Microbiology (1997), **143**, 1959-1967 Printed in Great Britain

The symbiotic phenotypes of exopolysaccharide-defective mutants of *Rhizobium sp.* strain TAL1145 do not differ on determinate- and indeterminate-nodulating tree legumes

Nikhat Parveen,[1,2]† David T. Webb[3] and Dulal Borthakur'

Author for correspondence: Dulal Borthakur. Tel: +1 808 956 6600. Fax: +1 808 956 3542.
e-mail: dulal@hawaii.edu

Department of Plant Molecular Physiology, Department of Microbiology[2], and Department of Botany[3], University of Hawaii, Honolulu, HI 96822, USA

Three classes of exopolysaccharide (EPS) defective mutants were isolated by Tn3Hogus-insertion mutagenesis of *Rhizobium sp.* strain TAL1145, which nodulates tree legumes. The class I and class III mutants produced 10-22% of the EPS produced by TAL1145 and appeared partially mucoid while the class II mutants formed small, opaque and non-mucoid colonies. Size-fractionation of the soluble EPSs made by these mutants in the culture supernatant indicated that the class I and the class III mutants produced reduced levels of both highand low-molecular-mass EPSs while the class II mutants lacked both these EPSs but produced a small amount of a medium-molecular-mass anthrone-reactive EPS. The succinyl and acetyl substituents observed in the TAL1145 EPS were absent in the EPS of the class II mutants. When examined under UV, the class I and class III mutants grown on Calcofluor-containing YEM agar showed dim blue fluorescence, compared to the bright blue fluorescence of the wild-type strain, whereas the class II mutants did not fluoresce. While the dim blue fluorescence of the class III mutants changed to yellow-green after 10 d, the fluorescence of the class I mutants did not change after prolonged incubation. Unlike the EPS-defective mutants of other rhizobia, these mutants did not show different symbiotic phenotypes on determinate- and indeterminate-nodulating tree legumes. The class I and the class III mutants formed small ineffective nodules on both types of legumes whereas the class II mutants formed normal nitrogen-fixing nodules on both types. The genes disrupted in the class I and class III mutants form a single complementation group while those disrupted in the class II mutants constitute another. All the three classes of EPS-defective mutants were located within a 10.8 kb region and complemented by two overlapping cosmids.

Keywords: nodulation, nitrogen fixation, exo genes

INTRODUCTION

Leguminous plants are capable of symbiotic nitrogen fixation in association with the root-nodule bacteria of the genera *Rhizobium, Bradyrhizobium, Sinorhizobium* and *Azorhizobium*. Specific interactions between rhizobial and host factors are essential for nodule formation and nitrogen fixation. *Rhizobium* exopolysaccharides (EPSs) have been shown to play important functions during symbiotic interactions with the legume hosts (for reviews see Leigh & Coplin, 1992; Leigh & Walker, 1994). Studies with *Sinorhizobium* (formerly *Rhizobium) meliloti, R. loti, R. leguminosarum* and *Rhizobium sp.* strain NGR234 and GRH2 have shown that rhizobial EPSs are essential for the infection of *Leucaena, Medicago, Pisum, Trifolium* and *Vicia spp.* that form indeterminate type of nodules (Borthakur *et*

† Present address: Department of Molecular Genetics and Microbiology, University of Massachusetts Medical Center, Worcester, MA 01655, USA.

Abbreviation: EPS, exopolysaccharide.

al., 1986; Chen *et al.,* 1985, Diebold & Noel, 1989; Hotter & Scott, 1991; Ko & Gadya, 1990; Lopez-Lara *et al.,* 1993). Empty nodules, devoid of bacteroids, are formed in these legumes by the EPS-defective mutants of different *Rhizobium spp.,* indicating that the infection is aborted at an early stage of symbiosis. However, nodule development in the determinate-nodule-forming hosts *Phaseolus vulgaris, Glycine max* and *Lotus* spp. is generally unaffected by mutations in the genes involved in EPS synthesis (Borthakur *et al.,* 1986; Diebold & Noel. 1989; Kim *et al.,* 1989; Ko & Gadya,1990; LopezLara *et al.,* 1993). The EPS-defective *exoB* mutants of *Bradyrbizobium japonicum* produced dissimilar results with *Glycine sofa* and *G. max,* although both legumes form determinate nodules. While these mutants formed effective nodules on *G. max,* they induced the formation of white, uninfected and ineffective nodule-like structures on *G. soja* (Parniske *et al.,* 1994).

Calcofluor-binding acidic EPS of various *Rhizobium* spp. exhibits blue-green fluorescence under UV when grown on Calcofluor-containing media. By screening the mutants for changes in this fluorescence, several genes involved in succinoglycan metabolism have been identified in S. *meliloti* (Long *et al.,* 1988). Most of the genes involved in succinoglycan synthesis, polymerization and secretion were found in clusters in *S. meliloti* and *Rhizobium sp.* strain NGR234 (Long *et al.,* 1988; Zhan *et al.,* 1990). Genes involved in the synthesis of an alternative EPS, known as EPS 11, in S. *meliloti* are located in a different cluster (Glazebrook & Walker, 1989). Functional homology between certain *exo* genes of *S. meliloti* and *Rhizobium sp.* NGR234 has been observed (Zhan *et al.,* 1990).

Rhizobium sp. strain TAL1145 forms effective nodules on indeterminate-nodulating tree legumes such as *Leucaena* and *Calliandra spp.,* and determinate-nodulating tree legumes such as *Gliricida sepium* (George *et al.,* 1994; Pooyan *et al.,* 1994). It produces large amounts of EPS in media containing mannitol (Parveen & Borthakur, 1994). Phylogenetically, it is different from other tree legume-nodulating species such as *R. tropici* and *Rhizobium sp.* strain NGR234 (George *et al.,* 1994). One characteristic of this strain that distinguishes it from *R. tropici is* that it catabolizes mimosine, a toxin present in large amounts in *Leucaena spp.* (Soedarjo *et al.,* 1994). In this work we isolated three classes of EPS defective mutants of TAL1145 and tested them on both indeterminate- and determinate-nodulating hosts for nodulation and nitrogen fixation. In contrast to the *exo* mutants of NGR234 or *R. leguminosarum,* the *exo* mutants of TAL1145 reported here showed similar nodulation phenotypes on both determinate- and indeterminate-nodulating hosts. Moreover, unlike S. *meliloti* EPS-deficient mutants, which failed to nodulate the indeterminate-nodulating host alfalfa, a class of EPS-deficient mutants of TAL1145 that did not produce the high-molecular-mass EPS, formed effective nodules on *Leucaena,* which forms indeterminate nodules. A cluster of genes involved in EPS synthesis in the strain TAL1145 was identified and mapped within a 10-8 kb region.

METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids are listed in Table 1. Rhizobia were routinely grown as previously described (Parveen *et* al., 1996). Rhizobia were also grown in minimal medium which contained the following components per litre of deionized water: 2-5 g sodium glutamate, 2-0 g sodium succinate, 0-5 g KNO_3, 250 mg K_2HPO_4, 100 mg KCI, 10 mg Na EDTA, 8 mg $FeCl_2$, 1 ml micronutrient solution, 0-5 ml 1 M $CaCl_2$, 1 ml 1 M $MgSO_4$ and 5 ml vitamin solution. The micronutrient solution contained the following salts per litre of deionized water: 1-5 g $MnSO_4$, 1-1 g $ZnS0_4$, 170 mg $CuC1_2.2H_2O$, 50 mg $Na_2MoO_4.2H_2O$ and 10 mg $CoC1_2.2H_2O$. The vitamin solution contained per litre of deionized water: 100 mg biotin, 100 mg thiamin, and 100 mg DL-pantothenate. Sterile stock solutions of $CaCl_2$, $MgSO_4$ and vitamins were added to the medium after autoclaving. The pH of the medium was adjusted to 6.8 before autoclaving.

Site-directed mutagenesis. The transposon Tn3Hogus, which is a derivative of Tn3-HoHo1 (Stachel *et* al., 1985), is 6.62 kb in size and was constructed in the laboratory of Brian Staskawicz by replacing the *lac*ZYA genes with a promoterless *gus* gene and a kanamycin resistance gene (B. Staskawicz, personal communication). The promoterless *gus* gene near the left inverted repeat (IR,,) makes transcriptional fusions with genes if it is inserted in the correct orientation. Cloned *Rhizobium* DNA in plasmids pUHR221 and pUHR222 was mutagenized with Tn3Hogus insertions using the same method as for Tn3-HoHol (Stachel *et* al., 1985) except that kanamycin was used for the selection of the transposon. Tn3Hogus insertions in these two plasmids were homogenotized to the wild-type *Rhizobium* strain TAL1145 by marker exchange (Ruvkun & Ausubel, 1981). The positions of the Tn3Hogus insertions in the mutants were determined by Southern hybridization. The Tn3Hogus insertions of three selected mutants were transferred to the complementing plasmid pUHR221 by homologous recombination.

β-Glucuronidase (GUS) activity assay. Preliminary selection of mutants for GUS activity was done by streaking the mutants on YEM agar (Vincent, 1970) containing 10 µg ml^{-1} 5-bromo-4-chloro-3-indolyl ß-D-glucuronic acid (X-G1cA) (Sigma). Fluorometric assay for GUS activity using 4-methylumbelliferyl ß-D-glucuronide (MUG) (Sigma) was done according to Jefferson *et* al. (1987). The fluorescent product 7-hydroxy4-methylcoumarin (MU) is produced through hydrolysis of MUG by GUS.

DNA techniques. Genomic and plasmid DNA preparations, electrophoresis and Southern hybridizations were carried out as previously described (George *et* al., 1994). Restriction enzymes were obtained from Promega.

Extraction, purification and analysis of EPS and LPS. EPS and LPS were extracted and purified as previously described (Parveen *et* al., 1996) except that minimal medium supplemented with 1 % mannitol was used to grow the cultures for extraction of EPS and LPS. LPS was analysed by PAGE on 18% acrylamide gels with 10% SDS and 0-5% deoxycholic acid as detergents. The gels were stained by the Alcian blue/silver stain method (Corzo *et* al., 1991). Proton NMR spectra of total EPS were recorded at 500 MHz in D_20 using a General Electric GN Omega 500 spectrometer at the NMR facility, Department of Chemistry, University of Hawaii. Free

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Rhizobium		
TAL1145	Wild-type strain, nodulates tree legumes, EPS$^+$ Rif^r Str^r	George *et al.* (1994)
NP85–NP149	Tn*3Hogus* insertion, EPS-defective mutants of TAL1145, Rif^r Str^r Kan^r	This work
RUH123, RUH124 and RUH125	Spontaneous EPS$^-$ mutants of TAL1145, Rif^r Str^r	This work
Plasmids		
pUHR221–pUHR225	Cosmids containing cloned TAL1145 DNA in cosmid vector pLAFR3, complement mutants NP85–NP149	This work
pRK404	Broad-host-range cloning vector, Tet^r IncP	Ditta *et al.* (1985)
pUHR230	2·2 kb *Eco*RI fragment of pUHR221 cloned in pRK404	This work
pUHR236	1·8 kb *Eco*RI fragment of pUHR221 cloned in pRK404	This work
pUHR237	Plasmid containing a 3·4 kb insert obtained by deleting the *Eco*RI fragments of pUHR221; this 3·4 kb fragment is homologous to a 4·4 kb *Eco*RI fragment in pUHR223	This work
pUHR256	7·6 kb *Hin*dIII fragment of pUHR221 cloned in pRK404	This work
pUHR257	7·0 kb *Hin*dIII fragment of pUHR221 cloned in pRK404	This work
pUHR258	4·6 kb *Hin*dIII fragment of pUHR221 cloned in pRK404	This work
pEX312	Plasmid containing *exo* genes of *S. meliloti*	Long *et al.* (1988)
R′3222	Plasmid containing *exo* genes of *Rhizobium* sp. strain NGR234	Gray *et al.* (1991)
pIJ1427	Plasmid containing the *pss* genes of *R. leguminosarum* bv. *phaseoli*	Borthakur *et al.* (1988)
pRK2013	RK2 derivative, Kan^r Tra$^+$	Figurski & Helinski (1979)

decay signal was sampled at a block size of about 16000 over 5 Hz spectral width. Typically, 2000 transients were recorded with an acquisition time of 3.28 s. Assignments of resonance were based on those reported by McNeil et al. (1986).

Plant experiments. Nodulation and nitrogen fixation assays on all three legumes, *Gliricida sepium*, *Leucaena leucocepbala* and *Pbaseolus vulgaris*, were performed in growth pouches as described by George et al. (1994). Two-day-old seedlings were inoculated with approximately 10^7 c.f.u. ml^{-1} of rhizobial culture suspension. Plants were observed 4 weeks after inoculation for nodulation and nitrogen fixation. All plant inoculation experiments were done at least twice.

Microscopic studies. Nodules were fixed in 2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 6-8), followed by post-fixation in buffered 2% osmium tetroxide. These were dehydrated in ethanol and embedded in Epon resin. Sections were cut with a Sorval microtone and stained with toludine blue.

RESULTS

Isolation of EPS-clefective mutants

Three spontaneous EPS-defective colonies were isolated by screening approximately 10000 colonies of *Rhizobium* sp. strain TAL1145 on YEM agar. The small rough colonies of the mutants showed dim bluish fluorescence under UV compared to the bright bluish fluorescence of TAL1145 on Calcofluor-containing YEM agar. After 10d incubation colonies showed a bright yellowish-green fluorescence, which is not a characteristic of TAL1145. To complement these mutants for the EPS defects, a cosmid clone library of TAL1145 (George et al., 1994) was transferred to the three mutants separately and a number of transconjugants showing the EPS phenotype of the wild-type were selected on YEM medium. Five different overlapping cosmid clones containing an 18 kb common DNA region were isolated from these transconjugants. When these clones, pUHR221-pUHR225, were again transferred to the three mutants, their EPS defects were complemented since all transconjugants produced EPS like TAL1145. Two of the five cosmids that complemented the three EPS defective mutants, pUHR221 and pUHR222, were used to obtain site-directed mutants of TAL1145 using the transposon Tn3Hogus as described in Methods. Fifty-seven EPS-defective mutants were selected that formed rough, opaque and smaller colonies as compared to the mucoid, translucent and large colonies of the wild-type. The mutants were streaked on YEM medium containing Calcofluor and on the basis of the colony morphology and fluorescence they were divided into three classes (Fig. 1). The class I and the class III mutants produced reduced amounts of EPS and appeared partially mucoid while the colonies of the class II mutants were non-mucoid, small and opaque. When examined under UV, the class I and class III mutants on Calcofluor-containing YEM agar showed dim blue fluorescence compared to the bright blue fluorescence of the wild-type strain whereas the class II mutants did not fluoresce. However, unlike the class I mutants or the wild-type strain, the fluorescence of the class III mutants changed to yellowish-green when the colonies were incubated for 10 d or longer.

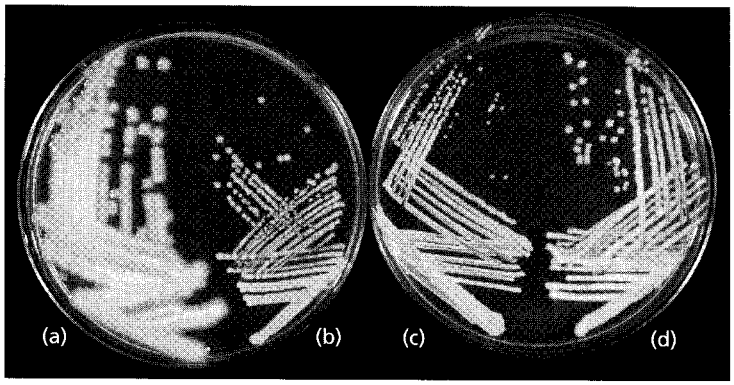

Fig. 1. Photograph showing the colonies of the wild-type strain TAL1145 (a), class I mutant NP98 (b), class II mutant NP91 (c) and class III mutant NP95 (d), grown on YEM agar for 1 week.

Table 2. Amount of ethanol-precipitable EPS produced by three classes of Tn*3Hogus* insertion *exo* mutants of TAL1145

Class	Strain	Dry weight of EPS* [μg (mg protein)$^{-1}$]	EPS %
Wild-type	TAL1145	3595 ± 66	100
I	NP98	455 ± 20	13
	NP118	550 ± 29	15
	NP120	355 ± 113	10
II	NP86	< 10	< 0·3
	NP88	< 10	< 0·3
	NP91	< 10	< 0·3
	NP125	26 ± 19	0·7
	NP146	< 10	< 0·3
III	NP92	710 ± 17	20
	NP95	790 ± 103	22
	NP97	680 ± 75	19

* Mean ± SE of three replicates.

Quantification and analysis of EPS of some selected mutants

Dry weights of EPS obtained by ethanol-precipitation of the culture supernatants of the wild-type and some selected mutants are shown in Table 2. None of the class II mutants showed a detectable level of the high-molecular-mass EPS, which is precipitable by ethanol. The mutants belonging to class I and class III produced 10-22 % of the EPS produced by the wild-type. Size-fractionation of the soluble EPS of the wild-type in the culture supernatant by column chromatography indicated that it contained a high- and a low-molecular-mass fraction. Results of column chromatography of one mutant each of class I, II and III are indicated in Fig. 2. (Note that the amounts of material loaded for the mutants in the column were ten times that for the wildtype.) The class I mutant (NP98) and class III mutant (NP95) were found to produce reduced levels of high-molecular-mass EPS. Although these mutants showed a relatively large peak for the low-molecular-mass EPS, this corresponded to only one-tenth the amount produced by the wild-type. The class II mutant (NP88) lacked both the high- and low-molecular-mass fractions present in the wild-type but contained a peak for an intermediate-size EPS in fractions 44-52. This intermediate-size EPS peak was observed when the lyophilized culture supernatants of three other class II mutants, NP125, NP86 and NP91 were fractionated (data not shown).

NMR spectra of the EPS of TAL1145 and three mutants are shown in Fig. 3. The multiplets between 2-48 and 2-63 p.p.m. represented the methylene protons of the succinyl group, and the signals between 2.1 and 2.2 were assigned to the methyl protons of O-acetyl substituents. The peaks at 1.46 p.p.m. in all four spectra indicated the presence of methyl protons of 1-carboxyethylidene or pyruvate groups. Neither succinyl nor acetyl resonances were apparent in the spectrum of the class II mutant, NP88. The succinyl peak at 2.5 p.p.m. was also absent in the spectra for NP95 (class I1I). The spectra for mutant NP98 (class I) did not show major differences from that of TAL1145 in the carbohydrate ring protons.

The LPS profiles of some selected mutants of all three classes were examined by SDS/deoxycholate/PAGE.
The LPS gel pattern of the mutants did not show significant differences from that of the wild-type (data not shown).

Localization of Tn3Hogus insertions in the mutants Fig. 4(a) shows the restriction map of the plasmids pUHR221 and pUHR222. The positions of the transposon insertion in the mutants were determined by the sizes of the shifted bands in Southern hybridization and are shown in Fig. 4(b). The Tn3Hogus insertions in the class I and class III mutants were located within a 4.0 kb region whereas the class II mutants were located 2.4 kb apart from the class III mutants on a 4.4 kb EcoRI fragment. Mutants containing active gus fusions are indicated in Fig. 4(b). The direction of these active gus fusions suggests that the class I and class III genes are transcribed from left to right as shown in Fig. 4(b) whereas the direction of the class II genes is from right to left.

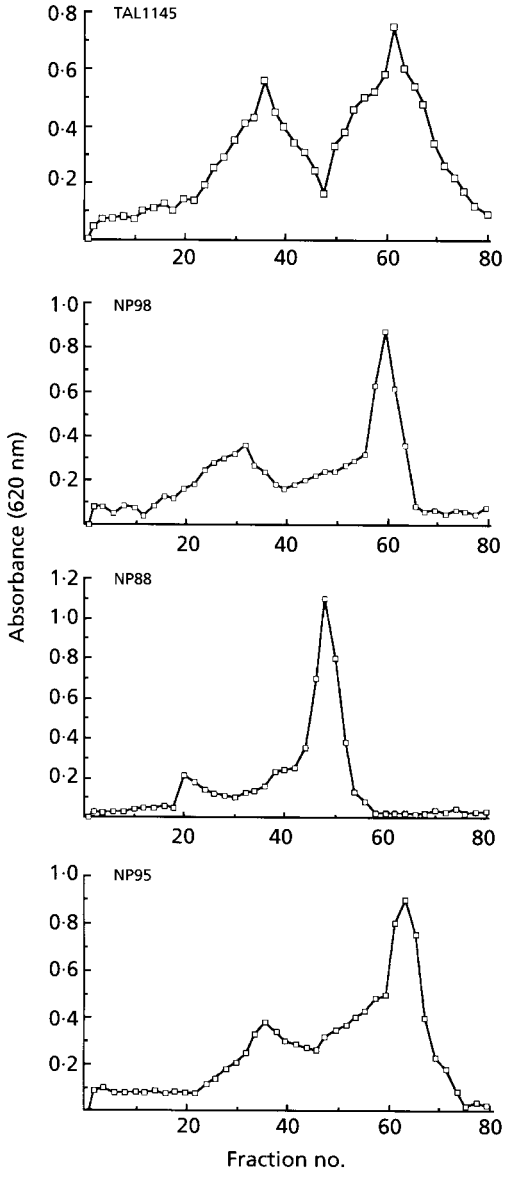

Fig. 2. Gel filtration of the culture supernatants of TAL1145 and the class I (NP98), class II (NP88) and class III (NP95) mutants. *Rhizobium* cultures were grown in minimal medium supplemented with 1% mannitol. Lyophilized culture supernatants of TAL1145 (20 mg) and the mutants (200 mg) were loaded on the column. The hexose contents of the samples were determined as previously described (Parveen *et al.*, 1996).

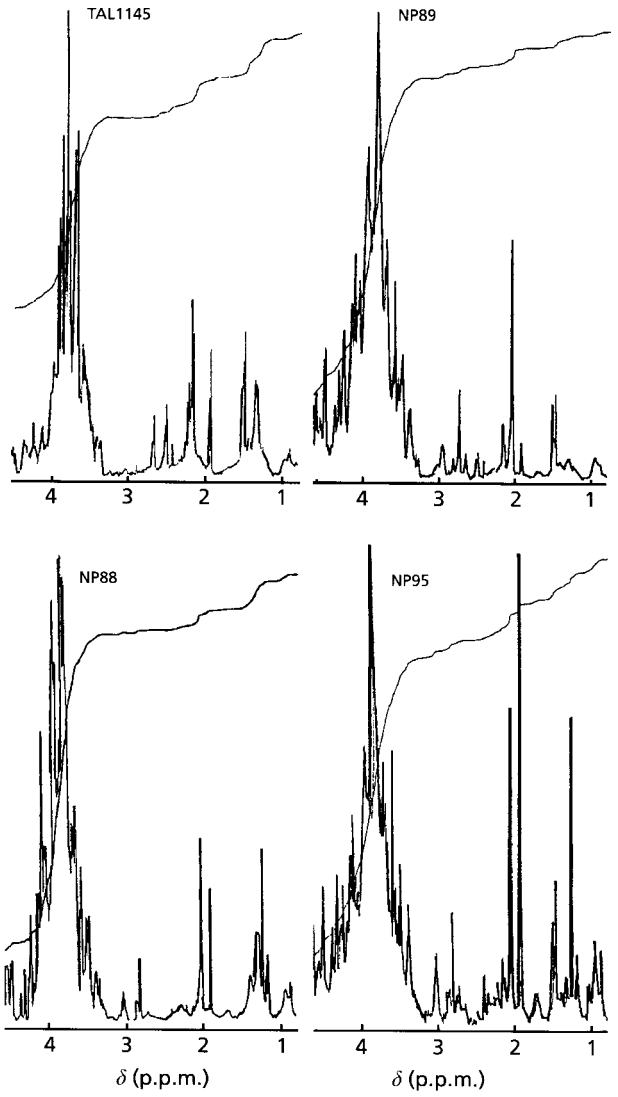

Fig. 3. Proton NMR spectra of ethanol-precipitated EPS of TAL1145 and three EPS-defective mutants, NP98 (class I), NP88 (class II) and NP95 (class III). Samples were dissolved in D_2O and spectra were obtained at 500 MHz.

Symbiotic phenotypes of the mutants

The EPS-defective mutants were used to inoculate common bean (P. *vulgaris)* and two tree legumes, one forming determinate nodules (G. *sepium)* and the other forming indeterminate nodules (L. *leucocephala).* The class I and the class III mutants formed ineffective small nodules on all three legumes whereas the class II mutants showed normal nodulation and nitrogen fixation on these species. The small nodules formed by one class I mutant (NP98) and one class III mutant (NP95) on *L. leucocephala* are shown in Fig. 5. Microscopic observation of the sections of small *Leucaena* nodules formed by the class I and class III mutants did not show the presence of any bacteroid or invasion by rhizobia (Fig. 6). The nodules made by the class 11 mutants contained well-developed bacteroids.

Complementation of the mutants

All three classes of EPS-defective mutants were complemented for EPS synthesis by pUHR221. Plasmid pUHR222 also complemented all mutants except a few class 11 mutants located within the 1.4 kb *Hind*III fragment, a part of which is absent in this plasmid (Fig. 4a). The complemented EPS^+ derivatives of the class I and class III mutants formed normal nitrogen-fixing nodules on all three legumes tested (Fig. 5). The class I

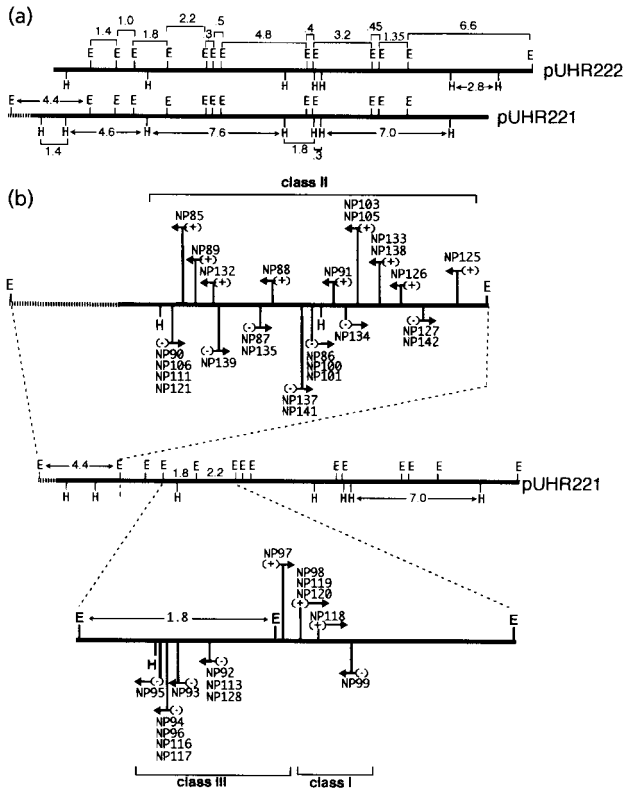

Fig. 4. (a) Restriction map of the cloned TAL1145 DNA in two overlapping cosmid clones, pUHR221 and pUHR222. Restriction sites for *Eco*RI (E) and *Hin*dIII (H) are shown. The numbers indicate the sizes of the fragments in kb. Plasmid pUHR221 does not contain the complete 4·4 kb *Eco*RI fragment on the left; the missing part is shown as a broken line and was obtained from the homologous 4·4 kb *Eco*RI fragment in the overlapping plasmid pUHR223. (b) Location of the Tn*3*Ho*gus* insertions in the different EPS-defective mutants of TAL1145. The positions of the various mutants in the chromosome are indicated as relative positions in the cloned DNA in cosmid pUHR221. The 4·4 kb *Eco*RI fragment showing the positions of the Tn*3*Ho*gus* insertions in the class II mutants, and the 1·8 and the 3·3 kb fragments showing the positions of the class III and class I mutants, are amplified. Note that a part of the 4·4 kb *Eco*RI fragment is missing in pUHR221 which is indicated with a broken line. The horizontal arrows indicate directions of the *gus* gene in different mutants while the plus or the minus signs behind the arrows indicate high or undetectable GUS activities, respectively.

mutants that were mapped on a 22 kb EcoRl fragment and a 7.6 kb *HindIII* fragment were not complemented by either pUHR230 or pUHR256, which contained the 2.2 and 7.6 kb cloned DNA fragments, respectively. Similarly, the class III mutants were not complemented by the cloned 1.8 kb EcoRI fragment in pUHR236. The class II mutants were complemented by pUHR237 containing a 3.4 kb insert of pUHR221, which is homologous to the 4.4 kb EcoRl fragment of pUHR223.

pUHR221: :Tn3Hogus-92, which is a derivative of pUHR221 carrying the mutant allele from the class III mutant NP92, did not complement the class I mutants. Similarly, pUHR221: : Tn3Ho*gus*-98, containing the mutant allele from the class I mutant NP98, failed to restore the wild-type phenotype to class III mutants. Thus, genes on these two EcoRI fragments are placed in the same complementation group. pUHR221: : Tn*3*Ho*gus*-87, a derivative of pUHR221 containing the mutant allele from the class II mutant NP87, complemented both class I and class III mutants but did not complement other class II mutants indicating that mutants located within the 4-4 kb EcoRI fragment constitute another complementation group.
The cosmid R'3222 containing the *exo* region of *Rhizobium sp.* strain NGR234, the cosmid pEX312 containing the cluster of *exo* genes of S. *meliloti,* and pIJ1427 containing *pss* genes of *R. leguminosarum* bv. *phaseoli,* failed to complement these mutants.

DISCUSSION

In media containing mannitol, *Rhizobium sp.* strain TAL1145 makes large amounts of EPS which can be separated into high- and low-molecular-mass fractions (Parveen *et al.,* 1996). We observed that the relative amounts of the high- and low-molecular-mass EPS made by this strain depended on the amount of mannitol in the medium. When we used higher amounts of mannitol in the growth medium, we observed a relatively larger peak for the high-molecular-mass EPS. In the present study, a cluster of genes involved in the synthesis of both high- and low-molecular-mass acidic EPS in TAL1145 has been identified by isolating three classes of EPS-defective mutants within a 10.8 kb region. The class I and class III mutants were present in one complementation group within a 4.0 kb region while the class II mutants, constituting another complementation group, were located in a 4.4 kb fragment.

The class II mutants did not produce detectable levels of ethanol-precipitable EPS and formed small rough colonies on YEM agar. They produced small amounts of a medium-molecular-mass anthrone-reactive material, which was not precipitated with ethanol. Previously, Zevenhuizen & van Neerven (1983) also observed that only the high-molecular-mass, but not the low-molecular-mass EPSs are precipitated by 2-5 vols ethanol or acetone. The absence of both acetate and succinate groups in the EPS of NP88, as observed in the NMR analysis, also suggests that the EPS produced by the class 11 mutants is different from that of the wildtype. The class II mutants resemble S. *meliloti* mutants in the *exoA, exoB, exoF, exoL, exoM, exoP, exoQ, exoT* or *exoY* genes that form small EPS colonies (Leigh *et al.,* 1985, 1987; Leigh & Lee, 1988; Long *et al.,* 1988; Leigh & Walker, 1994). In *S. meliloti* strain Rm1021, Leigh & Lee (1988) and Long *et al.* (1988) showed that *exoP, exoM, exoA, exoL, exoF, exoQ* and *exoB* mutants produced negligible amounts of EPS in the culture supernatant and showed non-fluorescent phenotype on Calcofluor-containing medium like the class II mutants in the present study. Interestingly, these mutants formed normal nitrogen-fixing nodules on both indeterminate and determinate-nodulating hosts. This is in contrast

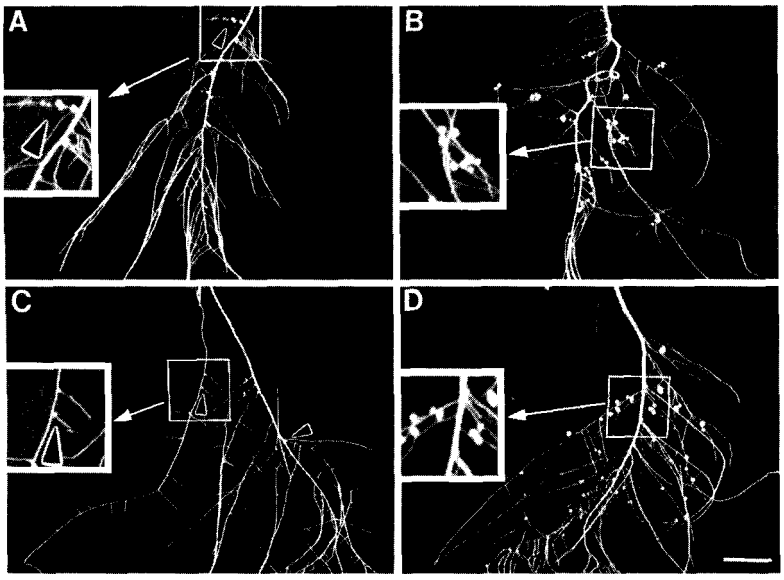

Fig. 5. Small nodules formed by *L.* leucocephala plants inoculated with the class I mutant NP98 (A) and the class III mutant NP97 (C) compared to the normal nitrogen-fixing nodules formed by their transconjugants NP98(pUHR221) (B) and NP97(pUHR221) (D). Bar, 2.5 cm. The boxed areas of the root systems are shown at x 2 magnification in the insets on the left-hand side of each photograph.

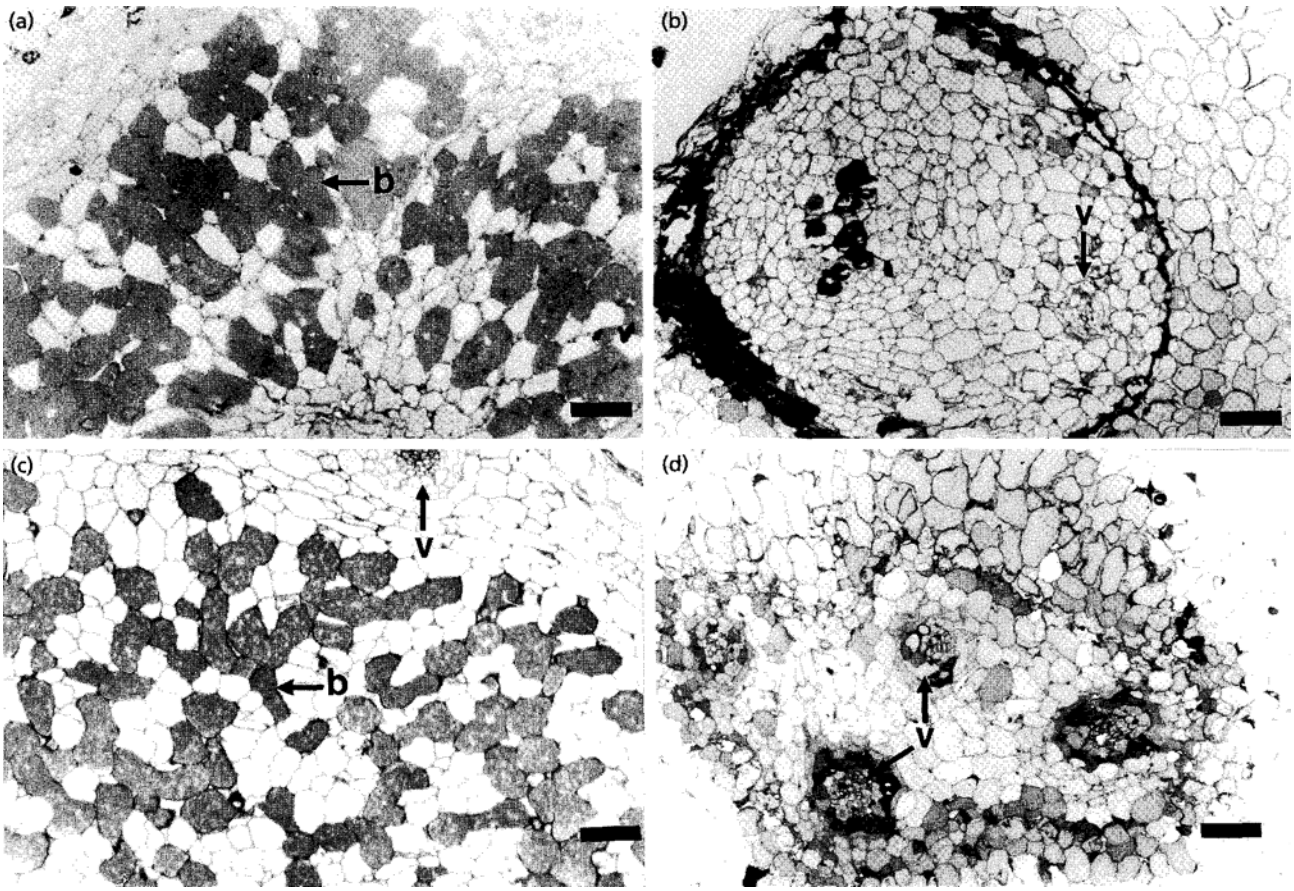

Fig. 6. Light microscopy of sections, 1 µm thick, of 5-week-old *L.* leucocephala root nodules. (a) TAL1145, (b) class I mutant NP98, (c) class II mutant MP88 and (d) class III mutant NP95. The bacteroid-filled cells, labelled 'b', are seen as darkly stained regions in panels (a) and (c). The small nodules formed by NP98 and NP95 contained characteristic vascular bundles (v) in the cortex but lacked bacteroids. With NP88 vascular bundles were present outside the zone of bacteroid as with TAL1145. The darkly stained areas in (b) do not represent cells with bacteroids but are due to tannin or other reactive chemicals. Bars, 60 µm in (a) and (c); 100 µm in (b) and (d).

with the previous reports with *R. leguminosarum, R. loti, S. meliloti* and *Rhizobium sp.* strain NGR234 EPSdefective mutants that were defective for nodulation on indeterminate hosts such as *Pisum sativum, Vicia hirsuta, Medicago sativa, Trifolium spp.* and *L. leucocepbala* (Borthakur *et al.,* 1986, 1988; Chen *et al.,* 1985; Diebold & Noel, 1989; Gray *et al.,* 1991; Hotter & Scott, 1991, Leigh *et al.,* 1985; Long *et al.,* 1988; Lopez-Lara *et al.,* 1993). It is possible that the small amount of the medium-molecular-mass EPS present in these mutants substitutes for the absence of the low- and high-molecular-mass EPS. Reuhs *et al.* (1995) observed that a capsular polysaccharide in RM41, named KDOPS, could surmount the symbiotic defect of certain EPS mutants in the S. *meliloti* strain RM41. A component similar to KDOPS described in S. *meliloti* strain RM41 may also be produced by TAL1145 and the class II mutants.

The difference between class I and class III mutants is that the class III mutants showed a yellowish fluorescence after 10d incubation while the colour of the Calcofluor-fluorescence of class I mutants did not change on prolonged incubation. Both produced reduced quantities of high- and low-molecular-mass EPS fractions compared to the wild-type. The reduced amounts of ethanol-precipitatable EPS made by the class I and the class III mutants are represented by the high-molecular-mass peaks in Fig. 2. The low-molecular-mass EPS fractions, represented by the relatively larger peaks, are not precipitable with ethanol. The difference in the chemical structure of EPS of class I and class III mutants has not been established in this study, but their peaks for the large- and small-molecular-mass fractions coincided with those of the EPS produced by the wildtype. Both class I and class III mutants formed small ineffective nodules on all three legume hosts. Lack of normal EPS in these mutants might have prevented normal bacteroid development, resulting in the Fix phenotype. Leigh *et al.* (1987) observed that S. *meliloti exoH* mutants that failed to succinylate their EPS formed empty nodules in alfalfa. Those mutants also showed invasion-deficiency similar to the class I and class III *exo* mutants of TAL1145 in the present study.

Several previous studies with *R. leguminosarum* and *R. loti* EPS-defective mutants showed that the mutants had different symbiotic phenotypes on indeterminate- and determinate-nodulating hosts. Based on those results, it was expected that the EPS-defective mutants of TAL1145 would nodulate *Gliricidia* and beans but not *Leucaena.* The structure of the *Gliricidia* nodules is similar to those of beans with spherical meristems although the *Gliricidia* nodules are much larger than bean nodules. Surprisingly, the EPS-defective mutants of TAL1145 have the same phenotypes on both *Gliricidia* and *Leucaena.* In contrast to the EPS-defective mutants, an LPS-defective mutant of TAL1145 nodulated *Leucaena* but not *Gliricidia* or beans (Parveen *et al.,* 1996).

The EPS of TAL1145 shows some similarities with both *S. meliloti* and *R. leguminosarum* EPS. NMR analysis of TAL1145 EPS indicated the presence of a succinate group as in the EPS of S. *meliloti* strains (Leigh *et al.,* 1985, 1987). The peaks between 1.2 and 1.4 p.p.m. in the NMR spectrum of TAL1145 EPS indicated the presence of a-glycosyl residues and O-(3-hydroxybutanol) groups, which are also reported in the EPS of *R. leguminosarum* (McNeil *et al.,* 1986; Hollingsworth & Dazzo, 1988). Further comparison of the EPS of TAL1145 and other rhizobia is not possible at this stage because the structural composition of the TAL1145 EPS has not been elucidated.

The *exo* genes of TAL1145 may be functionally different from those in S. *meliloti* and *Rhizobium sp.* strain NGR234 because (i) none of the EPS-defective mutants of TAL1145 in this study could be complemented by plasmids pEX312, R'3222 or pIJ1427 that contain the *exo* genes of S. *meliloti* Rm1021, *Rhizobium sp.* NGR234 and *R. leguminosarum* bv. *phaseoli* 8002, respectively; (ii) the yellowish-green Calcofluor-fluorescence observed upon prolonged incubation of class III EPS-defective mutants of TAL1145 has not been reported in any other *Rhizobium sp.,* which suggests that the EPS in TAL1145 may be qualitatively different from the EPS of other *Rhizobium spp.;* and (iii) the nodulation phenotypes of the three classes of mutants are similar for both the indeterminate- and determinate-nodulating tree legumes. Phylogenetically, TAL1145 is closer to *R. tropici* and *R. leguminosarum* than to S. *meliloti* and *Rhizobium sp.* strain NGR234 (George *et al.,* 1994).

ACKNOWLEDGEMENTS

We thank Brian Staskawicz for providing the transposon Tn3Hogus and Leslie R. Berger for a critical review of the manuscript. This work was supported partially by a subgrant SM-CRPS-023 under the grant no. DAN-1311-G-00-1049-00 from USAID to the NifTAL Project and partially by a HITAHR Minigrant no. HAW0671-H from the University of Hawaii. Paper 4263 of the College of Tropical Agriculture and Human Resources, University of Hawaii, Honolulu, USA.

REFERENCES

Borthakur, D., Barber, C. E., Lamb, J. W., Daniels, M. J., Downie, J. A. & Johnston, A. W. B. (1986). A mutation that blocks exopolysaccharide synthesis prevents nodulation of peas by *Rhizobium leguminosarum* but not of beans by *Rhizobium phaseoli* and *is* corrected by cloned DNA from *Rhizobium* or the phytopathogen *Xanthomonas. Mol Gen Genet 203,* 320-323.

Borthakur, D., Barker, R. F., Latchford, 1. W., Rossen, L. & Johnston, A. W. B. (1988). Analysis of pss genes of *Rhizobium leguminosarum* required for exopolysaccharide synthesis and nodulation of peas: their primary structure and their interaction with psi and other nodulation genes. *Mol Gen Genet 213,* 155-162.

Chen, H., Batley, M., Redmond, J. & Rolfe, B. G. (1985). Alteration of the effective nodulation properties of a fast-growing broad host range *Rhizobium* due to changes in exopolysaccharide synthesis. *J Plant Physiol 120,* 331-349.

Corzo, J., Pdrez-Galdona, R., Leon-Barrios, M. & Gutidrrez-Navarro, A. M. (1991). Alcian blue fixation allows staining of the isolated polysaccharide component of bacterial lipopolysaccharides in polyacrylamide gels. *Electrophoresis 12,* 4391441.

Diebold, R. & Noel, K. D. (1989). *Rhizobium leguminosarum* exopolysaccharide mutants: biochemical and genetic analyses and symbiotic behavior on three hosts. J *Bacteriol 171,* 4821-4830.

Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, X.-W., Finlay, D. R., Guiney, D. & Helinski, D. R. (1985). Plasmids related to the broad host-range vector, pRK290, useful for gene cloning and monitoring gene expression. *Plasmid 13,* 149-153.

Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci USA 76,* 1648-1652.

George, M. L. C., Young, 1. P. W. & Borthakur, D. (1994). Genetic characterization of *Rbizobium sp.* strain TAL1145 that modulates tree legumes. *Can* J *Microbiol 40,* 208-215.

Glazebrook, J. & Walker, G. C. (1989). A novel polysaccharide can function in place of the Calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rbizobium meliloti. Cell 56,* 661-672.

Gray, J. X., Zhan, H., Levery, S. B., Battisti, L., Rolfe, B. G. & Leigh, J. A. (1991). Heterologous exopolysaccharide production in *Rbizobium sp.* strain NGR234 and consequences for nodule development. J *Bacteriol 173,* 3066-3077.

Hollingsworth, R. I. & Dazzo, F. B. (1988). The complete structure of the trifoliin: a lectin-binding capsular polysaccharide of *Rbizobium trifolii* 843. *Carbobydr Res 172,* 97-112.

Hotter, G. S. & Scott, D. B. (1991). Exopolysaccharide mutants of *Rbizobium loti* are fully effective on a determinate nodulating host but are ineffective on an indeterminate nodulating host. J *Bacteriol 173,* 851-859.

Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. (1987). GUS fusions: f-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J 6,* 3901-3907.

Kim, C.-H., Tully, R. E. & Keister, D. L. (1989). Exopolysaccharidedeficient mutants of *Rhizobium fredii* HH303 which are symbiotically effective. *Appl Environ Microbiol 55,* 1852-1854.

Ko, Y. H. & Gadya, R. (1990). Nodule formation in soybeans by exopolysaccharide mutants of *Rbizobium fredii* USDA191. J *Gen Microbiol 136,* 105-113.

Leigh, 1. A. & Coplin, D. L. (1992). Exopolysaccharides in plant-bacterial interactions. *Annu Rev Microbiol 46,* 307-346.

Leigh, J. A. & Lee, C. C. (1988). Characterization of polysaccharides of *Rbizobium meliloti exo* mutants that form ineffective nodules. J *Bacteriol 170,* 3327-3332.

Leigh, J. A. & Walker, G. C. (1994). Exopolysaccharides of *Rbizobium:* synthesis, regulation and symbiotic function. *Trends Genet 10,* 63-67.

Leigh, J. A., Signer, E. R. & Walker, G. C. (1985). Exopolysaccharide-deficient mutants of *Rbizobium meliloti* that form ineffective nodules. *Proc Natl Acad Sci USA 82,* 6231-6234.

Leigh, J. A., Reed, J. W., Hanke, J. F., Hirsch, A. M. & Walker, G. C. (1987). *Rbizobium meliloti* mutants that fail to succinylate their calcofluor-binding exopolysaccharide are defective in nodule invasion. *Cell 51,* 579-587.

Long, S., Reed, J. W., Himawan, J. & Walker, G. C. (1988). Genetic analysis of a cluster of genes required for the synthesis of the calcofluor-binding exopolysaccharide of *Rbizobium meliloti. J Bacteriol 170,* 4239-4248.

Lopez-Lara, I. M., Orgambide, G., Dazzo, F. B., Olivarez, J. & Toro, N. (1993). Characterization and symbiotic importance of acidic extracellular polysaccharides of *Rhizobium sp.* strain GRH2 isolated from *Acacia* nodules. J *Bacteriol 10,* 2826-2832.

McNeil, M., Darvill, J., Darvill, A. G. & Albersheim, P. (1986). The discernible structural features of the acidic polysaccharides secreted by different *Rbizobium* species are the same. *Carbobydr Res 146,* 307-326.

Parniske, M., Schmidt, P. E., Kosch, K. & MOller, P. (1994). Plant defense responses of host plants with determinate nodules induced by EPS-defective *exoB* mutants of *Bradyrbizobium japonicum. Mol Plant-Microbe Interact 7,* 631-638.

Parveen, N. & Borthakur, D. (1994). Construction of a single-transposon-insertion mutant in *Rbizobium sp.* strain TAL1145 from a double-insertion mutant. *LettApp1 Micro biol 19,* 142-145.

Parveen, N., Webb, D. T. & Borthakur, D. (1996). *Leucaena leucocepbala* nodules formed by a surface polysaccharide defective mutant of *Rbizobium sp.* strain TAL1145 are delayed in bacteroid development and nitrogen fixation. *Mol Plant-Microbe Interact 9,* 364-372.

Pooyan, S., George, M. L. C. & Borthakur, D. (1994). Isolation and characterization of a gene for nodule development linked to the *ndvA* and *ndvB* genes in *Rbizobium sp.* strain TAL1145. *Symbiosis 17,* 201-215.

Reuhs, B. L., Williams, M. N. V., Kim, J. S., Carlson, R. W. & Cote, F. (1995). Suppression of the Fix$^-$ phenotype of *Rhizobium meliloti exoB* mutants by lpsZ is correlated to a modified expression of K polysaccharide. *J Bacteriol 177,* 4289-4296.

Ruvkun, G. B. & Ausubel, F. M. (1981). A general method for sitedirected mutagenesis in prokaryotes. *Nature 289,* 85-88.

Soedarjo, M., Hemscheidt, T. K. & Borthakur, D. (1994). Mimosime, a toxin present in leguminous trees *(Leucaena spp.)* induces a mimosine-degrading enzyme activity in some *Rbizobium* strains. *Appl Environ Microbiol 60,* 426814272.

Stachel, S. E., An, G., Flores, C. & Nester, E. W. (1985). A Tn3lacZ transposon for the random generation of f-galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium. EMBO J 4,* 891-898.

Vincent, J. M. (1970). *A Manual for the Practical Study o f Root-Nodule Bacteria (IBP* Handbook no. 15), p. 164. Oxford: Blackwell Scientific Publications.

Zhan, H., Gray, J. X., Levery, S. B., Rolfe, B. G. & Leigh, J. A. (1990). Functional and evolutionary relatedness of genes for exopolysaccharide synthesis in *Rhizobium meliloti* and *Rbizobium sp.* strain NGR234. *J Bacteriol 172,* 5245-5253.

Zevenhuizen, L. P. T. M. &van Neerven,A. R. W. (1983). (1 -> 2)fD-glucan and acidic oligosaccharides produced by *Rhizobium meliloti. Carbobydr Res 118,* 127-134.

Received 11 September 1996; revised 29 January 1997; accepted 13 February 1997.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Rhizobium		
TAL1145	Wild-type strain, nodulates tree legumes, EPS$^+$ Rif' Strr	George et al. (1994)
NP85-NP149	Tn3Hogus insertion, EPS-defective mutants of TAL1145, Rifr Str`	This work
RUH123, RUH124 and RUH125	Spontaneous EPS$^-$ mutants of TAL1145, Rif' Str"	This work
Plasmids		
pUHR221-pUHR225	Cosmids containing cloned TAL1145 DNA in cosmid vector pLAFR3, complement mutants NP85-NP149	This work
pRK404	Broad-host-range cloning vector, Tet` IncP	Ditta et al. (1985)
pUHR230	22 kb EcoRI fragment of pUHR221 cloned in pRK404	This work
pUHR236	1-8 kb EcoRI fragment of pUHR221 cloned in pRK404	This work
pUHR237	Plasmid containing a 3-4 kb insert obtained by deleting the EcoRl fragments of pUHR221; this 3-4 kb fragment is homologous to a 4-4 EcoRl fragment in pUHR223	This work
pUHR256	7-6 kb HindlIl fragment of pUHR221 cloned in pRK404	This work
pUHR257	7-0 kb HindlII fragment of pUHR221 cloned in pRK404	This work
pUHR258	4-6 kb HindIII fragment of pUHR221 cloned in pRK404	This work
pEX312	Plasmid containing exo genes of S. meliloti	Long et al. (1988)
R'3222	Plasmid containing exo genes of *Rhizobium sp.* strain NGR234	Gray et al. (1991)
p1J1427	Plasmid containing the pss genes of *R. leguminosarum* bv. pbaseoli	Borthakur et al. (1988)
pRK2013	RK2 derivative, Kan' Tra$^+$	Figurski & Helinski (1979)

decay signal was sampled at a block size of about 16000 over 5 Hz spectral width. Typically, 2000 transients were recorded with an acquisition time of 328 s. Assignments of resonance were based on those reported by McNeil et al. (1986).

Plant experiments. Nodulation and nitrogen fixation assays on all three legumes, Gliricida sepium, Leucaena leucocephala and Phaseolus vulgaris, were performed in growth pouches as described by George et al. (1994). Two-day-old seedlings were inoculated with approximately 10' c.f.u. ml-' of rhizobial culture suspension. Plants were observed 4 weeks after inoculation for nodulation and nitrogen fixation. All plant inoculation experiments were done at least twice.

Microscopic studies. Nodules were fixed in 2-5 % glutaraldehyde and 2 % paraformaldehyde in 0-1 M sodium cacodylate buffer (pH 6-8), followed by post-fixation in buffered 2% osmium tetroxide. These were dehydrated in ethanol and embedded in Epon resin. Sections were cut with a Sorval microtone and stained with toludine blue.

RESULTS

Isolation of EPS- defective mutants

Three spontaneous EPS-defective colonies were isolated by screening approximately 10000 colonies of *Rhizobium sp.* strain TAL1145 on YEM agar. The small rough colonies of the mutants showed dim bluish fluorescence under UV compared to the bright bluish fluorescence of TAL1145 on Calcofluor-containing YEM agar. After 10 d incubation colonies showed a bright yellowish-green fluorescence, which is not a characteristic of TAL1145. To complement these mutants for the EPS defects, a cosmid clone library *of* TAL1145 (George *et* al., 1994) was transferred to the three mutants separately and a number of transconjugants showing the EPS phenotype of the wild-type were selected on YEM medium. Five different overlapping cosmid clones containing an 18 kb common DNA region were isolated from these transconjugants. When these clones, pUHR221-pUHR225, were again transferred to the three mutants, their EPS defects were complemented since all transconjugants produced EPS like TAL1145.

Two of the five cosmids that complemented the three EPS defective mutants, pUHR221 and pUHR222, were used to obtain site-directed mutants of TAL1145 using the transposon Tn3Hogus as described in Methods. Fifty-seven EPS-defective mutants were selected that formed rough, opaque and smaller colonies as compared to the mucoid, translucent and large colonies of the wild-type. The mutants were streaked on YEM medium containing Calcofluor and on the basis of the colony morphology and fluorescence they were divided into three classes (Fig. 1). The class I and the class III mutants produced reduced amounts of EPS and appeared partially mucoid while the colonies of the class 11 mutants were non-mucoid, small and opaque. When examined under UV, the class I and class III mutants on Calcofluorcontaining YEM agar showed dim blue fluorescence compared to the bright blue fluorescence of the wildtype strain whereas the class 11 mutants did not fluoresce. However, unlike the class I mutants or the wild-type strain, the fluorescence of the class III mutants changed to yellowish-green when the colonies were incubated for 10 d or longer.