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Rhizobium leguminosarum Exopolysaccharide Mutants: Biochemical and Genetic Analyses and Symbiotic Behavior on Three Hosts

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Ten independently generated mutants of *Rhizobium leguminosarum* biovar *phaseoli* CFN42 isolated after Tn*5* mutagenesis formed nonmucoid colonies on all agar media tested and lacked detectable production of the normal acidic exopolysaccharide in liquid culture. The mutants were classified into three groups. Three mutants harbored Tn*5* insertions on a 3.6-kilobase-pair *Eco*RI fragment and were complemented to have normal exopolysaccharide production by cosmids that shared an *Eco*RI fragment of this size from the CFN42 genome. The Tn*5* inserts of five other mutants appeared to be located on a second, slightly smaller *Eco*RI fragment. Attempts to complement mutants of this second group with cloned DNA were unsuccessful. The mutations of the other two mutants were located in apparently adjacent *Eco*RI fragments carried on two cosmids that complemented those two mutants. The latter two mutants also lacked O-antigen-containing lipopolysaccharides and induced underdeveloped nodules that lacked nitrogenase activity on bean plants. The other eight mutants had normal lipopolysaccharides and wild-type symbiotic proficiencies on bean plants. Mutants in each of these groups were mated with *R. leguminosarum* strains that nodulated peas (*R. leguminosarum* biovar *viciae*) or clovers (*R. leguminosarum* biovar *trifolii*). Transfer of the Tn*5* mutations resulted in exopolysaccharide-deficient *R. leguminosarum* biovar *viciae* or *R. leguminosarum* biovar *trifolii* transconjugants that were symbiotically deficient in all cases. These results support earlier suggestions that successful symbiosis with peas or clovers requires that rhizobia be capable of acidic exopolysaccharide production, whereas symbiosis with beans does not have this requirement.

All well-characterized rhizobia produce exopolysaccharide (EPS) (9). Secreted molecules are logical candidates for playing important roles in the interactions between plants and rhizobia, and a number of studies have correlated the production of EPS with the ability to induce nodules in different rhizobia-plant interactions.

Mutants of *Rhizobium meliloti* SU47 that lack the acidic EPS (Exo^-) induce uninfected nodules on alfalfa (18, 25, 30). Normal nodulation is restored by complementation of the Exo^- mutants with cloned DNA. The *exo* alleles are classified into 14 complementation groups, most of which are extrachromosomal (26). Noncarbohydrate substituents of the *R. meliloti* acidic EPS appear to be important for normal nodule development. *R. meliloti exoH* mutants, which secrete EPS that is not succinylated, induce empty alfalfa nodules with aborted infection threads (24). Another *R. meliloti* mutant class produces excessive amounts of EPS that lacks the terminal pyruvate residue on the side chain of the repeating octasaccharide unit (30). These strains induce alfalfa nodules that lack infection threads. Interestingly, production of a second acidic EPS, which is normally cryptic in strain SU47, suppresses the symbiotic defect of mutants that lack the normal acidic EPS (20, 45).

A number of Exo^- mutants of broad-host-range *Rhizobium* strain NGR234 form nonmucoid colonies and induce defective nodules on *Leucaena leucocephala* (14). These mutants lack the acidic EPS, as shown by biochemical analyses. Enhancement of nodulation has been observed when the Exo^- mutants and purified EPS from the parental strain are coinoculated onto *L. leucocephala* plants (17).

Very few Exo^- mutants of *Rhizobium leguminosarum* have been well characterized. Exo^- mutants of *R. leguminosarum* biovar *viciae* 128C53 elicit very limited nodule development on peas (31). Biochemical analysis has shown that one of these mutants (strain EXO-1) does not produce the normal acidic EPS (11). However, these mutants have not been studied genetically, and it is not known whether Exo^- and the symbiotic defect are caused by the same mutation. On the other hand, three *R. leguminosarum* Exo^- (nonmucoid) mutants have been studied genetically, but their EPS deficiencies have not been characterized biochemically. One of these mutants, which was derived from *R. leguminosarum* biovar *trifolii* ANU794, elicits clover nodules in which the bacteria occupy infection threads, but later infection events are incomplete (13). The other two mutants, which were derived from *R. leguminosarum* 8002, were given different host range capacities by introducing either an *R. leguminosarum* biovar *phaseoli* or an *R. leguminosarum* biovar *viciae* Sym (symbiosis) plasmid (5). The *R. leguminosarum* biovar *viciae*derivatives failed to nodulate peas. These three mutants have been complemented with wild-type DNA to give Exo^+ (mucoid) derivatives with wild-type nodulation proficiencies on clovers or peas (5, 13). In the case of strain 8002, two closely linked *exo* genes on the complementing DNA, *pss-1* and *pss-2*, have been sequenced recently (6). Gene *psi*, which is located on *R. leguminosarum* biovar *phaseoli* Sym plasmid pPR2JI and which is presumed to regulate EPS synthesis during symbiosis, has been sequenced also (7).

Whereas these studies suggest that Exo^- mutants cannot nodulate clovers and peas properly, there is no consistent correlation between rhizobial EPS production and nodulation of beans, the host of *R. leguminosarum* biovar *phaseoli*. Some Exo^- mutants of *R. leguminosarum* biovar *phaseoli* 127K26 nodulate beans as well as the parental strain does, whereas others do not induce normal nodules (38, 39). The

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two nonmucoid mutants of *R. leguminosarum* biovar *phaseoli* 8002 induce normal nodules on beans, even though they are unable to nodulate peas when an *R. leguminosarum* biovar *viciae* Sym plasmid is introduced (5). These studies suggest that EPS production is not essential for complete nodule development on beans. However, in neither study was the EPS of the mutants analyzed chemically. Deficiency in EPS was inferred from colony morphology or the amount of hexose found in the culture supernatant.

To examine more thoroughly the connection between rhizobial EPS production and bean nodulation, a number of Exo^- mutants of *R. leguminosarum* CFN42 were isolated in the present study, and genetic and biochemical analyses were undertaken. Additionally, the influence of the same *exo* mutations on pea and clover nodulation was determined.

MATERIALS AND METHODS

Bacterial strains. All strains with the prefix CE (see Table 1) were derived from *R. leguminosarum* biovar *phaseoli* wild-type isolate CFN42 (35), which induces well-developed nitrogen-fixing nodules on beans. The Exo^- mutants were isolated by selecting for nonmucoid colony phenotype on AMA agar (described below) after Tn*5* mutagenesis. Strains with the prefix RL (see Table 5) were derived from wild-type *R. leguminosarum* biovar *viciae* 128C569. Strains with the prefix BT (see Table 5) were derived from wild-type *R. leguminosarum* biovar *trifolii* 162BB1. Strains 128C569 and 162BB1 were obtained from Nitragin Company, Inc. (Milwaukee, Wis.).

Bacterial growth conditions and media. All *Rhizobium* strains were grown at 30°C. Rich medium (TY) contained 0.5% tryptone (Difco Laboratories, Detroit, Mich.), 0.3% yeast extract (Difco), and 10 mM $CaCl_2$ (3). Minimal medium (Y) consisted of 0.4 mM $MgSO_4$, 1.25 mM K_2HPO_4, 1 mM $CaCl_2$, 3.7 mM disodium succinate or 55 mM mannitol (as the carbon source), 3.7 mM monosodium glutamate (as the nitrogen source), 0.15 mM $FeCl_3$, 1 mg of biotin per liter, 1 mg of pantothenic acid per liter, and 1 mg of thiamine per liter. Phosphate-buffered yeast extract-mannitol salts medium (AMA) was prepared as described previously (44). Agar medium contained 1.5% Bacto-Agar (Difco). *Escherichia coli* strains were grown on LB medium (27). The following antibiotics (Sigma Chemical Co., St. Louis, Mo.) were used at the indicated concentrations (per liter): kanamycin, 30 mg; erythromycin, 10 mg; nalidixic acid, 30 mg; tetracycline, 5 mg (*R. leguminosarum*) or 15 mg (*E. coli*); and streptomycin, 200 mg.

Plant tests. Seeds of *Phaseolus vulgaris* cv. Midnight, *Trifolium repens* cv. Ladino, and *Pisum sativum* cv. Wando were surface sterilized with commercial hypochlorite bleach solution diluted 1:1 with water and germinated for 2 days at 30°C in sterile glass petri dishes covered at the bottom with filter paper saturated with water. Bean plants were grown in pouches (34) (Northrup King) with nitrogen-free nutrient solution (RBN [44]). Peas were grown in vermiculite in modified Leonard jars (2). Clover plants were grown on RBN agar slants by a previously described method (29). Nitrogenase activity was measured by acetylene reduction.

***Rhizobium* matings.** Erythromycin-sensitive (Ery^s) *R. leguminosarum* biovar *phaseoli* donor strains harboring *exo*::Tn*5* mutations and the conjugative plasmid pJB3 were grown overnight in liquid TY and mixed on TY agar with equal volumes of erythromycin-resistant (Ery^r) recipient cultures (*R. leguminosarum* biovars *trifolii*, *viciae*, or *phaseoli*) that also had been grown overnight in TY liquid medium. Such a mating plate was incubated at 30°C overnight, and then the mixed culture was suspended in 3 ml of 0.1 M $MgSO_4$. Transconjugants were selected on Y agar containing mannitol, erythromycin, and kanamycin. Exo^- transconjugants were detected as nonmucoid colonies on this medium. The recipient genetic background was indicated by Ery^r and by analyzing proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (32).

Isolation of cosmids that restored the Exo^+ phenotype. The library of total genomic DNA of strain CE3 in cosmid pLAFR1 was maintained in *E. coli* HB101. Its construction has been described previously (33). Intergeneric triparental matings (16) were carried out to complement the Exo^- mutants to Exo^+ by mixing 0.2 ml each of fully grown *E. coli* donor (harboring the cosmid library) and *E. coli* HB101 carrying Tra^+ helper plasmid pRK2013 with 0.5 ml of *R. leguminosarum* Exo^- recipients that were fully grown in TY liquid medium. The mixture was spread onto TY agar and incubated overnight at 30°C. The matings were suspended in 0.1 M $MgSO_4$, and a dilution series was plated onto Y-mannitol agar plates containing tetracycline. The restoration of a mucoid colony phenotype was the criterion for complementation. Cosmids were isolated from the complemented Exo^+ *Rhizobium* strains by a small-scale alkaline lysis procedure (27). The isolated cosmids were then reintroduced into competent *E. coli* HB101 cells by transformation (27). Selection for transformants was on LB agar containing tetracycline. These purified HB101 transformants carrying the isolated cosmids were used in all subsequent complementation experiments by the procedure described above.

DNA isolation and manipulation. Total *Rhizobium* DNA was isolated by the method of Meade et al. (29). Restriction enzymes were used according to the instructions of the manufacturer (Pharmacia P-L Biochemicals, Inc., Milwaukee, Wis.). Plasmids were isolated by a small-scale alkaline lysis procedure (27). DNA was labeled with [^{32}P]dCTP (Dupont, NEN Research Products, Boston, Mass.) with a nick-translation kit (Amersham Corp., Arlington Heights, Ill.). Southern hybridizations were performed at a high stringency (27).

EPS analysis. Bacteria were grown at 30°C in 500 ml of liquid mannitol-Y medium for 2 days. The CFU in the cultures was measured by plating dilutions onto TY agar with appropriate antibiotics. Cells were pelleted at 16,000 × *g* for 30 min and washed three times by suspension and centrifugation in 100 ml of 0.17 M NaCl. The supernatants from the washes and culture fluid were combined and lyophilized. Lyophilized material was dissolved in 50 ml of water and then precipitated with four volumes of ethanol. The ethanol precipitate was dissolved in water, dialyzed against several changes of water for 3 days, and lyophilized. For analysis by gel filtration chromatography, this lyophilized material was dissolved in column buffer (100 mM EDTA and 300 mM triethylamine) at a concentration of 2 mg/ml. A 2-ml portion of this solution was applied to a Sepharose 4B column (1.5 by 22.5 cm). Fractions of 1 ml were eluted with the column buffer. The hexose content was measured by the anthrone method (41). Uronic acid was assayed by reaction with *m*-hydroxydiphenyl (4). The 3-deoxy-D-manno-2-octulosonic acid content was measured by a thiobarbituric acid assay (22).

The hexose compositions of total EPS or the Sepharose 4B fractions were determined by acid hydrolysis, reduction of the monosaccharides to alditols with $NaBH_4$, preparation

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Symbiotic phenotype[a]	Reference[b]
R. leguminosarum biovar *phaseoli*[c]			
CE3	*str-1* (Str^r derivative of CFN42)	Ndv$^+$ Fix$^+$	35
CE8	*ery-1* (Ery^r derivative of CFN42)	Ndv$^+$ Fix$^+$	
	Class A1 Exo$^-$ strains		
CE338	*exo-338*::Tn*5* *str-1*	Ndv$^+$ Fix$^+$	
CE339	*exo-339*::Tn*5* *str-1*	Ndv$^+$ Fix$^+$	
CE341	*exo-341*::Tn*5* *str-1*	Ndv$^+$ Fix$^+$	
	Class A2 Exo$^-$ strains		
CE301	*exo-301*::Tn*5* *str-1*	Ndv$^+$ Fix$^+$	
CE307	*exo-307*::Tn*5* *str-1*	Ndv$^+$ Fix$^+$	
CE308	*exo-308*::Tn*5* *str-1*	Ndv$^+$ Fix$^+$	
CE330	*exo-330*::Tn*5* *str-1*	Ndv$^+$ Fix$^+$	
CE342	*exo-342*::Tn*5* *str-1*	Ndv$^+$ Fix$^+$	
	Class B Exo$^-$ strains		
CE320	*exo-320*::Tn*5* *str-1* (Exo$^-$ Lps$^-$)	Ndv$^-$ Fix$^-$	
CE343	*exo-343*::Tn*5* *str-1* (Exo$^-$ Lps$^-$)	Ndv$^-$ Fix$^-$	
R. leguminosarum biovar *viciae* 128C569	Ery^r Exo$^+$	Ndv$^+$ Fix$^+$	
R. leguminosarum biovar *trifolii* BT2	Ery^r derivative of 162BB1, Exo$^+$ *ery-1*	Ndv$^+$ Fix$^+$	
E. coli HB101	RecA$^-$ Str^r, Ery^r		27
Plasmids			
pJB3	R68.45 derivative, Tc^r		8
pRK2013	Tra$^+$ Km^r		16
pLAFR1	pRK290::*cos*		19
pSUP2021	pSUP202::Tn*5*		40

[a] Abbreviations: Exo$^+$, Mucoid colony phenotype on agarose plates; Exo$^-$, nonmucoid colony phenotype on agarose plates; Lps$^-$, lacks the LPS which contains the O antigen of strain CNF42; Ndv$^+$, normal nodule development; Ndv$^-$, underdeveloped nodules; Fix$^+$, nodules have nitrogenase activity; Ery, erythromycin; Str, streptomycin; Km, kanamycin.
[b] If a reference is not given, the strain was isolated in this study.
[c] All strains with the prefix CE were derived from *R. leguminosarum* biovar *phaseoli* wild-type isolate CFN42 (35).

of the alditol acetates, and analysis by gas chromatography on a column packed with SP2330 (Supelco) (1). Uronic acids were identified by reaction of the polysaccharides with methanol in dilute acid followed by reduction with $NaBH_4$ and acetylation (10).

Gel electrophoresis. SDS extracts of bacterial cells were prepared as described previously (12). Following discontinuous SDS-PAGE (12), gels were stained with a silver staining kit (Bio-Rad Laboratories, Richmond, Calif.) by the instructions of the supplier, with the following modification. After fixation, gels were treated with 0.7% sodium metaperiodate for 5 min followed by a 30-min wash in glass-distilled water. The staining of lipopolysaccharides (LPSs) was much enhanced and the staining of proteins was much diminished by this treatment.

RESULTS

***R. leguminosarum exo* mutants.** After Tn*5* mutagenesis of *R. leguminosarum* CE3, 10 mutants designated as Exo$^-$ were isolated (Table 1). These mutants arose from independent mutagenesis events. They formed small, nonmucoid colonies on agar or agarose plates containing minimal nutrients only, yeast extract with mannitol, or tryptone-yeast extract. Each strain grew well in liquid minimal medium.

The Exo$^-$ strains were tested for their ability to nodulate bean plants. Eight of the Exo$^-$ strains nodulated beans in a manner indistinguishable from that of wild-type strain CE3 and were designated class A mutants (Exo$^-$ Ndv$^+$ Fix$^+$) (Table 1). Two of the Exo$^-$ strains, CE320 and CE343, gave rise to small, white bumps on beans and were designated class B mutants (Exo$^-$ Ndv$^-$ Fix$^-$). The nodules induced by the class A mutants were crushed, and the released bacteria were streaked onto AMA agar plates. The reisolated bacteria retained the nonmucoid phenotype and kanamycin resistance of the mutant inoculants.

Because the class B Exo$^-$ mutants CE320 and CE343 induced nodules on beans that were similar to those induced by Lps$^-$ mutants of *R. leguminosarum* biovar *phaseoli* (12, 35), these strains were tested for the presence of LPS by SDS-PAGE (Fig. 1A, lanes 2 and 3). The class B mutants lacked an LPS band known as LPS I, which contains the O antigen of strain CFN42 (10). All of the class A mutants produced LPS I (Fig. 1B).

Genetic analysis of the Exo$^-$ mutants. The class A *exo*::Tn*5* mutations were transferred in matings that were mediated by conjugative plasmid pJB3 to strain CE8, an Ery^r Exo$^+$ Lps$^+$ CFN42 derivative. All of the more than 1,000 resultant Ery^r Km^r transconjugants were Exo$^-$ (nonmucoid). Therefore, the *exo* mutation was at least closely linked to the Tn*5* insertion. When the class B *exo*::Tn*5* mutations were transferred to CE8, the transconjugants were Exo$^-$ and Lps$^-$.

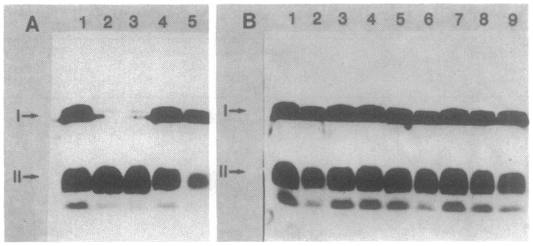

FIG. 1. Extracts of Exo$^-$ mutant cultures subjected to SDS-PAGE and stained for carbohydrate by the periodate-silver procedure. (A) Lanes: 1, CE3; 2, CE320; 3, CE343; 4, CE320 (pCOS320.1); 5, CE343(pCOS320.1). (B) Lanes: 1, CE3; 2, CE301; 3, CE307; 4, CE308; 5, CE330; 6, CE338; 7, CE339; 8, CE341; 9, CE342. The positions of LPS I and LPS II are indicated. (LPS II does not contain O-antigen-specific sugars.)

Therefore, it is likely that both Exo$^-$ and Lps$^-$ were the result of the Tn*5* insertion in each class B mutant.

Each of the *exo*::Tn*5* mutations appeared to be chromosomally located. Indigenous plasmids of the Exo$^-$ strains were separated on Eckhardt gels (36). The DNA remaining in the well and the diffuse band of sheared DNA hybridized with a labeled Tn*5* probe, but none of the five plasmid bands did (data not shown).

The *exo*::Tn*5* mutations were classified into three groups by Southern hybridization and genetic complementation analyses. The class A mutants were subdivided in this way into two groups (A1 and A2). All of the A1 Exo$^-$ strains were complemented to Exo$^+$ by four cosmids isolated from a CFN42 genomic library (Table 2). The rhizobial DNA inserts of these cosmids shared three *Eco*RI fragments (bands at 5.0, 3.6, and 1.7 kilobase pairs [kb] in lanes 1, 2, 4, and 5 of Fig. 2). Southern blot hybridization revealed that the Tn*5* insertion of each class A1 strain was located in a 9.4-kb *Eco*RI fragment (Fig. 3, lanes 4 to 6). Since the Tn*5* insert was 5.7 kb, the corresponding wild-type *Eco*RI fragment would be 3.7 kb. Therefore, the 3.6-kb fragment common to the complementing cosmids appeared to carry the *exo* DNA that was mutated in the class A1 mutants.

The group A2 strains were not complemented to Exo$^+$ by any of the cosmids transferred en masse into these strains from the genomic library. Southern hybridization of *Eco*RI digests of total DNA from these mutants revealed that the Tn*5* insertions were located in a 9.2-kb *Eco*RI fragment (Fig. 3, lanes 3, 7, and 9 to 11). The corresponding wild-type fragment would be 3.5 kb.

Both of the class B strains CE320 and CE343 were restored to Exo$^+$ Lps$^+$ Ndv$^+$ by either of two cosmids isolated from the gene library (Table 2 and Fig. 1A, lanes 4 and 5). These results provide further evidence that the Exo$^-$, Lps$^-$, and Ndv$^-$ phenotypes are caused by a single mutation in each strain. Southern hybridization of *Eco*RI digests of total DNA revealed that the class B *exo*::Tn*5* insertions were located in a 7.4-kb *Eco*RI fragment in strain CE343 and a 15-kb *Eco*RI fragment in strain CE320 (Fig. 3, lanes 2 and 8). The corresponding wild-type *Eco*RI fragments would be 1.7 kb and approximately 9 kb, respectively. Restriction analyses and cross-hybridization of the two cosmids that complemented these strains revealed that the 9.1- and 1.7-kb fragments were the only *Eco*RI fragments shared by both cosmids (Fig. 4).

The DNAs of the class A1 and class B cosmids may be linked closely on the chromosome. Class A1 cosmid pCOS341.6 appeared to share the 9.2-kb *Eco*RI fragment of class B cosmids pCOS320.1 and pCOS320.3 (Fig. 2, lanes 2 and 3).

EPS biochemical characterization. The extracellular

TABLE 2. Cosmids complementing the Exo$^-$ mutants

Cosmids	Exo$^-$ strains complemented[a]
Class A1	
pCOS338.1	CE338, CE339, CE341
pCOS338.4	CE338, CE339, CE341
pCOS339.7	CE338, CE339, CE341
pCOS341.6	CE338, CE339, CE341
Class B	
pCOS320.1	CE320, CE343
pCOS320.3	CE320, CE343

[a] Exo$^-$ strains CE301, CE307, CE308, CE330, and CE342 (class A2) were not complemented to Exo$^+$ by the CE3 cosmid library. Complementation signifies restoration to mucoid colony character.

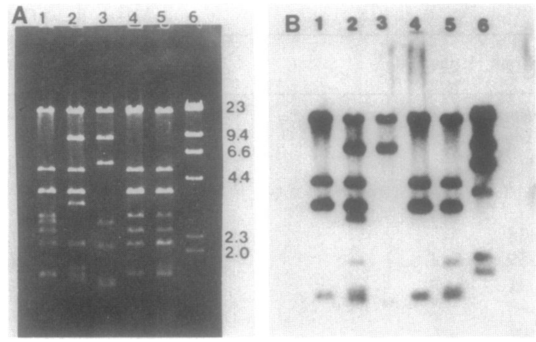

FIG. 2. (A) Agarose gel electrophoresis and ethidium bromide staining of *Eco*RI fragments of class A1 cosmids and class B cosmid pCOS320.1. Lanes: 1, pCOS339.7; 2, pCOS341.6; 3, pCOS320.1; 4, pCOS338.4; 5, pCOS338.1; 6, *Hin*dIII-digested phage lambda DNA, whose fragment sizes in kb are shown at the right. (B) Autoradiogram of the gel in panel A probed with radioactive pCOS341.6 DNA to determine which *Eco*RI fragments were common among the class A1 cosmids. The largest fragment in each lane is vector pLAFRI DNA. The standard fragments of lane 6 in panel A were visualized in panel B by the addition of ^{32}P-labeled phage lambda DNA.

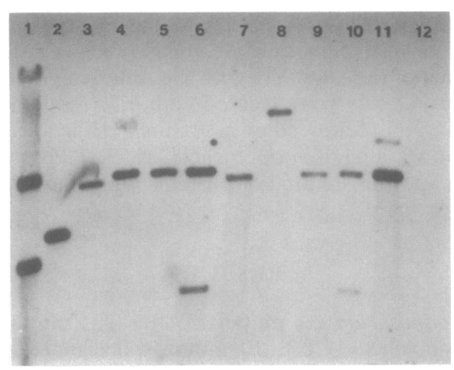

FIG. 3. Autoradiogram of a Southern blot of *Eco*RI-digested total DNA from the CFN42 Exo$^-$ mutants probed for Tn*5* content with plasmid pSUP2021 labeled with ^{32}P. Lanes: 1, the three largest fragments of *Hin*dIII-digested phage lambda DNA; 2, CE343; 3, CE342; 4, CE341; 5, CE339; 6, CE338; 7, CE330; 8, CE320; 9, CE308; 10, CE307; 11, CE301; 12, CE3.

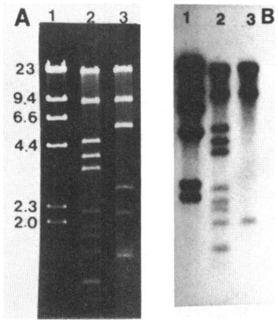

FIG. 4. (A) Ethidium bromide-stained gel of *Eco*RI digests of class B cosmids. Lanes: 1, *Hin*dIII-digested lambda DNA; 2, pCOS320.3; 3, pCOS320.1. Numbers to the left of the gel indicate standard sizes in kb. (B) Autoradiogram of the gel in panel A probed with ^{32}P-labeled pCOS320.3 and lambda DNA. The largest fragments in lanes 2 and 3 are vector DNA.

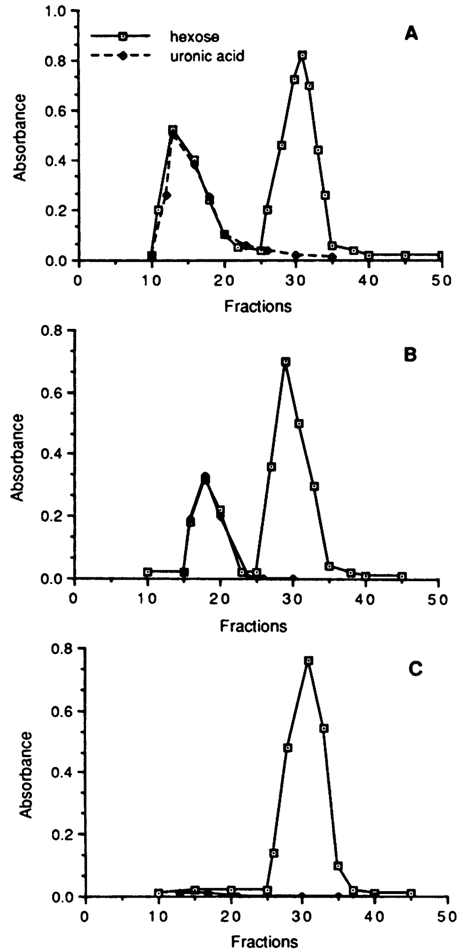

FIG. 5. Elution profiles of a Sepharose 4B gel filtration column of the ethanol-precipitated EPS from wild-type CE3 (A), the class A1 mutant CE339 (B), or the class B mutant CE320 (C). Hexose was measured by the anthrone assay, and uronic acid was measured by reaction with *m*-hydroxydiphenyl. In panels A and B the uronic acid peak and the first hexose peak were coincident; in panel C uronic acid was not detected. Note that the first peak in panel B was shifted to a lower molecular weight compared with its position in panel A.

polysaccharides from the parental and nonmucoid strains were isolated and characterized biochemically. After the cells were removed from 2-day-old cultures by centrifugation, the parental supernatant was much more viscous than were the supernatants from the mutants. Total EPS was isolated from the cell-free supernatant by ethanol precipitation. The Exo$^-$ mutants produced less than one-tenth of the amount of ethanol-precipitable material produced by the wild-type strain (Table 3). The precipitated EPSs from representative strains of each mutant class were analyzed further by Sepharose 4B gel filtration chromatography (Fig. 5). Pooled Sepharose 4B peak fractions were acid hydrolyzed, and the sugar compositions were analyzed (Table 4).

The EPS from the parental strain was separated into two peaks: a high-molecular-weight acidic EPS fraction and a low-molecular-weight neutral EPS fraction (Fig. 5A). The designation as acidic or neutral was made according to whether uronic acid was present. The parental acidic EPS fraction was composed of three sugars (galactose, glucose, and glucuronic acid) at a ratio of approximately 1:5:2 (Table 4). This result corresponded very well with the reported acidic EPS structures of other *R. leguminosarum* strains (28). Acid hydrolysis of the parental neutral EPS fraction yielded glucose almost exclusively (Table 4). The position of the Sepharose 4B peak and this composition suggest the presence of low-molecular-weight glucans.

The EPS from the class A strains was also separated into two Sepharose 4B peaks (Fig. 5B). However, the class A acidic fractions eluted from the column in a different position and were present in lower amounts than the wild-type acidic fraction was. Instead of having the composition characteristic of normal acidic EPS, the class A Sepharose 4B acidic fractions exhibited sugar compositions in close agreement with those of the parental LPS (Table 4). These sugars included *O*-methyl-deoxyhexose, fucose, mannose, and galacturonic acid, none of which are components of the parental acidic EPS. 3-Deoxy-D-manno-2-octulosonic acid (KDO) was present in these fractions also. The class A acidic fractions contained very low amounts of glucose, the predominant sugar of the parental acidic EPS. When the acidic fractions from the class A strains were subjected to SDS-PAGE, a staining pattern characteristic of LPS was observed (data not shown). The class A mutants apparently released intact LPS into the growth medium. Neither the parental strain nor the complemented class A1 strains released LPS into the medium, as shown by the sugar composition (Table 4). The EPS of A1 mutant CE338 comple-

TABLE 3. EPS yields

Strain	EPS produced (mg/10^{11} viable bacteria)[a]
CE3	315
CE301	22
CE320	18
CE330	21
CE338	22
CE339	28
CE343	21
CE338(pCOS338.1)	428
CE320(pCOS320.1)	368
CE343(pCOS320.1)	354

[a] Values are the total dry weight of dialyzed ethanol-precipitated material from culture supernatants.

TABLE 4. Relative sugar compositions isolated from the parent and exo⁻ strains

Strain	Class[a]	Amt of sugar[b]						
		OMDH	Fuc	Man	Gal	Glc	GalA	GlcA
CE3	Parent							
Acidic[c]		0	0	1	13	61	0	25
Neutral[c]		0	0	1	2	97	0	0
CE338	A1							
Acidic		17	25	13	12	2	19	11
Neutral		0	0	1	1	98	0	0
CE339	A1							
Acidic		13	26	13	19	6	14	8
Neutral		0	0	2	2	96	0	0
CE301, total[d]	A2	13	29	12	14	6	16	10
CE330, total	A2	14	29	14	12	6	17	9
CE320, total	B	0	0	1	1	98	0	0
CE343, total	B	0	0	2	5	92	0	0
CE338(pCOS338.1)	A1/+[e]							
Acidic		0	0	1	12	58	0	29
Neutral		0	0	1	1	98	0	0
CE320(pCOS320.1), acidic	B/+[e]	0	0	1	12	57	0	29
CE343(pCOS320.1), acidic	B/+[e]	0	0	13	10	56	0	21

[a] The mutant class to which the strain belonged.
[b] The amount of each sugar is given as a percentage of the total mass of the sugars detected by gas chromatography (rather than as a percentage of the total sample mass). Abbreviations: OMDH, *O*-Methyl-deoxyhexoses; Fuc, fucose; Man, mannose; Gal, galactose; Glc, glucose; GalA, galacturonic acid; GlcA, glucuronic acid; 0, not detected.
[c] Indicates Sepharose 4B acidic or neutral fractions.
[d] Total EPS was not fractionated further after ethanol precipitation and dialysis.
[e] These transconjugant strains carried cosmids which restored the mucoid colony phenotype (Exo⁺).

mented with cosmid pCOS338.1 (Table 1) was similar in composition to the wild-type EPS (Table 4).

The class B EPS did not contain acidic polysaccharides but did retain the low-molecular-weight neutral fraction (Fig. 5C). This fraction was composed almost entirely of glucose (Table 4). These mutants did not synthesize LPS I (Fig. 1A), and the components of LPS II (galacturonic acid, galactose, and mannose) apparently were not released into the medium. When the class B mutants were complemented to Exo⁺ by the class B cosmids, acidic EPS production was restored (Table 4).

Exo⁻ transconjugants of strains whose host is clovers or peas. In cases in which the host was peas or clovers, previously reported *R. leguminosarum* Exo⁻ mutants have been Nod⁻ or Ndv⁻ (5, 13, 31). In one study it was shown, however, that the same *exo* mutation that prevented nodulation of peas did not affect nodulation of beans (5). To determine whether the same was true of the *exo* mutations of the Exo⁻ mutants described above, the *exo*::Tn*5* alleles were transferred to closely related strains whose host was peas (*R. leguminosarum* biovar *viciae*) or clovers (*R. leguminosarum* biovar *trifolii*).

Transconjugants (Table 5) were selected as kanamycin-resistant colonies carrying the erythromycin resistance and SDS-PAGE protein profile of the *R. leguminosarum* biovar *viciae* or the *R. leguminosarum* biovar *trifolii* recipient. Since such colonies were also nonmucoid, it was inferred that an *exo*⁺ allele was replaced by an *exo*::Tn*5* allele. Class A1 and A2 *exo*::Tn*5* alleles were transferred into both *R. leguminosarum* biovar *trifolii* and *R. leguminosarum* biovar *viciae* strains. Class B *exo*::Tn*5* alleles were transferred only to *R. leguminosarum* biovar *viciae* 128C569 (Table 5).

The Exo⁻ transconjugants were tested for the inability to nodulate peas and clovers (Table 5). When any of the *R. leguminosarum* biovar *viciae* Exo⁻ transconjugants were

TABLE 5. Properties of the *R. leguminosarum* biovar *viciae* and *R. leguminosarum* biovar *trifolii* Exo⁻ transconjugants

Strain	Parental *exo* mutant[a]	Class	Exo[b]	Symbiotic phenotype[c]
R. leguminosarum biovar *viciae*				
128C569			+	Ndv⁺ Fix⁺
RL301	CE301	A2	−	Nod⁻ Fix⁻
RL320	CE320	B	−	Nod⁻ Fix⁻
RL320(pCOS320.1)		B/+	+	Ndv⁺ Fix⁺
RL330	CE330	A2	−	Nod⁻ Fix⁻
RL341	CE341	A1	−	Nod⁻ Fix⁻
RL341(pCOS338.1)		A1/+	+	Ndv⁺ Fix⁺
RL341(pCOS341.6)		A1/+	+	Ndv⁺ Fix⁺
RL343	CE343	B	−	Nod⁻ Fix⁻
RL343(pCOS320.1)		B/+	+	Ndv⁺ Fix⁺
R. leguminosarum biovar *trifolii*				
BT2			+	Ndv⁺ Fix⁺
BT307	CE307	A2	−	Ndv⁻ Fix⁻
BT338	CE338	A1	−	Ndv⁻ Fix⁻
BT338(pCOS338.1)		A1/+	+	Ndv⁺ Fix⁺
BT338(pCOS341.6)		A1/+	+	Ndv⁺ Fix⁺

[a] Each transconjugant was obtained by mating wild-type strain 128C569 or BT2 with an Exo⁻ mutant of strain CFN42 (see Table 1 and the text).
[b] Symbols: +, Mucoid colonies; −, nonmucoid colonies.
[c] Strains of *R. leguminosarum* biovar *viciae* were tested on peas (*Pisum sativum* cv. Wando). Strains of *R. leguminosarum* biovar *trifolii* were tested on clover (*Trifolium repens* cv. Ladino). Abbreviations: Ndv⁺, Normal nodule development; Ndv⁻, underdeveloped nodules; Nod⁻, no detectable nodule structures; Fix⁺, nitrogenase activity; Fix⁻, no nitrogenase activity.

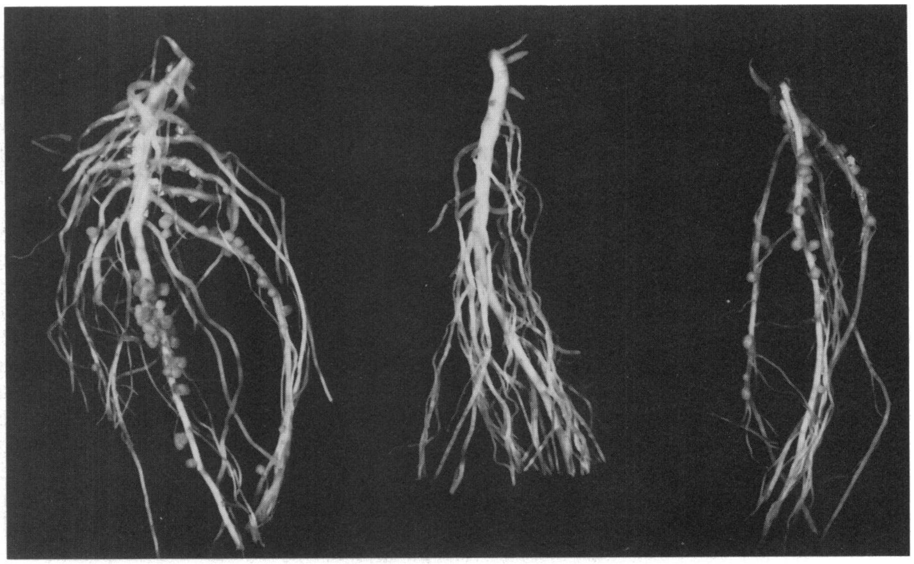

FIG. 6. Roots of 24-day-old peas (*Pisum satirum* cv. Wando) inoculated with strains 128C569 (left), RL320 (center), RL320(pCOS320.1) (right).

inoculated onto peas, no nodules were observed 24 days after inoculation, whereas the nodules induced by *R. leguminosarum* biovar parental *viciae* 128C569 were well developed at this time (Fig. 6) and exhibited nitrogenase activity.

Whereas the two class B mutant alleles *exo-320*::Tn*5* and *exo-343*::Tn*5* caused defective LPS biosynthesis in the genetic background of *R. leguminosarum* biovar *phaseoli* CFN42 (Fig. 1A, lanes 2 and 3), in the genetic background of *R. leguminosarum* biovar *viciae* 128C569 the mutations did not appear to affect LPS (Fig. 7, lanes 3 and 4). Therefore, the nodulation defect on peas caused by these mutations was due to the lack of acidic EPS rather than a defect in LPS production.

The *R. leguminosarum* biovar *viciae* Exo$^-$ transconjugants RL320 and RL343 were restored to Exo$^+$ (mucoid colony appearance) by cosmids pCOS320.1 and pCOS320.3. Exo$^-$ transconjugant RL341 was complemented to Exo$^+$ by cosmids pCOS338.1 and pCOS341.6 (Table 5). When transconjugants carrying complementing cosmids were tested on peas, wild-type nodulation (Fig. 6) and nitrogenase activity were restored. Because *exo*$^+$ DNA restored nodulation, the Nod$^-$ phenotype of the Exo$^-$ *R. leguminosarum* biovar *viciae* strains was inferred to be caused by the *exo*::Tn*5* mutations.

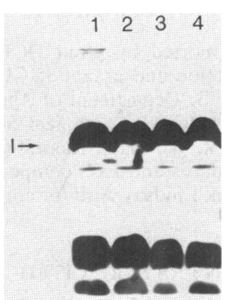

FIG. 7. Silver stain profiles by SDS-PAGE of extracts of cultured *R. leguminosarum* biovar *viciae* transconjugants harboring *exo*::Tn*5* alleles. Lanes: 1, 128C569 (wild type); 2, RL341; 3, RL320; 4, RL343. The position of LPS I, the O-antigen-containing LPS, is indicated.

A somewhat different nodulation phenotype was observed on clover plants that were inoculated with the *R. leguminosarum* biovar *trifolii* Exo$^-$ transconjugants (Table 5). After 24 days there were many small white bumps scattered along the roots. These bumps emerged later than did the nodules induced by the wild type. After 24 days roots inoculated with parental strain *R. leguminosarum* biovar *trifolii* BT2 had nodules that were large, pink, and present on the uppermost portion of the root. After 4 weeks clover plants inoculated with the Exo$^-$ transconjugants were smaller than plants inoculated with the wild type and exhibited a chlorotic appearance, which is indicative of nitrogen deficiency. Exo$^-$ *R. leguminosarum* biovar *trifolii* BT338 was complemented to Exo$^+$ (mucoid colony appearance) and normal nodulating ability by cosmids pCOS338.1 and pCOS341.6 (Table 5).

DISCUSSION

The results from a number of previous studies have indicated that rhizobial EPS production is necessary for successful nodulation of alfalfa, peas, clovers, and *Leuceana leucocephala* (5, 13, 14, 25, 31). The results from this study also support the idea that acidic EPS production by *R. leguminosarum* strains is necessary for normal nodule development on clovers and peas. However, acidic EPS did not seem to be essential for bean nodulation. The results, therefore, agree fully with and expand upon the work of Borthakur et al. (5).

The results also are consistent with those of an earlier study in which about half of the Exo$^-$ mutants of a *R. leguminosarum* biovar *phaseoli* strain were nodulation defective (39). In the present study the two class B Exo$^-$ mutants of *R. leguminosarum* biovar *phaseoli* CFN42 were Ndv$^-$, whereas the eight class A Exo$^-$ mutants were Ndv$^+$. Both class B mutants also lacked O-antigen-containing LPS, whereas class A mutants were Lps$^+$. Based on previous work with Lps mutants of strain CFN42 (10, 12, 35), the LPS defect is sufficient to explain why the class B mutants were

Ndv$^-$. Perhaps the Nod$^-$ Exo$^-$ mutants of an earlier study (39) also were defective in LPS.

Transconjugants carrying the *exo*::Tn*5* alleles induced defective nodules on clovers and peas. Since cosmids complementing A1 and B mutants restored the wild-type proficiency for the nodulation of clovers and peas, as well as EPS production, the mutations causing Exo$^-$ were responsible for the defective nodule development on clovers and peas. It is still possible that the Exo$^-$ phenotype itself is not the cause of the nodulation defects; an *exo* mutation may have pleiotropic effects. However, by mutating three distinct loci, the same correlation between EPS production and nodulation on clovers and peas resulted.

Most previous studies of Exo$^-$ *R. leguminosarum* mutants have been based on only one mutant per study. One mutant (*R. leguminosarum* biovar *viciae*) has been studied biochemically (11) but not genetically. Conceivably, its defect in pea nodulation could be due to a different mutation than the one that caused the Exo$^-$ defect. Mutants that have been studied genetically have not been studied biochemically (5, 13). In this study the mutants were analyzed biochemically as well as genetically. The EPS of the wild type was characterized by gel filtration and sugar composition. This type of analysis showed that the *exo*::Tn*5* mutations eliminated the production of the well-known *R. leguminosarum* acidic EPS containing galactose, glucose, and glucuronic acid (11, 28). The mutations did not eliminate neutral EPS. The acidic EPS was restored when plasmids containing the presumptive wild-type *exo* alleles were introduced into class A1 and B mutants. Therefore, mucoid or nonmucoid character was due to the presence or absence of the acidic EPS found in other strains of *R. leguminosarum* that have been analyzed chemically (11, 28).

Whereas the fluorescent dye calcofluor has been very useful in isolating and studying Exo$^-$ mutants of *R. meliloti*, it is not useful for the same purpose in *R. leguminosarum*. Colonies of Exo$^-$ mutants of strain CFN42 are stained more intensely than wild-type colonies on agar containing calcofluor (33). Calcofluor appears to bind to something that is tightly associated with *R. leguminosarum* cells, e.g., cellulose (33). Previously reported mutants (43) of strain CFN42 whose colonies exhibited weak fluorescence in the presence of calcofluor on minimal agar but whose colonies exhibited normal fluorescence when yeast extract was present were shown subsequently to be purine and pyrimidine auxotrophs (33). The weak fluorescence and translucent appearance of the colonies was due to poor growth on purines and pyrimidines contaminating the agar, whereas the almost normal size of the colonies was due to overproduction of EPS under purine starvation conditions (33). Therefore, contrary to previous speculation (43), the symbiotic defect of these auxotrophic mutants was not due to an EPS deficiency.

Class A Exo$^-$ strains released LPS into the growth medium. Carlson and Lee (11) have reported a similar result with an Exo$^-$ mutant derived from a different *R. leguminosarum* strain. It may be that the Exo$^-$ mutants actively increase the production of LPS to compensate for the loss of EPS or that the lack of EPS causes a physical change in the cell surface that results in the loss of LPS from the outer membrane.

Exo$^-$ mutants of class A2 were not complemented by the cosmid library. It is quite conceivable that the library is incomplete. More exotic possibilities are that the mutations are dominant (15) or that multiple copies of this region are lethal or interfere with EPS synthesis because of imbalances in enzyme concentrations (42).

Aside from investigating the mutant EPS composition more closely, the present study complements a previous report of an *R. leguminosarum* biovar-specific EPS symbiotic role (5) in another way. In the previous study (5), host-bacteria combinations were altered by the elegant technique of introducing different symbiotic plasmids into the same *exo* (*pss*) genomic background. The differences in symbiotic performance between such *R. leguminosarum* biovar *viciae* and *R. leguminosarum* biovar *phaseoli* constructs were due either to the hosts (peas and beans) or the Sym plasmids. However, one could not exclude the possibility that the apparent difference in host requirement for EPS was predicated on the particular *R. leguminosarum* genomic background involved (i.e., strain 8002). The approach used in this study was based on an earlier demonstration of haploid recombination between different wild-type isolates of the species *R. leguminosarum* (21). The weakness of this approach is that differences may be due to either the different rhizobial isolates or the hosts. For example, strain CFN42 may produce a polysaccharide (perhaps induced by symbiosis) that suppresses the symbiotic phenotype of the *exo* mutant, regardless of the host. However, the composite observations in various laboratories strongly favor the conclusion that differences in the requirement for acidic EPS depend upon either the Sym plasmid or the host of an *R. leguminosarum* strain.

Generally, it appears that rhizobial acidic EPS production is necessary for indeterminate nodulation but is not essential for determinate nodulation. All plants known to require Exo$^+$ microsymbionts (alfalfa, peas, clovers, *Leuceana* spp.) form indeterminate nodules, whereas on two determinate plants (beans and soybeans), Exo$^-$ strains elicit normal nodule development (5, 23). This generalization also could reflect the promiscuity of the host plants. Soybeans and beans each appear to be nodulated by a wider range of rhizobial species compared with temperate legumes. Alternatively, the varied requirements for EPS may indicate that other bacterial surface components (such as LPS) carry out the symbiotic functions of EPS in different rhizobium-legume interactions. The suppression of an *exo* mutant by another rhizobial surface component may even occur in the same rhizobium-legume combination. For example, isolates of *R. meliloti* 41 apparently lacking the normal acidic EPS form effective nodules on alfalfa (37). *R. meliloti* SU47 mutants lacking this EPS also can induce nitrogen-fixing alfalfa nodules if a normally cryptic different acidic EPS is produced (20, 45).

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