mutations are located as determined by complementation or hybridization. Horizontal lines below the restriction maps delineate subcloned DNA (pDEL23 and pE168) that complemented all four of the mutations in the respective regions. Numbers below the maps indicate sizes of *Eco*RI fragments in kilobase pairs.

of 20 times with the *lps* mutation. Furthermore, symbiotically proficient revertants were isolated from rare normal-appearing nodules after inoculation of plants with CE166. In these revertants, the Tn*5* insertion of strain CE166 had been excised and inserted elsewhere. The resulting strains not only elicited complete nodule development but also had the wild-type LPS banding pattern on SDS-PAGE.

Plant tests. The 10 Lps mutants described in Table 1 were tested on bean plants for nodulation. In each case, the roots developed numerous nodules that were small, white, and devoid of nitrogenase activity. No infected cells were observed after extensive examination of 14-day-old nodules elicited by the mutants. Developing nodules induced by four strains, CE121, CE148, CE166, and CE168, were examined 8 days after inoculation and found to contain aborted infection threads in the root hairs. In other words, the symbiotic behavior was identical to that of previously reported Lps mutants CE109 and CE113 (29).

Complementation to Lps$^+$ by pCOS126 or pCOS309 allowed the transconjugation strain to elicit nodules that were identical in development and nitrogenase activity to nodules elicited by the wild type. On the other hand, strains comple-

mented to Lps$^+$ by pCOS109.11, including CE166(pCOS 109.11), induced nodules that generally exhibited little or no nitrogenase activity, although such nodules developed further than did the nodules induced by the mutants when not carrying complementing DNA. Similar results were obtained when strains CE109 and CE125 containing pE109 were inoculated on bean plants. The numbers of bacteria found within many of these nodules were comparable to the number (10^8) found in nodules harboring the wild-type strain, whereas nodules induced by the mutant strains had only 10^4 bacteria (presumably contained only in the distended, abortive infection threads [29]). Bacteria isolated from the nodules retained their original genetic markers, including Tc^r of pCOS109.11.

DISCUSSION

This work supports the hypothesis that LPS I is required for complete development of the symbiosis between bean plants and *R. leguminosarum* CFN42. The eight Ndv$^-$ mutants isolated in this study (Table 1) were each in some manner altered in LPS I synthesis. In view of the genetic cloning and complementation experiments, there seems little doubt that a single mutation can cause both the symbiotic and LPS defects. Although other formal possibilities still exist, the most obvious explanation is that the mutations affect nodule development because LPS I content or structure is altered.

Mutations in at least three distinct genetic regions conferred these phenotypes (Ndv$^-$ and deficient LPS I). Mutations in two of these regions were complemented by the respective cloned wild-type DNA (on pCOS126 or the 309 cosmids) to result in apparently normal LPS and symbiotic proficiency. Mutations in the third region could be complemented by pCOS109.11 to give apparently normal LPS I, but symbiotic capability was restored only partially. Mutants of this third class also grew more slowly ex planta when carrying pCOS109.11 or pE109. Perhaps the multiple copies of this region lead to overproduction of O-antigen precursors or some other imbalance in LPS synthesis. In certain *Salmonella* Rfb$^-$ mutants, accumulation of O-antigen precursors is speculated to tie up the available pool of undecaprenol carrier lipid, thereby inhibiting peptidoglycan synthesis and cell growth (22). Possible overproduction of O antigen may be responsible for the suspected partial suppression of the CE166 mutation by pCOS109.11. If elevated gene dosage leads to larger quantities of O-antigen substrate, it might
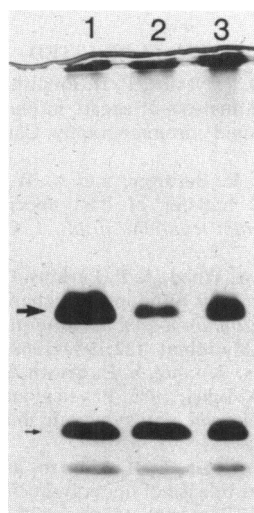

FIG. 6. Partial complementation of strain CE166 by pCOS109.11 as shown by SDS-PAGE. Upper arrow, LPS I; lower arrow, LPS II. Lanes: 1, CE3; 2, CE166; 3, CE166(pCOS109.11).

accelerate the inefficient catalysis of LPS I synthesis in this mutant.

The exact role of LPS I in symbiosis remains to be elucidated. It is not required for induction of nodule meristematic activity, in a host-specific manner or for the initiation of infection thread formation (29). Soon after being started, however, thread development goes awry in nodules elicited by mutants with undetectable or diminished amounts of LPS I. Nodules elicited by the four mutants whose nodulation was examined microscopically in this study and two mutants studied previously (29) all exhibited this property. The aborted infection threads were greatly swollen but did not appear to release bacteria into root hair cytoplasm (29).

A hypothesis consistent with these observations is that LPS I protects against host defenses that confront the bacteria only after the infection thread is initiated. It may be that LPS I in sufficient quantities masks features which elicit host defenses that stop the infection process. Another possibility is that LPS prevents entry of toxic molecules from the host. It is known that *Salmonella* mutants with severely truncated polysaccharide have increased sensitivity to dyes and detergents (27). Recently, a polysaccharide in *Rhizobium loti* was shown to protect this bacterium from a bacteriocidal flavolan (15). An attractive feature of this hypothesis is that LPS could serve the same function in pathogenic as well as mutualistic symbioses.

Other roles are certainly possible. Brewin et al. (3) have suggested that the LPS structure changes between the free-living and the bacteroid state. The possibility exists that these changes are required during the course of infection and that the mutants are incapable of making the required structures. It may be that LPS I serves as a signal molecule to trigger a step in infection thread synthesis. Strain CE166 may be informative in this regard. The results of SDS-PAGE, immunoblots using polyclonal antisera, and sugar composition allow the possibility that the LPS I of this strain is identical to the wild-type LPS. If borne out by detailed chemical analysis of the LPS of strain CE166, this finding would contradict the functioning of LPS I as a cell-bound positive signal, since strain CE166 should have enough LPS I to be recognized as a signal. However, if LPS I must be released from the bacterial cells (5) to act as a signal, a minimum level might be required. The alternative idea of a protective role against host defenses is consistent with the properties of CE166; the significantly lower LPS I content of this strain might not provide sufficient protection.

Given the known properties of the wild-type LPS, SDS- or DOC-PAGE clearly was sufficient to establish that the mutants had alterations in LPS biosynthesis. Moreover, the PAGE data strongly suggest that the LPS biosynthetic disruptions in mutants CE109, CE121, CE166, and CE309 are different from each other and from the specific LPS defects of the other mutants. However, chemical analyses of both mutant and wild-type LPS will be necessary to define precisely the specific biosynthetic defects. Conversely, in the absence of further analysis, it should not be assumed that an LPS in a mutant which comigrates with a wild-type LPS actually is structurally identical, even if it cross-reacts antigenically. Specifically, it would be rash to suggest that mutants whose LPS II seems to comigrate with wild-type LPS II are defective in O-antigen synthesis rather than LPS II synthesis. Mutant CE166, as stated above, is another case in which detailed chemical analysis is required to establish that its LPS I is structurally unaltered.

The *R. leguminosarum* LPS structure seems to differ fundamentally in its polysaccharide portion from the paradigm of enteric bacteria (8, 22). The mutants of this study could aid in determining this LPS structure and its biosynthesis. For instance, the loss of LPS I in the mutants was coupled with a significant increase in the amount of LPS II. One possibility is that LPS II (or a molecule comigrating with it) is a precursor of LPS I that accumulates when a block in complete synthesis occurs. Another is that LPS II is synthetically independent and derepressed when LPS I is absent. The LPS III of strain CE109 (9) and the LPS IV produced by strain CE121 also may represent normal intermediates in LPS I biosynthesis that accumulate because of particular blocks. Therefore, detailed structural analysis of the LPS of the mutants and use of mutant LPS as substrates in vitro should contribute greatly to the understanding of LPS biosynthesis in this species.

Whereas there have been numerous chemical studies of rhizobial LPS, the genetics have received much less attention. Recent work has begun to address this imbalance. *lps* DNA has been cloned from one region of the genome of *R. leguminosarum* bv. *viciae* VF39. Lps mutants of this strain are defective in eliciting complete nodulation of *Vicia hirsuta* (33). DNA regions required for both LPS and exopolysaccharide biosynthesis of *R. meliloti* wild-type isolate SU47 have been identified (19). A nonnodulating mutant of *R. leguminosarum* reportedly undergoes changes in LPS when its ability to nodulate clover is restored by rhizobial DNA carried on a hybrid plasmid (36).

This study has identified three genetic regions in *R. leguminosarum* CFN42 that are necessary for the synthesis of an LPS molecule required for symbiosis with bean plants. Frequency and clustering of mutations indicate that two of these regions may be rather extensive. It remains to be seen whether these gene clusters represent distinct functions on the order of the *rfa* and *rfb* clusters of enteric bacteria (22).

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