JOURNAL OF BACTERIOLOGY, Nov. 1989, p. 6161–6168
0021-9193/89/116161-08$02.00/0

Vol. 171, No. 11

Genes Involved in Lipopolysaccharide Production and Symbiosis Are Clustered on the Chromosome of *Rhizobium leguminosarum* Biovar *viciae* VF39

URSULA B. PRIEFER

Lehrstuhl für Genetik, Fakultät für Biologie, Universität Bielefeld, Postfach 8640, D-4800 Bielefeld 1, Federal Republic of Germany

Received 18 May 1989/Accepted 23 August 1989

Four mutants of *Rhizobium leguminosarum* biovar *viciae* VF39 altered in lipopolysaccharide (LPS) synthesis were isolated upon random Tn*5* mutagenesis. These mutants produced matt colonies on TY medium and showed autoagglutination and loss of motility. On sodium dodecyl sulfate-polyacrylamide gels, they lacked a slow-migrating carbohydrate band, corresponding to the complete LPS (LPSI). All four mutants formed small white nodules on *Vicia hirsuta*. These nodules were infected but showed no nitrogen-fixing activity and senesced prematurely. Three of the mutants were complemented by a wild-type cosmid to synthesis of normal LPS and induction of nitrogen-fixing nodules. By hybridization and in vivo cloning experiments, the mutations were mapped within different *Eco*RI fragments which could be localized on the VF39 chromosome. Cross-complementation analyses revealed that the three mutants were affected in different transcriptional units. The results indicate that a cluster of genes necessary for LPSI production and symbiotic efficiency is located within a defined region of 20 kilobases on the *R. leguminosarum* bv. *viciae* chromosome.

The establishment of nitrogen-fixing nodules in the *Rhizobium*-legume symbiosis is a complex multistep interaction between microsymbiont and its specific host plant (for a review, see reference 2). Early events involve recognition and deformation or curling of root hair cells. Cortical cell division, leading to the formation of a unique organ, the nodule, is induced probably by diffusible bacterial substances. Invasion of the nodule by the bacteria is initiated by penetration of the root hair cell wall and the formation of an infection thread, in which the bacteria are carried towards the dividing root cortex cells. Bacteria are eventually released from the infection thread into the plant cells. They remain separated from the host cytoplasm by peribacteroid membranes and differentiate into bacteroids able to reduce atmospheric nitrogen.

This complex developmental process obviously requires continuous signal exchange between plant and bacterial cells. Undoubtedly, bacterial cell surface components play an important role in this specific interaction; particularly extracellular polysaccharides (EPSs) and lipopolysaccharides (LPSs) have been hypothesized to be involved in the symbiotic process (for reviews see references 5 and 15).

The LPS of gram-negative bacteria consists of lipid A, a core oligosaccharide and an O-antigenic side chain. The *Rhizobium* LPSs are very heterogenous molecules and vary greatly among different species as among strains of a single species (6, 49). Therefore, rhizobial LPS has been suggested to be involved in specific recognition and attachment of bacteria to the root hair cells of compatible host plants, mediated by the binding of lectins (47). While recent work (12) supports an essential function of lectins in host-plant specificity, a role of LPS in host-specific nodulation remains unclear.

Differences in LPS composition were described between nodulating and nonnodulating strains of *Rhizobium leguminosarum* bv. *trifolii* (previously called *R. trifolii*) (8, 34, 35). Similarly, quantitative differences in the LPS of a Nod^- mutant of a fast-growing *Rhizobium japonicum* strain were observed (9). However, in both cases, the alterations were caused not by single mutations but by the elimination of a plasmid from the nodulating strain. Thus, these data do not necessarily imply a role of the LPS in the nodulation ability. Also, mutants of *Bradyrhizobium japonicum* were found to be defective in both nodulation and LPS synthesis, but again, it was not shown that these phenotypes originated from single mutations (24, 42).

Defined single mutations leading to simultaneous defects in both LPS synthesis and symbiotic ability were described for *R. leguminosarum* bv. *phaseoli* (10, 28) and recently also for *R. leguminosarum* bv. *viciae* 248 (11).

The studies presented here have concentrated on the isolation of defined mutants of *R. leguminosarum* bv. *viciae* VF39 with altered surface polysaccharides. Tn*5*-induced mutants were isolated which exhibited both defects in LPS production and symbiotic nitrogen fixation. The pleiotropic mutant phenotype was shown to be due to single transposon insertions, localized within a defined region of the *R. leguminosarum* chromosome.

(Preliminary aspects of this work were presented at the 8th Nitrogen Fixation Congress in Cologne, Federal Republic of Germany, 1988 [33], and at the 4th International Meeting on Microbe-Plant Interaction in Acapulco, Mexico, 1988 [31]).

MATERIALS AND METHODS

Bacterial strains and plasmids. *R. leguminosarum* VF39 was isolated from nodules of field-grown *Vicia faba* (cultivar Kristall) in Bielefeld, Federal Republic of Germany. It contains six plasmids (pVF39a through pVF39f) in the range between 90 and 400 megadaltons (MDa). The strain used in this study is a spontaneous streptomycin-resistant derivative of VF39. Strain UBAPF2 (19) is a plasmid-free derivative of *Agrobacterium tumefaciens*. Upon insertion of Tn*5*-Mob (36), the VF39 plasmids were individually introduced into UBAPF2, resulting in strains UBAPF2(pVF39a) through UBAPF2(pVF39f) (17). The *Escherichia coli* strains and

TABLE 1. *E. coli* strains, plasmids, and phages

Strain, plasmid or phage	Relevant characteristics	Source or reference
Strains		
S17-1	*E. coli* 294, RP4-2 (Tc::Mu) (Km::Tn*7*) integrated in the chromosome	38
CSH56	*nalA*	26
K802	*hsdR supE*	48
Plasmids		
pSUP1021	pACYC184 derivative Mob, Tn*5*, Cm, Tc, Km	37
pSUP205	pBR325 derivative Mob, cos, Cm, Tc	38
pME206	RP4 derivative, deletion in Tc gene, Ap, Km	D. Haas, ETH Zürich
RP4 (Km::ISR*1*) (Tc::Tn*5*-Gm)	RP4 derivative with ISR*1* (32) insertion in Km gene and Tn*5*-Gm in Tc gene, Ap, Gm	22
Recombinant plasmids		
pCos4	38-kilobase DNA of *R. leguminosarum* VF39 cloned in pSUP205	This work
pCos4-23	Tn*5*-carrying pCos4 derivative isolated from LpsI mutant VF39-23	This work
pCos4-32	Tn*5*-carrying pCos4 derivative isolated from LpsI mutant VF39-32	This work
pCos4-51	Tn*5*-carrying pCos4 derivative isolated from LpsI mutant VF39-51	This work
pSUP205-E6	6.3-kilobase *Eco*RI fragment from pCos4 cloned into pSUP205	This work
pSUP205-E8	9.5-kilobase *Eco*RI fragment from pCos4 cloned into pSUP205	This work
Phages		
λ cI ts	Lambda mutant, *c*I857	26
λ::Tn*5*	Transposon Tn*5* inserted on lambda mutant genome	3
M13mp8-IS*50*	M13 derivative carrying part of the inverted repeats of Tn*5*	37

plasmids used and constructed in this work are listed in Table 1.

Media, growth conditions, and bacterial matings. *E. coli* strains were grown in Penassay broth (Difco Laboratories) or LB medium (26). *R. leguminosarum* VF39 and *A. tumefaciens* strains were usually cultured in tryptone-yeast (TY) medium (4) at 30°C. Mutants of VF39 were identified on minimal medium (45) or yeast-mannitol medium (46). To test for auxotrophy, the mutants were grown on minimal medium prepared with agarose instead of agar (15 g/liter). Antibiotics were used at the following concentrations: neomycin, 100 μg/ml; tetracycline, 5 μg/ml; kanamycin, 25 μg/ml; streptomycin, 400 μg/ml; nalidixic acid, 100 μg/ml; gentamicin, 25 μg/ml for *Rhizobium* spp. and 10 μg/ml for *E. coli*. Bacterial crosses were performed on membrane filters (pore size, 0.2 μm) as described elsewhere (36).

Plant tests and microscopic studies. Nodulation and nitrogen-fixing ability of bacterial strains were tested on *Vicia hirsuta*. Seeds were surface sterilized by hydrochloride or H_2SO_4 treatment and were germinated in the dark for 3 days (44). Seedlings were then transferred onto nitrogen-free salt medium (44), inoculated with bacteria, and grown for 21 to 25 days. Nitrogenase activity was assayed by acetylene reduction activity. For microscopic analyses of nodules, longitudinal semithin sections were prepared and stained as described (1).

Tn*5* mutagenesis. Streptomycin-resistant *R. leguminosarum* VF39 was mated with S17-1(pSUP1021), and Tn*5*-carrying transconjugants were selected on TY medium supplemented with neomycin and streptomycin. Nm^r transconjugants were tested on tetracycline-containing medium for retention of the transposon carrier and on minimal medium for auxotrophic mutants. Colony morphology mutants were identified visually on yeast-mannitol and TY media. Fragment-specific mutagenesis with Tn*5* was basically carried out as described elsewhere (21, 39).

Complementation analyses. Since the VF39 wild-type cosmid library was constructed in pSUP205 (Tc), which is not able to replicate in *Rhizobium* strains, complementation of the mutants was performed in two different ways. These were (i) RP4-cosmid cointegrate formation and (ii) integration into the genome.

(i) RP4-cosmid cointegrate formation. The principle of this method is described elsewhere (37). In the work presented here, the VF39 wild-type cosmid gene bank or individual cosmid clones were introduced into *E. coli* CSH56 carrying the Tc^s RP4 derivative pME206. Because of homologous DNA regions on RP4 and the cosmid vector pSUP205, single crossover events can occur, resulting in fused molecules. These RP4-cosmid cointegrates were conjugally transferred into *R. leguminosarum* mutants selecting for the vector-encoded tetracycline resistance. Tc^r transconjugants were then tested for complementation of the mutant phenotype. Complementing cointegrates were transferred back into *E. coli* K802 (λ cI ts), where spontaneous resolution of the cointegrates could occur. Heat induction of the λ *c*I ts prophage resulted in packaging of the complementing cosmid.

(ii) Integration into the genome. Alternatively, cosmids were introduced into *E. coli* S17-1 and mobilized into the *R. leguminosarum* mutants. Selection for the vector-encoded tetracycline resistance resulted in transconjugants carrying the cosmid integrated into the genome via single crossover events. The merodiploid transconjugants were then tested for complementation of their mutant phenotype.

In vivo cloning of the Tn*5* insertions. The Tn*5* insertions were cloned from the mutant genomes by in vivo cloning basically as described elsewhere (22, 27). The wild-type cosmid pCos4 was integrated into the genomes of mutant strains, and Tn*5*-carrying derivatives were transferred back with the helper plasmid RP4(Km::ISR1) (Tc::Tn*5*-Gm). As *E. coli* recipient, a λ cI ts lysogenic K802 derivative was used, so that the reisolated cosmids could be packaged in vivo into lambda bacteriophage particles by heat induction.

Reverse mutagenesis (homogenotization). Tn*5*-carrying cosmids or subcloned DNA fragments were introduced into *E. coli* S17-1 and mobilized into VF39 (Sm) wild type. Nm^r Sm^r transconjugants were tested for loss of the vector-encoded tetracycline resistance. Recombination of the Tn*5*-carrying region into the genome via double crossover was confirmed by hybridization.

Preparation of LPS and SDS-PAGE. For the isolation of crude LPS, cells were grown in TY medium and harvested in stationary phase. After enzymatic digestion with lysozyme,

RNase, and DNase, hot phenol-water extractions were performed (20). The aqueous phase was dialyzed exhaustively against water and freeze dried.

For miniscale preparations, 1.5 ml of stationary cultures were pelleted, suspended in sodium dodecyl sulfate (SDS) sample buffer (23), and denatured at 100°C for 3 min. These extracts were either directly subjected to SDS-polyacrylamide gel electrophoresis (PAGE) or treated with proteinase K in order to digest proteins (16). After discontinuous SDS-PAGE (23), gels were stained by Coomassie blue for proteins or by periodic acid-silver for carbohydrates (43).

DNA biochemistry. Routine visualization of plasmids was accomplished by a modified Eckhardt technique (13, 18, 19). Isolation and restriction enzyme analyses of genomic and plasmid DNA were carried out as described previously (14, 29, 30). Cloning and transformation experiments were performed by established protocols (25). The cosmid library of *R. leguminosarum* VF39 wild-type genome was constructed as follows: total DNA of VF39 was prepared, partially digested with *Eco*RI, and cloned into the mobilizable vector pSUP205 basically as described previously (30). Upon in vitro packaging, recombinant molecules were introduced into *E. coli* S17-1 and identified by their tetracycline resistance and chloramphenicol sensitivity. The cosmid bank was maintained as a phage lysate after in vivo packaging by λ cI ts.

For hybridizations, DNA was transferred from agarose gels onto nitrocellulose filters by Southern (blot hybridization) transfer (41). DNA probes were labeled with biotin-ATP, and hybridizations were carried out as recommended by the manufacturer (BlueGene system; Bethesda Research Laboratories, Inc.).

RESULTS

Isolation and characteristics of Lps mutants. *R. leguminosarum* VF39 (Sm) was mutagenized with Tn*5*, and Nm^r transconjugants were isolated at a frequency of 10^{-4} per recipient. Integration of the Tn*5* carrier vector, identified by retention of the tetracycline resistance marker, was observed at a frequency of approximately 10%, and auxotrophic mutations occurred within the range of 0.5 to 1%.

Mutants with altered colony morphology were identified visually. Besides colonies which were altered in slime production on yeast-mannitol and minimal media, clones were isolated that generated colonies on TY plates which appeared less glossy than the wild-type strain. These mutant strains were designated VF39-23, VF39-32, VF39-51 and VF39-C86. In liquid minimal medium, they showed growth comparable to that of the parent strain. In TY liquid medium, the mutants exhibited the tendency to precipitate (autoagglutination [Fig. 1]). When tested on TY medium containing only 0.3% agar, the wild-type strain was able to swarm, whereas the mutants had lost their motility (Fig. 1).

These characteristics were expressed, dependent on the Ca^{2+} concentration in the medium. Under low Ca^{2+} concentrations, the mutants no longer showed autoagglutination and rough colonies on TY plates. Ca^{2+} also influenced the motility of the *R. leguminosarum* VF39 wild type. Concentrations of 1 mM or less resulted in loss of motility, and the wild type behaved like the mutant strains.

Total DNA of the mutants was digested with *Eco*RI and hybridized to M13mp8-IS*50* labeled with biotin-ATP. This DNA probe contains part of the inverted repeat of Tn*5* (IS*50*) cloned into M13mp8. For each mutant, only one fragment hybridized, indicating single Tn*5* insertions; moreover, independent IS*50* transpositions could be excluded. Since the size of the hybridizing fragments differed from mutant to mutant, it could be concluded that they represented independent mutations (data not shown).

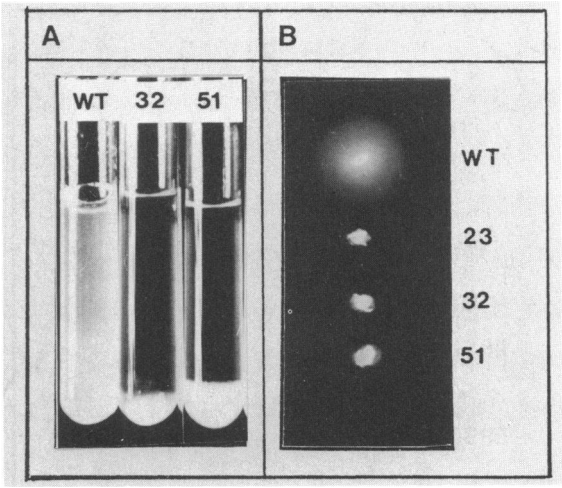

FIG. 1. Growth characteristics of wild-type VF39 and LpsI mutants. (A) Strains were grown in 10 ml of TY liquid medium; after 3 days, the LpsI mutants showed autoagglutination. (B) Swarm behavior of VF39 wild-type and mutant strains on TY soft agar (0.3% agar). WT, Wild type; 23, VF39-23; 32, VF39-32; 51, VF39-51.

Crude cell extracts of wild-type and mutant strains were separated on SDS-polyacrylamide gels and stained with Coomassie blue for proteins. No differences between mutant and wild-type strains could be detected. Coomassie blue staining was eliminated after digestion with proteinase K or when LPS samples obtained by hot phenol-water extraction were subjected to SDS-PAGE. When the gels were stained by the periodic acid-silver procedure, commonly used to stain LPSs, a slow-migrating carbohydrate band (LPSI) present in the wild type was missing in the mutants, whereas the low-molecular-weight band (LPSII) was present in both parent and mutant strains (Fig. 2). It also appeared that the low-molecular-weight band was much more intense in mutant strains, compared with the wild-type, which often showed only a very faint low-molecular-weight band. The results indicate that the *R. leguminosarum* mutants isolated in this study are affected in normal LPS production. As revealed by silver staining, they lack or produce at least a very reduced amount of LPSI and are therefore designated as LpsI mutants.

The four LpsI mutants were tested on *V. hirsuta* plants. All of them induced many nodules which were round, small, and white, compared with the reddish, long-shaped nodules induced by the VF39 wild-type strain. They were also devoid of nitrogenase activity as determined by acetylene reduction assay.

The morphology of a nodule, induced by mutant VF39-32, is shown in Fig. 3. Light microscopic analysis of semithin sections, 3 weeks after inoculation, revealed the presence of an apical meristem, a thread invasion zone with ramifying infection threads, and an infected zone with mutant bacteria released into plant cells. The nodules, despite exhibiting the typical morphology of indeterminate nodules, remained small and spherical (maximum size was one-third that of wild-type nodules) and senesced prematurely.

Complementation of *R. leguminosarum* LpsI mutants. A wild-type cosmid gene bank of *R. leguminosarum* VF39 was

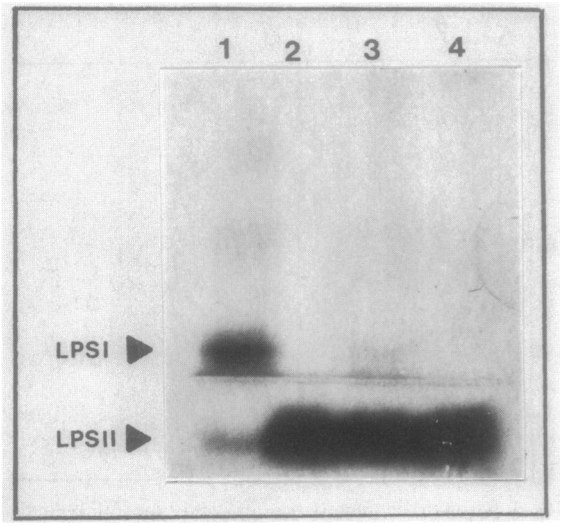

FIG. 2. LPS profiles on SDS-polyacrylamide gels. Periodic acid-silver stained SDS-polyacrylamide gel (15%) of proteinase K-treated SDS extracts of *R. leguminosarum* VF39 wild type (lane 1), VF39-23 (lane 2), VF39-32 (lane 3), and VF39-51 (lane 4). The arrows indicate the positions of suspected forms of LPS.

constructed in the mobilizable vector pSUP205 and maintained packaged in λ phage particles. Upon infection of *E. coli* CSH56(pME206), the plasmids were introduced en masse into *R. leguminosarum* mutant VF39-32 by conjugation. Cointegrates between RP4 and the cosmids selected by Tc^r transconjugants occurred at a high frequency. The presence of cointegrates in the mutant strain was verified by a modified Eckhardt gel electrophoresis. Most of the transconjugants carried an additional band of the size expected for a plasmid composed of the RP4 derivative and the cosmid; only a few colonies contained a plasmid comigrating with RP4, indicating that the cosmid had integrated into the genome of the mutant via homologous recombination.

Transconjugants carrying cointegrates were tested for their colony morphology on TY plates, their agglutination behavior, and their ability to swarm on soft agar. From 20 colonies which appeared to be complemented, the cosmids were rescued by transferring the cointegrates from complemented strains back into *E. coli* and isolating the cosmid by in vivo packaging. On the basis of their *Eco*RI restriction pattern, the cosmids could be classified into three different groups which shared a number of fragments in common (Fig. 4A).

When representatives of these three groups (i.e., cosmids pCos4, pCos15, and pCos17) were reintroduced into mutant VF39-32 and integrated into its genome, the complementation results obtained with the RP4-cosmid cointegrates could be confirmed.

The three cosmid clones were also integrated into the genomes of the other LpsI mutants isolated in this study.

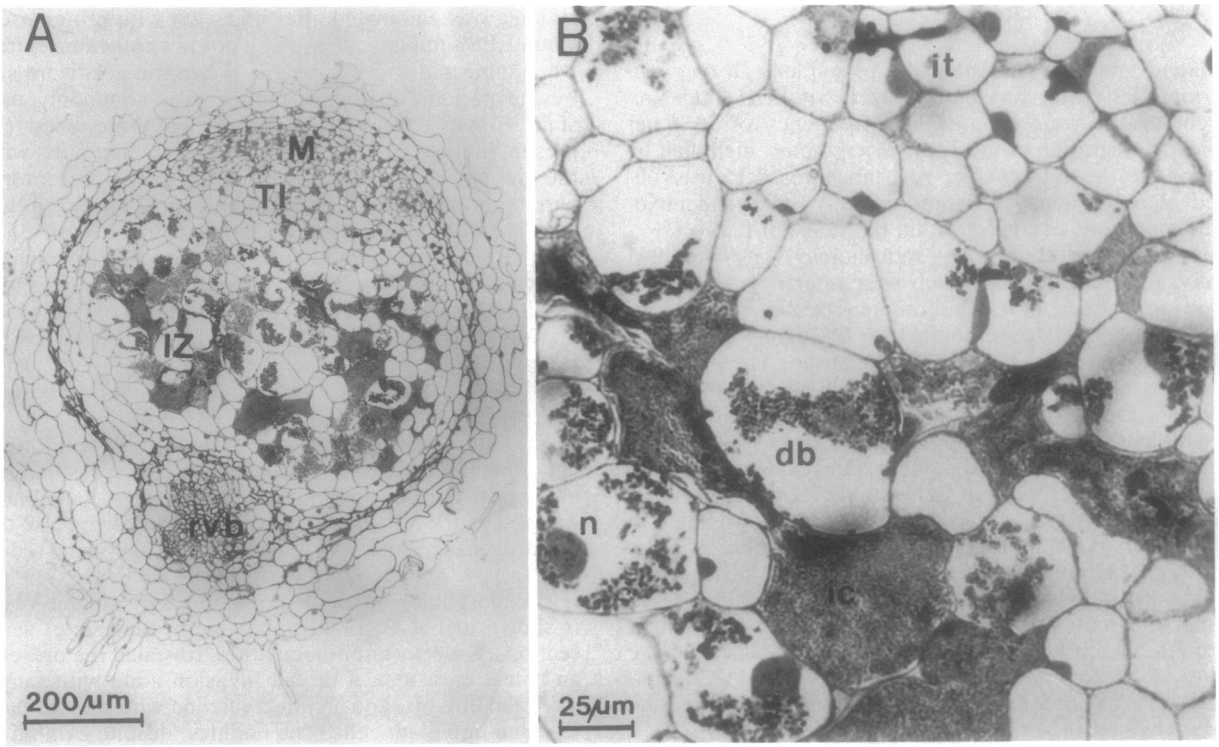

FIG. 3. Morphology of a nodule induced by LpsI mutant VF39-32 on *V. hirsuta*. Light micrograph of a longitudinal section (A) and enlarged detail (B) of the nodule, 3 weeks after inoculation. The nodule shows a meristematic zone (M), a thread invasion zone (TI) containing infection threads (it), and an infected zone (IZ) with infected plant cells (ic). Most of the bacteroids were already degenerating (db). The length of the nodule is approximately one-third of the wild-type nodule (not shown). rvb, Root vascular bundle; n, nucleus. Experiment and photographs by D. Kapp.

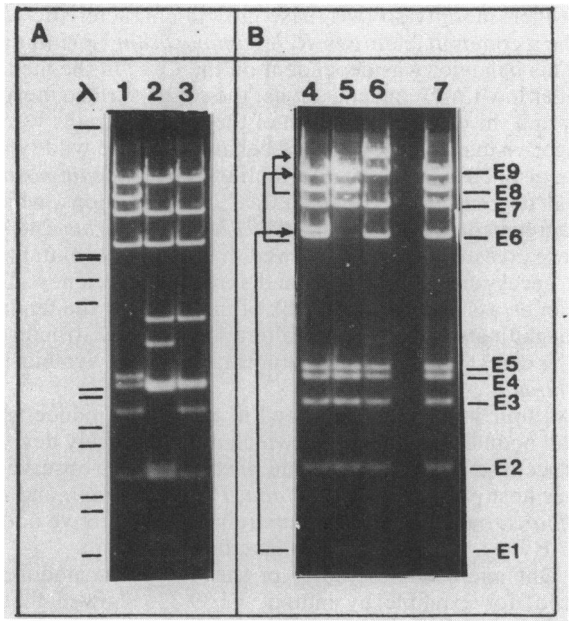

FIG. 4. Electrophoretic analysis of *Eco*RI-digested VF39 cosmids isolated from complemented LpsI mutants. (A) *Eco*RI digest of three overlapping cosmids able to restore the wild-type phenotype in mutant VF39-32. Lanes: 1, pCos4; 2, pCos15; 3, pCos17. (B) *Eco*RI pattern of Tn*5* carrying derivatives of pCos4 (lane 7; E1 to E9, E7 corresponds to the cosmid vector pSUP205). In pCos4-23 (lane 4), Tn*5* is inserted in fragment E1 (0.6 kilobases) and increases this fragment by approximately 6 kilobases (→ 6.6 kilobases); pCos4-32 (lane 5) carries the Tn*5* in fragment E6 (6.3 kilobases → 12.3 kilobases), and pCos4-51 (lane 6) contains the Tn*5* insertion in fragment E8 (9.5 kilobases → 15.5 kilobases). The λ DNA fragments generated by *Eco*RI-*Hin*dIII double digestion are indicated on the left (lane λ).

Two further mutants, VF39-23 and VF39-51, could be complemented to wild-type characteristics (i.e., normal growth and swarm behavior, normal LPS profiles on SDS-PAGE, and induction of nitrogen-fixing nodules on *V. hirsuta*). None of the three cosmids could complement mutant VF39-C86.

Since cosmid pCos4 was able to complement the three LpsI mutants VF39-23, VF39-32, and VF39-51, it was chosen for further characterization.

Hybridization of pCos4 DNA to total DNA isolated from VF39 (Sm) and digested with different enzymes confirmed that the DNA inserted in pCos4 was colinear with the VF39 genome (data not shown). The *Eco*RI restriction map of the *R. leguminosarum* VF39 DNA cloned in pCos4 is shown in Fig. 5.

Localization of the *lps* gene mutations in independent chromosomal transcription units. In order to correlate the DNA region cloned in pCos4 to either the chromosome or to one of the six plasmids endogenous in *R. leguminosarum* VF39, DNA of pCos4 was hybridized to *Eco*RI-digested total DNA of VF39 (Sm) wild type and of *A. tumefaciens* UBAPF2 carrying one of the VF39 plasmids from pVF39a through pVF39f. No hybridization corresponding to pCos4 could be detected with either of the UBAPF2 derivatives, suggesting that the DNA cloned in pCos4 originated from the VF39 chromosome (data not shown).

The *lps* mutations were mapped to specific *Eco*RI fragments of pCos4 by the following strategy. Cosmid pCos4 was integrated into the genomes of the LpsI mutants VF39-23, VF39-32, and VF39-51 by single crossovers. Second recombination events may lead either to the original or a Tn*5*-carrying derivative of pCos4. Both plasmid types can be mobilized back into *E. coli* by a helper RP4 plasmid. Tn*5*-carrying pCos4 derivatives are recognized by their tetracycline and kanamycin-neomycin resistance, whereas wild-type pCos4 confers only tetracycline resistance to the *E. coli* recipient. By this in vivo cloning procedure, the original pCos4 and the Tn*5*-carrying derivatives pCos4-23, pCos4-32, and pCos4-51 were isolated from the mutants. Comparison of the respective *Eco*RI restriction pattern (Fig. 4B) revealed that mutant VF39-32 carried the Tn*5* insertion in a 6.3-kilobase *Eco*RI fragment (E6), the Tn*5* in mutant VF39-51 could be localized on a 9.5-kilobase *Eco*RI fragment (E8), whereas the mutation in VF39-23 was due to a Tn*5* insertion in a very small *Eco*RI fragment of about 0.6 kilobases (E1). This result was confirmed by hybridizing pCos4 to *Eco*RI-digested total DNA from the mutant strains (data not shown).

The Tn*5*-carrying pCos4 derivatives pCos4-32 and pCos4-51 were introduced into the VF39 wild-type strain. Homogenotization of the Tn*5*-carrying regions into the wild-type genome generated phenotypes identical to those of the original mutants, i.e., matt colonies on TY, autoagglutination, loss of motility, lack of LPSI, and Fix$^-$ nodules on *V. hirsuta*. This experiment proved that the mutant phenotypes were caused by the identified Tn*5* insertions.

The three Tn*5*-carrying cosmids were integrated into the genome of mutant VF39-51, and transconjugants were tested for their growth and symbiotic phenotype. As expected,

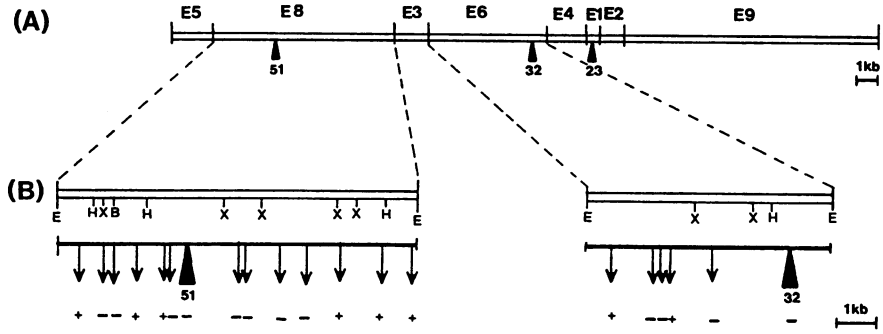

FIG. 5. Map of the *R. leguminosarum* VF39 DNA cloned in pCos4. (A) *Eco*RI restriction map. ▲, Positions of the original Tn*5* insertions in mutants VF39-23, VF39-32, and VF39-51. (B) Restriction map of the *Eco*RI (E) fragments E6 and E8 for the enzymes *Hin*dIII (H), *Xho*I (X), and *Bam*HI (B) and location of transposon insertions obtained after site-specific mutagenesis (↓). The phenotype of the corresponding homogenotes is indicated: (+) wild-type phenotype; (−) pleiotropic mutant characteristics.

pCos4-51 was not able to restore wild-type characteristics in mutant VF39-51, whereas VF39-51, containing either pCos4-32 or pCos4-23, behaved like the parental wild-type strain. Similarly, the other LpsI mutants could be cross-complemented by the respective Tn*5*-carrying cosmid derivatives. These results suggest that the Tn*5* insertions in mutants VF39-23, VF39-32, and VF39-51 have occurred in different transcriptional units.

The wild-type and the Tn*5*-carrying *Eco*RI fragments were cloned into pSUP205, and the Tn*5* insertion sites were mapped by restriction enzyme analysis within E6 and E8. Their positions are indicated in Fig. 5. When pSUP205-E6 was introduced into the genome of mutant VF39-32 by a single crossover, it was able to restore wild-type phenotype. Similarly, fragment E8, cloned into pSUP205, was sufficient for full complementation of mutant VF39-51.

The fragments E6 and E8 were mutagenized by Tn*5* and introduced into the VF39 wild-type genome by reverse mutagenesis. The occurrence of double crossover events, identified by neomycin resistance and tetracycline sensitivity, was confirmed by hybridization. The phenotypes of the respective homogenotes were tested in terms of their growth characteristics (rough surface, agglutination, motility) and LPS profiles on SDS-PAGE. The results are summarized in Fig. 5. In addition to the original mutations, further transposon insertions were identified which resulted in an LpsI phenotype. Some of them were separated by insertions which had no effect on growth characteristics and LPS synthesis. Thus, it appears that a cluster of genes involved in complete LPS production is located within the region cloned in pCos4.

DISCUSSION

This study describes the isolation and characteristics of four *R. leguminosarum* bv. *viciae* VF39 mutants which are altered in LPS synthesis. On SDS-polyacrylamide gels stained with periodic acid-silver, they lack a slow-migrating band present in the VF39 wild-type strain. This band is not stained by Coomassie blue, usually applied to detect proteins, and is insensitive to pronase or proteinase treatment. For *R. leguminosarum* bv. *phaseoli* and *R. leguminosarum* bv. *trifolii*, it was shown that this high-molecular-weight fraction corresponds to LPSI containing the O-antigen side chain (7, 8). To decide whether the mutants described here completely lack the LPSI or produce very reduced quantities, a more sensitive immunoblotting assay would be necessary. However, although confirmation by biochemical analysis is required, it is very much likely that they are altered in the synthesis of a normal O-antigen polysaccharide.

The fact, that the low-molecular-weight band appears to be more intense than that of the wild type would imply that the overall amounts of LPS in mutant and parent strains are identical. Similar observations have been reported for *R. leguminosarum* bv. *phaseoli* (10).

The defect in carbohydrate production is correlated with a number of other mutant phenotypes, such as rough colony surface, autoagglutination, loss of motility, and the lack of the ability to generate an effective symbiosis. In terms of their rough colony appearance and their agglutination in TY medium, the VF39 LpsI mutants resemble mutants of *R. leguminosarum* bv. *phaseoli* (10, 28). Similarly, some mutants of *R. leguminosarum* bv. *viciae*, isolated recently (11) and shown to be altered in LPSI, obviously have the tendency to clump; these mutants were also reported to have lost their motility so that these growth characteristics seem to be a common feature of *R. leguminosarum* LpsI mutants.

This behavior was dependent on the Ca^{2+} in the medium. Under low Ca^{2+} concentrations, the rough surface morphology and the autoagglutination of the mutant strains were no longer visible and the mutants behaved like the wild type. It was also observed that the motility of *R. leguminosarum* VF39 decreased with reduced Ca^{2+} concentrations, which is in agreement with findings for *R. leguminosarum* 248 (40). These investigations also showed that nonmotile mutants did not necessarily lack flagella and symbiotic efficiency. Thus, it can be assumed that the lack of motility and the tendency to agglutinate are due to the alterations in LPS structure, as is the defect in establishing a normal effective symbiosis on *V. hirsuta*.

All four mutants investigated in this study induce white, small nodules on *V. hirsuta*, which are completely devoid of nitrogenase activity. The same phenotype was observed on other host plants such as *V. faba*, *Pisum sativum*, and *Lens culinaris*, on which the parent strain forms effective nodules (M. Hynes, personal communication).

Light microscopic studies of the *V. hirsuta* nodules induced, for example by mutant VF39-32, showed that the defect occurs relatively late in symbiotic development: the nodules contain infection threads from which bacteria are released into host cells and become enclosed in peribacteroid membranes (data not shown). However, the nodules remain small and senescence occurs prematurely. This phenotype is similar to that of the *R. leguminosarum* 248 Lps mutants (11), whereas the mutants described for *R. leguminosarum* bv. *phaseoli* show no bacterial release at all (10, 28).

Interestingly, all genetically defined *R. leguminosarum* LpsI mutants published until now (10, 11, 28) are able to induce nodules but are unable to establish an effective symbiosis. The first mutants of *R. leguminosarum* bv. *phaseoli* were primarily selected for abnormal nodule development (28); however, all other Lps mutants, including those described here, were selected for LPS deficiency and were afterwards found to be all Nod^+ but Fix^-. This would imply that the LPSI of *R. leguminosarum* is required not in early recognition and specific attachment but in later steps of nodule development.

Until now, it has not been shown that the symbiotic defect is the direct consequence of the altered LPS. It is possible, that simultaneous changes in outer membrane composition causes the symbiotic phenotype. However, one can speculate about a possible function of LPS during symbiosis. One hypothesis is that LPSI physically masks other cell surface components, thereby protecting the bacterium from plant defense mechanisms which may occur, for example, during early steps of infection or at the stage of bacterial release. Another possibility could be that the appropriate LPSI serves actively as a signal for the plant. This hypothesis is supported by inoculation tests carried out by mixing VF39 LpsI mutants and Nod^- mutants of *R. leguminosarum* 8400 (pRL1JI) (kindly provided by A. W. B. Johnston). The combination of the two mutants was able to induce nitrogen-fixing nodules on *V. hirsuta* (data not shown). This result is consistent with the idea of LPSI being a signal which can be provided in trans by the Nod^- mutant.

The genetic data presented in this work demonstrate a strong correlation between growth behavior, LPSI structure, and symbiotic properties in the mutant strains: (i) hybridization of total DNA to IS*50* revealed the presence of only one hybridizing fragment, so that independent transposition of a

second Tn*5* or of IS*50* alone can be excluded; (ii) complementation experiments with cosmids and subcloned fragments led to simultaneous restoration of all mutant phenotypes; and (iii) introduction of the Tn*5* mutations into the wild-type genome by homogenotization generated the same pleiotropic phenotype as in the original mutant. These experiments prove that all features of a mutant strain are caused by a single Tn*5* insertion.

It could also be demonstrated that genes involved in LPS production are clustered on the chromosome of VF39. In cosmid pCos4, which contains about 38 kilobases of the VF39 chromosome, at least three separate genetic regions were identified to be necessary for LPSI production and symbiotic efficiency. Thus, the *lps* gene region identified on pCos4 appears to be rather extensive and to be organized in separate transcriptional units. However, there is at least one other DNA region in VF39 involved in LPS synthesis as demonstrated by mutant VF39-C86, which cannot be complemented by pCos4. Cloning and analysis of the respective wild-type DNA is currently being performed.

ACKNOWLEDGMENTS

I thank S. Preisler, and J. deWall for contributing to this report by carrying out the fragment-specific Tn*5* mutagenesis experiments, M. Hynes for providing unpublished information, and A. Kleickmann, M.-L. Wilke, and J. Meyer for photographic and technical assistance. I am especially indebted to D. Kapp for carrying out the microscopic studies. A. Pühler, R. Simon, and B. Kosier are acknowledged for their help and for reading and correcting the manuscript.

I also thank the European Community (grant no. BAP-0081-D) and the Deutsche Forschungsgemeinschaft (SFB 223) for their financial support.

LITERATURE CITED

1. **Aguilar, O. M., D. Kapp, and A. Pühler.** 1985. Characterization of a *Rhizobium meliloti* fixation gene (*fixF*) located near the common nodulation region. J. Bacteriol. **164**:245–254.
2. **Bauer, W. D.** 1981. Infection of legumes by *Rhizobia*. Annu. Rev. Plant Physiol. **32**:407–449.
3. **Berg, D. E.** 1977. Insertion and excision of the transposable kanamycin resistance determinant Tn*5*, p. 205–212. *In* A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), DNA insertion elements, plasmids, and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
4. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84**:188–198.
5. **Carlson, R. W.** 1982. Surface chemistry, p. 199–234. *In* W. J. Broughton (ed.), Nitrogen fixation, vol. 2. *Rhizobium*. Clarendon Press, Oxford.
6. **Carlson, R. W.** 1984. Heterogeneity of *Rhizobium* lipopolysaccharides. J. Bacteriol. **158**:1012–1017.
7. **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.
8. **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.
9. **Carlson, R. W., and M. Yadav.** 1985. Isolation and partial characterization of the extracellular polysaccharides and lipopolysaccharides from fast-growing *Rhizobium japonicum* USDA 205 and its Nod$^-$ mutant, HC205, which lacks the symbiotic plasmid. Appl. Environ. Microbiol. **50**:1219–1224.
10. **Cava, J. R., P. M. Elias, D. A. Turowski, and D. K. Noel.** 1989. *Rhizobium leguminosarum* CFN42 genetic regions encoding lipopolysaccharide structures essential for complete nodule development on bean plants. J. Bacteriol. **171**:8–15.
11. **DeMaagd, R. A., A. S. Rao, I. H. M. Mulders, L. Goosen-de Roo, M. C. M. VanLoosdrecht, 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.
12. **Diaz, C. L., L. S. Melchers, P. J. J. Hooykaas, B. J. J. Lugtenberg, and J. W. Kijne.** 1989. Root lectin as a determinant of host-plant specificity in the *Rhizobium* legume symbiosis. Nature (London) **338**:579–581.
13. **Eckhardt, T.** 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid **1**:584–588.
14. **Grönger, P., S. S. Manian, H. Reiländer, M. O'Connell, U. B. Priefer, and A. Pühler.** 1987. Organization and partial sequence of a DNA region of the *Rhizobium leguminosarum* symbiotic plasmid pRL6JI containing the genes *fixABC*, *nifA*, *nifB* and a novel open reading frame. Nucleic Acids Res. **15**:31–49.
15. **Halverson, L. J., and G. Stacey.** 1986. Signal exchange in plant-microbe interaction. Microbiol. Rev. **50**:193–225.
16. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154**:269–277.
17. **Hynes, M. F., K. Brucksch, and U. Priefer.** 1988. Melanin production encoded by a cryptic plasmid in a *Rhizobium leguminosarum* strain. Arch. Microbiol. **150**:326–332.
18. **Hynes, M. F., R. Simon, P. Müller, K. Niehaus, M. Labes, and A. Pühler.** 1986. The two megaplasmids of *Rhizobium meliloti* are involved in the effective nodulation of alfalfa. Mol. Gen. Genet. **202**:356–362.
19. **Hynes, M., R. Simon, and A. Pühler.** 1985. The development of plasmid-free strains of *Agrobacterium tumefaciens* by using incompatibility with a *Rhizobium meliloti* plasmid to eliminate pATC58. Plasmid **13**:99–105.
20. **Johnson, K. G., and M. B. Perry.** 1976. Improved techniques for the preparation of bacterial lipopolysaccharides. Can. J. Microbiol. **22**:29–34.
21. **Keller, M., P. Müller, R. Simon, and A. Pühler.** 1988. *Rhizobium meliloti* genes for exopolysaccharide synthesis and nodule infection located on megaplasmid 2 are actively transcribed during symbiosis. MPMI **1**:267–274.
22. **Klipp, W., B. Masepohl, and A. Pühler.** 1988. Identification and mapping of nitrogen fixation genes of *Rhodobacter capsulatus*: duplication of a *nifA-nifB* region. J. Bacteriol. **170**:693–699.
23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
24. **Maier, R. J., and W. J. Brill.** 1978. Involvement of *Rhizobium japonicum* O antigen in soybean nodulation. J. Bacteriol. **133**: 1295–1299.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Müller, P., M. Hynes, D. Kapp, K. Niehaus, and A. Pühler.** 1988. Two classes of *Rhizobium meliloti* infection mutants differ in exopolysaccharide production and in coinoculation properties with nodulation mutants. Mol. Gen. Genet. **211**:17–26.
28. **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.
29. **Priefer, U.** 1984. Isolation of plasmid DNA, p. 14–24. *In* A. Pühler and K. Timmis (ed.), Advanced molecular genetics. Springer-Verlag KG, Berlin.
30. **Priefer, U., R. Simon, and A. Pühler.** 1984. Cloning with cosmids, p. 190–201. *In* A. Pühler and K. Timmis (ed.), Advanced molecular genetics. Springer-Verlag KG, Berlin.
31. **Priefer, U. B., S. Preisler, C. Schmidt, D. Kapp, and A. Pühler.** 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. APS Press, St. Paul, Minn.

32. **Priefer, U. B., J. Kalinowski, B. Rüger, W. Heumann, and A. Pühler.** 1989. IRS1, a transposable DNA sequence resident in *Rhizobium* class IV strains, shows structural characteristics of classical insertion elements. Plasmid **21**:120–128.
33. **Pühler, A., B. Enenkel, A. Hillemann, D. Kapp, M. Keller, P. Müller, K. Niehaus, U. B. Priefer, J. Quandt, and C. Schmidt.** 1988. *Rhizobium meliloti* and *Rhizobium leguminosarum* mutants defective in surface polysaccharide synthesis and root nodule development, p. 423–430. *In* H. Bothe, F. J. de Bruijn, and W. E. Newton (ed.), Nitrogen fixation: hundred years after. Gustav Fischer Verlag, Stuttgart.
34. **Russa, R., T. Urbanik, E. Kowalczuk, and Z. Lorkiewicz.** 1982. Correlation between the occurrence of plasmid pUCS202 and lipopolysaccharide alterations in *Rhizobium*. FEMS Microbiol. Lett. **13**:161–165.
35. **Russa, R., T. Urbanik, W. Zurkowski, and Z. Lorkiewicz.** 1981. Neutral sugars in lipopolysaccharides of *Rhizobium trifolii* and its non-nodulating mutant. Plant and Soil **61**:81–85.
36. **Simon, R.** 1984. High frequency mobilization of gram negative bacterial replicons using the *in vitro* constructed Tn*5*-Mob transposon. Mol. Gen. Genet. **196**:413–420.
37. **Simon, R., M. O'Connell, M. Labes, and A. Pühler.** 1986. Plasmid vectors for the genetic analysis and manipulation of Rhizobia and other Gram-negative bacteria. Methods Enzymol. **118**:640–659.
38. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1**:784–791.
39. **Simon, R., J. Quandt, and W. Klipp.** 1989. New derivatives of transposon Tn*5* suitable for mobilization of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria. Gene **80**:161–169.
40. **Smit, G., J. W. Kijne, and B. J. J. Lugtenberg.** 1989. Roles of flagella, lipopolysaccharide, and a Ca^{2+}-dependent cell surface protein in attachment of *Rhizobium leguminosarum* biovar *viciae* to pea root hair tips. J. Bacteriol. **171**:569–572.
41. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
42. **Stacey, G., A. A. Paau, K. D. Noel, R. J. Maier, L. E. Silver, and W. J. Brill.** 1982. Mutants of *Rhizobium japonicum* defective in nodulation. Arch. Microbiol. **132**:219–224.
43. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
44. **Van Brussel, A. A. N., T. Tak, A. Wetselaar, E. Pees, and C. A. Wijffelman.** 1982. Small leguminosae as test plants for nodulation of *Rhizobium leguminosarum* and other Rhizobia and Agrobacteria harbouring a leguminosarum sym-plasmid. Plant Sci. Lett. **27**:317–325.
45. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria (IBP Handbook 15). Blackwell Scientific Publications, Ltd., Oxford.
46. **Wacek, T., and W. J. Brill.** 1976. Simple, rapid assay for screening nitrogen-fixing ability in soybean. Crop Sci. **16**:519–522.
47. **Wolpert, J. S., and P. Albersheim.** 1976. Host-symbionts interactions. I. The lectins of legumes interact with the O-antigen containing lipopolysaccharides of their symbiont rhizobia. Biochem. Biophys. Res. Commun. **70**:729–737.
48. **Wood, W. B.** 1966. Host specificity of DNA produced by *Escherichia coli*: bacterial mutations affected the restriction and modification of DNA. J. Mol. Biol. **16**:118–133.
49. **Zevenhuizen, L. P. T. M., I. Scholten-Koerselman, and M. A. Posthumus.** 1980. Lipopolysaccharides of *Rhizobium*. Arch. Microbiol. **125**:1–8.