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Characterization of a *Rhizobium etli* chromosomal gene required for nodule development on *Phaseolus vulgaris L.*

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A chromosomal gene, required for nodule development on *Phaseolus* bean, was characterized from *Rhizobium etli* strain TAL182. MLC640 is a Tn5 insertion mutant of TAL182 which shows decreased motility in soft TY agar and is defective in nodule development. The site of Tn5 insertion in MLC640 mapped to a 3.6-kb EcoRI chromosomal fragment. The 3.6-kb fragment was subcloned from the cosmid pUHR80 which complemented MLC640. Further subcloning and site-directed Tn5 mutagenesis localized the gene for nodule development to a 1.7-kb region within the 3.6-kb EcoRI fragment. Southern hybridization using the 3.6-kb EcoRI fragment as the probe against genomic DNA of several *Rhizobium* spp. indicated that this gene is conserved in different rhizobia.

Key words: Motility, *ndv* gene, nitrogen fixation, nodulation, *Rhizobium.*

Phaseolus vulgaris L. is effectively nodulated by strains of *Rhizobium leguminosarum* by *phaseoli, R. etli* and *R. tropici.* Previously, *R. etli* and *R. tropici* strains were known as *R. leguminosarum* by *phaseoli* type I and type II strains, respectively (Martinez-Romero *et al.* 1991; Segovia *et al.* 1993). On the basis of differences with *R. leguminosarum* strains in the 16S rRNA sequences and electrophoretic typing, bean-nodulating *Rhizobium* strains such as CFN42, CFNI and Viking 1 have recently been classified as *R. etli* (Segovia *et al.* 1993). TAL182 is classified as an *R. etli* strain on the basis of similar electrophoretic typing with CFN42 and Viking 1 (Pinero *et al.* 1988). In *R. leguminosarum* by *phaseoli, R. etli* and *R. tropici* strains, as in other fast-growing *Rhizobium spp.,* the genes for nodulation *(nod)* and N_2 fixation *(nif, fix)* are located in indigenous plasmids known as symbiosis plasmids (Lamb *et al.* 1982; Vargas *et al.* 1990, Vazquez *et al.* 1991). Besides the *nod, nif* and *fix* genes, the symbiosis plasmids of *R. leguminosarum* bv *phaseoli* strains contain other unique genes such as psi and *psr,* involved in the regulation of polysaccharide synthesis (Borthakur & Johnston 1987), *mel,* involved in melanin synthesis (Borthakur *et al.* 1987), and *nolE* and *no1D* with unknown functions (Davis & Johnston 1990).

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In various *Rhizobium spp.,* in addition to the plasmid-bome genes for nodulation and N_2 fixation, there are chromosomal genes which are required in the symbiosis process. The *ndvA* and *ndvB* loci of *R. meliloti,* which are similar to the *chvA* and *chvB* genes, respectively, of *Agrobacterium tumefaciens,* are examples of such chromosomal genes in *Rhizobium* which are involved in the nodule development (Dylan *et al.* 1986; Stanfield *et al.* 1988). Other genes, such as *exoR* and *exoS,* are located in the chromosome and are involved in exopolysaccharide synthesis and symbiotic N_2 fixation (Doherty *et al.* 1988). Similarly, genes involved in lipopolysaccharide synthesis are located in the chromosome of *R. etli* (Noel *et al.* 1986) and *R. leguminosarum* bv *viciae* (Priefer 1989). In *R. etli* strain CFN42, chromosomal mutants that formed ineffective nodules were reported (Vandenbosch *et al.* 1985). In the present study a chromosomal gene required for nodule development is characterized from the *R. etli* strain TAL182.

George & Robert (1991) isolated and characterized the mutant MLC640, a nodulation-defective mutant of *R. etli* strain TAL182. This mutant makes small non-fixing nodules on beans and fails to induce nodulation suppression in bean split-root systems. MLC640 was complemented by two overlapping cosmid clones pUHR80 and pUHR85 containing DNA from strain TAL182. The transconjugants of TAL182 containing either pUHR80 or pUHR85 formed N_2-fixing nodules on bean and induced nodulation suppres

Table 1. Bacterial strains and plasmids.

Strain/plasmid	Relevant characteristics	Reference
Rhizobium		
TAL182	*R. etli* wild-type strain	George *et al.* (1992)
MLC640	Nal^r, Kan^r, Tn*5*-insertion mutant of TAL182 defective in nodule development	George *et al.* (1992)
SP909	Rif^r, Kan^r, Tn*5*-insertion mutant of TAL182 defective in nodule development	This study
SP922	Rif^r, Kan^r, Tn*5*-insertion mutant of TAL182 defective in nodule development	This study
SP950	Rif^r, Kan^r, Tn*5*-insertion mutant of TAL182 defective in nodule development	This study
Escherichia coli		
DH5αMCR	Used for transformation	Bethesda Research Laboratories
C2110	Nal^r, *polA* mutation	Leong *et al.* (1982)
UNF510	Kan^r, Tn*5* integrated in the chromosome	Merrick *et al.* (1978)
Plasmids		
pRK2073	Sp^r, pRK2013::Tn7, Used for mobilizing cosmids	Leong *et al.* (1982)
pPH1JI	Gm^r, P1 group plasmid used to eliminate pLAFR3-based cosmid from *Rhizobium* strains	Beringer *et al.* (1978)
pRK404	Wide host range P1-group cloning vector	Ditta *et al.* (1985)
pRK404A	Derivative of pRK404 from which the second *Eco*RI site was removed, keeping the one in the multiple cloning site	W. J. Buikema*
pUHR80 and PUHR85	Tet^r, two cosmids with overlapping DNA fragments from *R. etli* strain TAL182 cloned in wide-host-range cloning vector pLAFRI	George *et al.* (1992)
pUHR122	3.6-kb *Eco*RI fragment of pUHR80 cloned in pUC19	This study
pUHR130 and pUHR136	Tet^r, the 3.6-kb *Eco*RI fragment from pUHR122 was cloned in either orientation in pRK404A	This study
pUHR179	Tet^r, the 2.9-kb *Hin*dIII-*Eco*RI fragment of pUHR136 cloned in pRK404A.	This study
pUHR180	Tet^r, the 2.6-kb *Pst*I-*Eco*RI fragment of pUHR136 cloned in pRK404A	This study

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sion in bean split-root systems (George et al. 1992). We report here the characterization of a gene for nodule development, contained in pUHR80, which was inactivated by the Tn5 insertion in the mutant MLC640.

Materials and Methods

Bacterial Strains and Plasmids

The *Rhizobium* and *Escherichia coli* strains and plasmids used are listed with their sources and relevant properties in Table 1. *Rhizobium* strains were grown at 28'C in TY (Beringer 1974) or YEM (Bohlool & Schmidt 1970) media. For the isolation of indigenous plasmids or total genomic DNA, *Rhizobium* strains were grown in PA medium (Hirsch *et al.* 1980). *Escherichia coli* strains were grown at 37°C in LB medium (Miller 1972). When selective conditions were required the media were supplemented with antibiotics to yield the following concentrations (μg ml^{-1}): kanamycin, 50; rifampicin, 50; spectinomycin, 50; tetracycline, 10; ampicillin, 100; or nalidixic acic, 50. Transfer of plasmids from *E.* coli to *Rhizobium* was carried out by patch cross (Johnston *et al.* 1978) or on membrane filters (Beringer & Hopwood 1976) using pRK2073 (Leong *et al.* 1982) as helper.

DNA Isolation and Manipulation

Genomic DNA from *Rhizobium* strains was prepared by modification of standard procedures (Ausubel *et al.* 1989). PA medium (30 ml) was inoculated with 1 ml of stationary phase *Rhizobium* culture and grown overnight. The cells were harvested and washed once with 20 ml 1 m NaCl and twice with 20 ml TES buffer (10 mm Tris, pH 8, 25 mm EDTA, 15 mm NaCl). The cells were resuspended in 4 ml TE_{25} buffer (10 mm Tris, pH 8, 25 mm EDTA) and treated with 0.5 ml lysozyme (2 mg ml^{-1}) at 37°C for 10 min. The cells were then lysed with 0.5 ml 20% Sarkosyl and 0.5 ml Pronase (5 mg ml^{-1}) which was pre-incubated for 1 h. at 37°C. The tube was then incubated at 37°C for 2 to 16 h for complete digestion of the proteins. The viscous lysate was extracted three to four times with an equal volume of phenol and once with an equal volume of chloroform. The DNA was precipitated with 0.1 volume of 3 M sodium acetate and 0.6 volume of propan-2-ol. The DNA thread was spooled with a hooked glass rod, washed with 70% ethanol, dried and resuspended in 1 ml of TE buffer (10 mm Tris, pH 8.0, 1 mm EDTA). Indigenous *Rhizobium* plasmids were isolated and visualized on horizontal agarose gels according to Hirsch *et al.* (1980). Standard procedures (Sambrook *et al.* 1989) were used for all DNA manipulations, including plasmid DNA extractions, restriction mapping and molecular cloning. Restriction enzymes and lygase were purchased from United States Biochemical, Cleveland, Ohio.

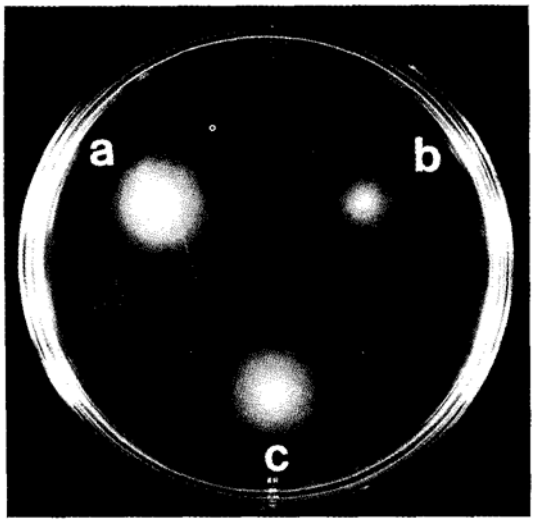

Figure 1. Photograph showing difference in motility between *R. etli* TAL182 (a) and mutant MLC640 (b) in 0.3% TY agar. The transconjugant of MLC640 containing pUHR80 (c) showed motility like the wild-type strain. The plates were incubated at 28°C for 2 days.

Southern Hybridization

Total genomic DNA was cleaved by restriction endonuclease and fragments were separated by electrophoresis overnight at 30 V through a 0.7% agarose gel. The DNA was transferred to Gene Screen Plus nylon membrane (Dupont, NEN Research Product) using an alkaline transfer procedure (Reed & Mann 1985). Probes for hybridization were made by the random priming method (Feinberg & Vogelstein 1983).

Tn5 Mutagenesis of Plasmid pUHR136 and Isolation of Site-directed Mutants

Tn5 mutagenesis of the 3.6 kb cloned DNA in the plasmid pUHR136 was done in *E.* coli strain UNF510 (Merrick et al. 1978) which contains a copy of the Tn5 in the chromosome. Plasmid pUHR136 was used to transform UNF510. Tn5 insertions into pUHR136 were isolated by selecting for joint transfer of resistances to kanamycin (specified by Tn5) and tetracycline (specified by pUHR136) in conjugal crosses into *E.* coli strain C2110 using pRK2073 as the helper. The pUHR136::Tn5 derivatives were transferred to the wild-type Rhizobium strain TALI82 and the Tn5 was homogenotized by marker exchange (Ruvkun & Ausubel 1981). The positions of the Tn5 in the marker-exchanged mutants were verified by Southern analysis using pUHR136 as the probe.

Plant Inoculation Tests

Phaseolus beans (variety Brazil 2) were grown in 250-ml flasks containing horticultural vermiculite (Star Garden, Honolulu) wetted with N-free plant nutrient solution (Hoagland & Amon 1938) as described by George & Robert (1991). Approximately 200 cm^3 vermiculite were put into each flask and sterilized before 80 ml of sterile plant nutrient medium was added. Seeds of Phaseolus were surface-sterilized by immersing in commercial bleach for 5 min. The seeds were then washed five times in sterile water, transferred to TY plates and allowed to germinate for 2 days at room temperature in the dark. One germinated seed was transferred to each flask and allowed to grow until the plumule reached the neck of the flask. At this stage the shoots were pulled past the bungs, inoculated with 1 ml cell suspension (10^6 to 10^7 rhizobia) of the appropriate strain and allowed to grow in a growth chamber. The plants were grown for 4 weeks after inoculation for the nodulation test. The ability of nodulated plants to fix N_2 was assayed by acetylene reduction (Hardy et al. 1968). Rhizobia were re-isolated from the nodules as described by Somasagaran & Hoben (1985).

Results

Mutant MLC640 Shows Decreased Motility

Mutant ML640, when transferred on 0.3% TY agar, showed decreased motility compared with the wild-type strain TALI82 (Figure 1). The mean diameter of the circular swarming region around the spot after 2 days for MLC640 was less than half of that for TALI82. The transconjugants of MLC640 containing pUHR80 showed motility like the wild-type strain.

Cloned DNA in pUHR80 is *a part of the* TALI82 *Chromosome*

Strain TALI82 has four indigenous plasmids of sizes between 100 and 220 MDa and the genes for nodulation and N_2 fixation are located on the second largest plasmid, 200 MDa in size (George *et al.* 1992). To determine if the cloned DNA in the plasmid pUHR80 is located in one of these plasmids, radioactively-labelled pUHR80 DNA was used as a probe against the indigenous plasmids of TALI82 separated in an agarose gel and blotted onto a nitrocellulose membrane. Total genomic DNA of TALI82 was also loaded in one lane of the gel as a control. The probe hybridized with the genomic DNA but not with any of the plasmids, indicating that the cloned DNA is a part of the chromosome (Figure 2).

Localization of Tn5 in *the Mutant MLC640*

To localize the Tn5 in MLC640, plasmid pUHR80 was used as a probe in Southern hybridization against the EcoRl digested genomic DNA of TALI82 and MLC640 (Figure 3). The 3.6-kb-hybridizing band in TALI82 was replaced by a 9.3-kb band in MLC640, indicating that the Tn5 in this mutant was inserted in a 3.6-kb fragment. The 3.6-kb EcoRl fragment from pUHR80 was sub-cloned into pUC19 to obtain pUHR122. Plasmid pUHR122 was used as a probe against the genomic digest of TALI82 and MLC640. As seen in Figure 3b, the 3.6-kb EcoRl fragment of TALI82 was replaced by a 9.3-kb EcoRl fragment in MLC640, confirming that the Tn5 was inserted on the 3.6-kb fragment. Plasmid pUHR122 was digested with several enzymes and a restriction map of the 3.6-kb EcoRl fragment was developed (Figure 4). By using plasmid pUHR122 as a probe against *Hindlll*- and Hindlll plus EcoRl-digested genomic DNA of MLC640 and TALI82, the position of the

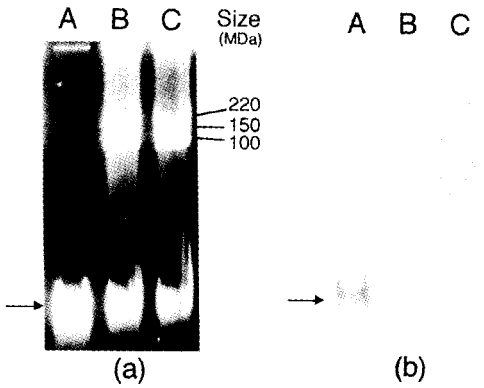

Figure 2. Southern hybridization of genomic and indigenous plasmid DNA of TAL182 with pUHR80. (a) Aproximately 2 μg of undigested genomic DNA (lane A) and indigenous plasmid DNA (lanes B and C) of TAL182 were separated on a 0.6% agarose gel at 30 V for 12 h at 4°C. The upper bands in lanes B and C represent covalently closed circular plasmid DNA. The largest two plasmids of TAL182, 220 and 200 MDa in size, appear as a single band in this gel. The arrow shows genomic DNA in lane A and linear plasmid DNA in lanes B and C. (b) The DNA of this gel was transferred to a nylon membrane and hybridized with labelled pUHR80 DNA.

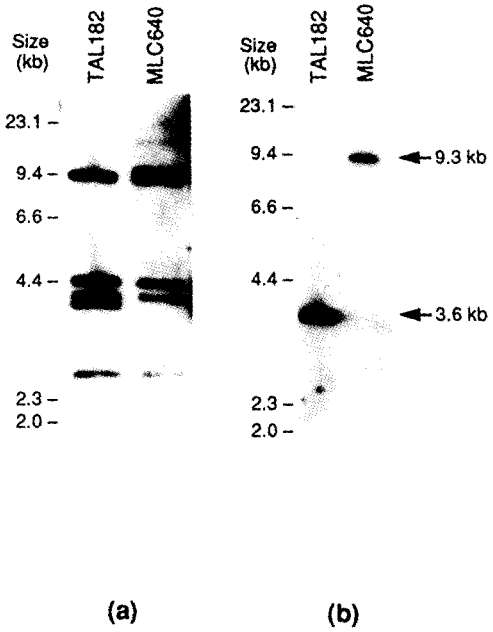

Figure 3. Localization of Tn5 in the mutant MLC640. Plasmids pUHR80 (a) and pUHR122 (b) were used as probes in Southern hybridization against *Eco*RI-digested genomic DNA of TAL182 and MLC640.

Tn5 in MLC640 was located 0.6 kb apart from the *HindIII* site in the 3.6-kb fragment, as shown in Figure 4.

Complementation of MLC640 with Subcloned Fragments

The 3.6-kb EcoRI fragment from pUHR122 was sub-cloned in the broad-host-range plasmid pRK404A in both orientations to get plasmids pUHR130 and pUHR136. These were

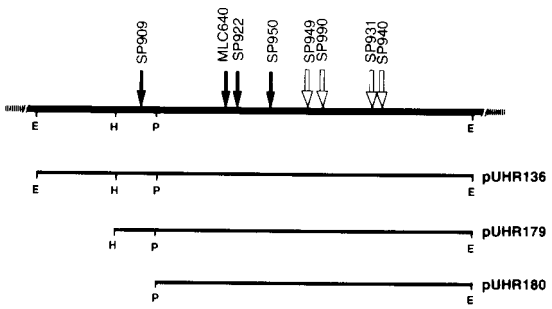

Figure 4. Restriction map of the 3.6-kb *Eco*RI fragment in pUHR136 and the positions of Tn5 insertions in mutants of TAL182. E—*Eco*RI; H—*Hin*dIII; P—*Pst*I. Insertions within the gene for nodule development are shown as solid arrows while those outside the gene are indicated by open arrows.

transferred to the mutant MLC640 and the transconjugants were used to inoculate beans. The derivatives of MLC640 containing either pUHR 130 or pUHR136 formed N_2-fixing nodules, indicating that the 3.6-kb cloned DNA in these two plasmids complemented the mutation in MLC640. Plasmids pUHR130 and pUHR136 also restored motility in the transconjugants of MLC640 to the wild-type level. The 2.9-kb *HindIII-EcoRI* and 2.6-kb *Pst*I-EcoRI fragments of pUHR136 were sub-cloned in pRK404A to obtain the plasmids pUHR179 and pUHR180, respectively (Figure 4). These plasmids were transferred to MLC640 and the transconjugants were used to inoculate beans. The transconjugants of ML640 containing pUHR179 formed N_2-fixing nodules on beans whereas those containing pUHR180 formed only small non-fixing nodules such as those made by MLC640, indicating that the gene involved in nodule development is located within this 2.9-kb fragment and the *Pst*I site is within the gene.

Site-directed Mutagenesis of Plasmid pUHR136

To localize the gene in this 3.6-kb fragment, plasmid pUHR136 was mutagenized with Tn5 and 11 insertions were isolated. These pUHR136::Tn5 derivatives were each transferred to TALI82 in order to homogenotize these insertions into the TALI82 chromosome. Homogenotes were obtained for eight insertions (Figure 4). These homogenotes were analysed by Southern hybridization using the 3.6-kb EcoRI fragment as the probe. In all of them the 3.6kb EcoRI fragment of TALI82 was replaced by a 9.3-kb fragment, indicating that the Tn5 insertions in these mutants occurred by homologous recombination between pUHR136::Tn5 derivatives and the 3.6-kb homologous DNA fragment of TALI82 (data not shown). These mutants were tested for motility on 0.3% TY agar and for nodulation and N_2-fixing abilities on beans. Three mutants, SP909, SP922 and SP950, were found to be defective in both motility and symbiosis. Like MLC640, these mutants showed reduced motility on TY soft agar and formed small

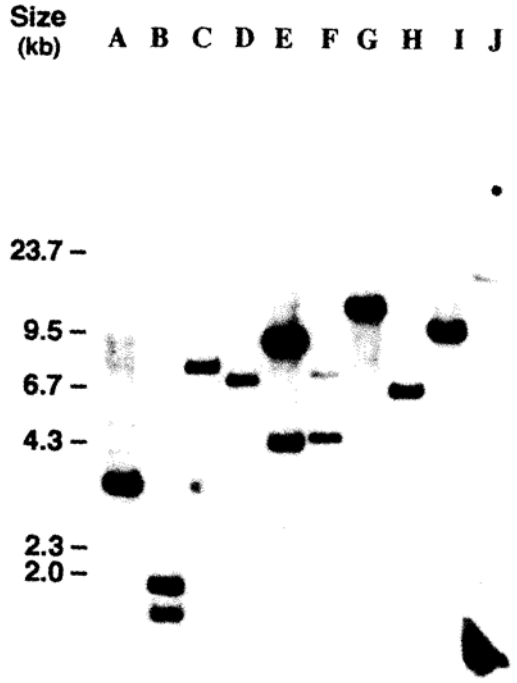

Figure 5. Hybridization of the 3.6-kb *Eco*RI fragment of pUHR136 with other rhizobia. Genomic DNA (2 μg) digested with *Hind*II was used for each strain. Methods for electrophoresis, blotting and hybridization were as described in Materials and Methods. Hybridization and washings were done at 60°C. Lanes A to D hold leucaena-nodulating *Rhizobium* strains, TAL1145 (A), TAL21 (B), TAL1143 (C) and TAL1381 (D). The other lanes hold *R. etli* TAL182 (E), *R. leguminosarum* bv *viciae* F4 (F), *R. leguminosarum* bv *trifolii* WU290 (G), *R. meliloti* NZP4013 (H), *R. fredii* USDA205 (I) and *B. japonicum* USDA110 (J).

white nodules on beans, indicating that the Tn5 insertions in these mutants may be on the same gene as in MLC640.

Homology with other Rhizobium

The *ndvA* (Dylan *et al.* 1986) and *ndvB* genes of *R. fredii* (Bhagwat *et al.* 1992) were used as the probes in Southern hybridization against pUHR80 and no hybridization was observed (data not shown), indicating that the cloned gene is not *ndvA* or *ndvB*. The 3.6-kb fragment of pUHR130 was used as the probe against the genomic DNA of *R. meliloti, R. leguminosarum* bv *viciae, R. leguminosarum* by *trifolii, R. tropici, Rhizobium* strains NGR234 and TAL1145 and *Bradyrhizobium japonicum* (Figure 5). The 3.6-kb fragment hybridized strongly with all *Rhizobium* species, indicating that the gene contained in the 3.6-kb EcoRI fragment of pUHR80 is conserved among *Rhizobium* species. The 3.6kb fragment hybridized weakly with *B. japonicum,* indicating that there may be a weaker DNA homology in the case of *B. japonicum*.

Discussion

Rhizobia interact with the roots of leguminous plants and stimulate them to develop nodules, which the bacteria invade, inhabit and use to fix N_2. Many *Rhizobium* genes are responsible for this differentiation process. The *Rhizobium* genome is composed of two types of replicons, the chromosome and several large plasmids (Long 1989). The *nod, nif* and fix genes are located on a single large plasmid, known as the symbiosis plasmid (pSym). In two *R. etli* strains, CFN42 and TALI82, the pSym has been identified (Girard *et al.* 1991; Vazquez *et al.* 1991; George of *al.* 1992). In the strain CFN42, some *nod* and *nif* genes involved in nodulation and N_2 fixation are reiterated (Brom *et al.* 1988). In fast-growing rhizobia there are also chromosomal genes which are necessary for nodule development or N_2 fixation. The *ndvA* and *ndvB* are such chromosomal genes required for nodule development in *Rhizobium* (Dylan *et al.* 1986; Stanfield *et al.* 1988). Similarly, the *exoR* and *exoS* genes, which are required for exopolysaccharide synthesis and N_2 fixation, are located in the chromosome whereas most other *exo* genes are located in a megaplasmid (Doherty *et al.* 1988). Here, a *R. etli* chromosomal gene involved in nodule development on beans has been characterized.

The mutants MLC640, SP909, SP922 and SP9250 form very small, non-fixing nodules on beans. Similar phenotypes have been reported for the *ndvB* mutants of *R. meliloti* (Dylan *et al.* 1986) and *R. fredii* (Bhagwat *et al.* 1992). This kind of phenotype has been reported for other classes of mutants of *R. meliloti* (Finan *et al.* 1985; Leigh *et al.* 1985) that cannot produce extracellular acidic heteropolysaccharide. Other *R. meliloti* mutants of this kind are ndvF::Tn5 mutants, which formed mere swellings on lateral roots or small ineffective nodules which varied in colour from white to light brown (Charles *et al.* 1991). The mutant MLC640 and three other site-directed mutants described in the present study produce normal amounts of exopolysaccharide and stain normally with the fluorescent dye Calcofluor. However, it is possible that these mutants do not produce a minor but essential exopolysaccharide component. The *ndvB* mutant of *R. meliloti* also produces the normal amount of Calcofluor-binding exopolysaccharide but is defective in synthesis of ß-1,2-glucan. Whether mutant MLC640 is unable to make ß-1,2-glucan or other minor polysaccharides was not investigated in this study. The *ndvA* mutant of *R. meliloti,* which was also unable to synthesise ß-1,2-glucan, formed small, white, empty nodules on alfalfa roots and exhibited reduced motility (Stanfield *et al.* 1988). The four mutants in the present study also showed reduced motility compared with the wild-type parental strain. However, the gene contained in the 3.6-kb EcoRI fragment is not *ndvA* or *ndvB* because probes containing these genes did not hybridize with the cloned DNA in the cosmid pUHR80. Since mutations in this gene lead to the incomplete development of nodules, this gene can be placed among a class of genes designated as *ndv* (affecting nodule development).

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Non-infective *R.* etli mutations which are not located on the symbiosis plasmid have been reported previously (Noel et al. 1984; Vandenbosch et al. 1985). Three mutants of *R.* etli strain CFN42 induced the formation of small, white swellings on the roots of beans which were similar to the small nodules formed by the ndv mutants in the present study. However, unlike these ndv mutants, the three mutants of CFN42 (Noel et al. 1984) had detectable differences in exopolysaccharide production and Calcofluor staining in agar culture. Noel et al. (1986) reported another class of *R.* etli mutants that have altered exopolysaccharide (LPS) and rough colony texture on TY agar medium. These mutants stained normally with Calcofluor and induced the formation of small, white, ineffective nodules on the roots of beans. Priefer (1989) reported four similar mutations in a *R.* leguminosarum by viciae strain which resulted in altered LPS profile, less glossy colony morphology on TY agar, autoagglutination in TY liquid medium and loss of motility on soft agar. These mutants formed small, round, white, ineffective nodules on Vicia hirsuta roots, compared with the reddish, long-shaped effective nodules induced by the wildtype strain. The mutants MLC640, SP909, SP922 and SP950 in the present study did not show rough colony morphology like strains CE109 and CE113 (Noel et al. 1986) or auto-agglutination like the R. leguminosarum by viciae mutants (Priefer 1989). The four ndv mutants in the present study showed reduced motility but not the complete loss of motility seen in the LPS mutants described by Priefer (1989).

The isolation of symbiotically defective mutants of Rhizobium has proved a useful tool for isolation of bacterial genes by complementation of the mutants and elucidating the function of genes involved in symbiosis. Phenotypic analysis of mutants and their complemented derivatives helps to elucidate the complexities of Rhizobium-plant interaction and determine the role of certain genes involved in this interaction. In the present study, by complementation, sub-cloning and Southern analysis of the nodulation-defective mutant MLC640, we have isolated a 3.6-kb DNA fragment containing the wild-type copy of the gene which complemented this mutation. The Pstl site in the 3.6-kb fragment is located within the gene because plasmid pUHR180 containing only the 2.6-kb Pstl-EcoRI fragment of pUHR136 did not complement MLC640. However, the 2.9-kb HindIII-EcoRI fragment from pUHR136 cloned in plasmid pUHR179 complemented MLC640, indicating that the HindIll site is outside the coding region. The coding region for the gene must be located within the 1.7-kb region between the site of Tn5 insertion in SP990 and the HindIII site in the 3.6-kb EcoRI fragment within which the Tn5 insertions for the nodulation-defective mutants MLC640, SP909, SP922 and SP950 are located.

Acknowledgement

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References

Ausubel, F.M., Brent, R., Kingston, R., Moore, D.D., Seidman, J.G., Smith, J.A. & Struhl, K. 1989 *Current Protocols in Molecular Biology.* New York: Wiley Interscience.

Beringer, J.E. 1974 R Factor transfer in *Rhizobium leguminosarum. Journal of General Microbiology 84,* 188-198.

Beringer, J.E., Beynon, J.L., Buchanan-Wollaston, A.V. & Johnston, A.W.B. 1978 Transfer of the drug-resistance transposon Tn5 *to Rhizobium. Nature 276,* 633-634.

Beringer, J.E. & Hopwood, D.A. 1976 Chromosomal recombination and mapping in *Rhizobium leguminosarum. Nature 264,* 291-293.

Bhagwat, A.A., Tully, R.A. & Keister, D.L. 1992 Isolation and characterization of an *ndvB locus* from *Rhizobium fredii. Molecular Microbiology 6,* 2159-2165.

Bohlool, B.B. & Schmidt, E.L. 1970 Immunofluorescent detection of *Rhizobium japonicum* in *soils. Soil Science 110,* 229-236.

Borthakur, D. & Johnston, A.W.B. 1987 Analysis of psi, a gene on symbiotic plasmid of *Rhizobium phaseoli* which inhibits exopolysaccharide synthesis and nodulation: determination of its sequence and demonstration that its transcription is inhibited *by psr,* another gene on the symbiotic plasmid. *Molecular and General Genetics 207,* 149-154.

Borthakur, D., Lamb, J.W. & Johnston, A.W.B. 1987 Identification of two classes of *Rhizobium phaseoli* genes required for melanin synthesis one of which is required for nitrogen fixation and activates the transcription of the other. *Molecular and General Genetics 207,* 155-160.

Brom, S., Martinez, E., Davila, G. & Palacios, R. 1988 Narrowand broad-host-range symbiotic plasmids of *Rhizobium spp.* strains that nodulate *Phaseolus vulgaris. Applied and Environmental Microbiology 54,* 1280-1283.

Charles, T.C., Newcomb, W. & Finan, T.M. 1991 *nodvF, a* novel locus located on megaplasmid pRmeSU47b (pEXO) of *Rhizobium meliloti, is* required for normal nodule development. *Journal of Bacteriology 173,* 3981-3992.

Davis, E.O. & Johnston, A.W.B. 1990 Regulatory functions of the three *nodD* genes of *Rhizobium leguminosarum* bv. *phaseoli. Molecular Microbiology 4,* 933-941.

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

Doherty, D., Leigh, J.A., Glazebrook, J. & Walker, G.C. 1988 *Rhizobium meliloti* mutants that overproduce the *R. meliloti* acidic Calcofluor-binding exopolysaccharide. *Journal of Bacteriology 170,* 4249-4256.

Dylan, T., Ielpi, L., Stanfield, S., Kashyap, L., Douglas, C., Yanofsky, M., Nester, E., Helinski, D.R. & Ditta, G. 1986 *Rhizobium meliloti* genes required for nodule development are related to chromosomal virulence genes in *Agrobacterium tumefaciens. Proceedings of the National Academy of Sciences of the United States of America 83,* 4403-4407.

Feinberg, A.P. & Vogelstein, B. 1983 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Annals of Biochemistry 132,* 6-13.

Finan, T.M., Hirsch, A.M., Leigh, J.A., Johansen, E., Kuldau, G.A., Deegan, S., Walker, G.C. & Signer, E.R. 1985 Symbiotic mutants

of Rhizobium meliloti that uncouple plant from bacterial differentiation. *Cell* 40, 869-877.

George, M.L.C. & Robert, F.M. 1991 Autoregulatory response of *Phaseolus vulgaris* L. to symbiotic mutants of *Rhizobium leguminosarum* bv. *phaseoli. Applied and Environmental Microbiology* 57, 2687-2692.

George, M.L.C., Robert, F.M. & Borthakur, D. 1992 Genetic analysis of *Rhizobium leguminosarum* bv. *phaseoli* mutants defective in nodulation and nodulation suppression. *Applied and Environmental Microbiology* 58, 1050-1053.

Girard, M. De L., Flores, M., Brom, S., Romero, D., Palacios, R. & Guillermo, D. 1991 Structural complexity of the symbiotic plasmid of *Rhizobium leguminosarum* bv. *phaseoli. Journal of Bacteriology* 173, 2411-2419.

Hardy, R.W.F., Holsten, R.D., Jackson, E.K. & Bums, R.C. 1968 The acetylene assay for nitrogen fixation: laboratory and field evaluation. *Plant Physiology* 43, 1185-1207.

Hirsch, P.P., Van Montagu, M., Johnston, A.W.B., Brewin, N.J. & Schell, J. 1980 Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of *Rhizobium leguminosarum. Journal of General Microbiology* 120, 403-412.

Hoagland, D.R. & Amon, D.I. 1938 The water culture method for growing plants without soil. *California Agricultural Experimental Station, Circular* 347, 39.

Johnston, A.W.B., Beynon, J.L., Buchanan-Wollaston, A.V., Setchell, S.M., Hirsch, P.R. & Beringer, J.E. 1978 High frequency transfer of nodulating ability between strains and species of *Rhizobium. Nature* 276, 634-636.

Lamb, J.W., Hombrecher, G. & Johnston, A.W.B. 1982 Plasmid-determined nodulation and nitrogen fixation abilities in *Rhizobium phaseoli. Molecular and General Genetics* 186, 449-452.

Leigh, J.A., Signer, E.R. & Walker, G.C. 1985 Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. *Proceedings of the National Academy of Sciences of the United States of America* 82, 6231-6235.

Leong, S.A., Ditta, G.S. & Helinski, D.R. 1982 Heme biosynthesis in *Rhizobium.* Identification of cloned coding for a-aminolevulinic acid synthetase from *Rhizobium meliloti. Journal of Biological Chemistry* 257, 8724-8730.

Long, S.R. 1989 *Rhizobium* genetics. *Annual Review of Genetics* 23, 483-505.

Martinez-Romero, E., Segovia, L., Mercante, F.M., Franco, A.A., Graham, P. & Pardo, M.A. 1991 *Rhizobium tropici,* a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena sp.* trees. *International Journal of Systematic Bacteriology* 41, 417426.

Merrick, M., Filser, M., Kennedy, C. & Dixon, R. 1978 Polarity of mutations induced by insertion of transposons Tn5, Tn7 and Tn10 into the *nif* gene cluster of *Klebsiella pneumoniae. Molecular and General Genetics* 165, 103-111.

Miller, J.H. 1972 *Experiments in Molecular Genetics.* New York: Cold Spring Harbor Laboratory.

Noel, K.D., Sanchez, L., Famandez, J., Leemans, J. & Cevallos, M.A. 1984 *Rhizobium phaseoli* symbiotic mutants with Tn5 insertions. *Journal of Bacteriology* 158, 148-155.

Noel, K.D., Vandenbosch, K. & Kulpaca, B. 1986 Mutations in *Rhizobium phaseoli* that lead to arrested development of infection threads. *Journal of Bacteriology* 168, 1392-1401.

Pinero, D., Martinez, E. & Selander, R.K. 1988 Genetic diversity and relationships among isolates of *Rhizobium leguminosarum* biovar *phaseoli. Applied and Environmental Microbiology* 54, 2825-2832.

Priefer, U.B. 1989 Genes involved in lipopolysaccharide production and symbiosis are clustered on the chromosome of *Rhizobium leguminosarum* bv. *viciae* VF39. *Journal of Bacteriology* 171, 6161-6168.

Reed, K.C. & Mann, D.A. 1985 Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Research* 13, 7207-7221.

Ruvkun, G.B. & Ausubel, F.M. 1981 A general method for site-directed mutagenesis in prokaryotes. *Nature* 289, 85-88. Sambrook, J., Fritsch, E.F. & Maniatis, T. 1989 *Molecular Cloning: a Laboratory Manual.* New York: Cold Spring Harbor Laboratory. Segovia, L., Young, J.P.W. & Martinez-Romero, E. 1993 Reclassification of American *Rhizobium leguminosarum* biovar *phaseoli* type I strains as *Rhizobium etli sp.* nov. *International Journal of Systematic Bacteriology* 43, 374-377.

Somasegaran, P. & Hoben, H.J. 1985 *Methods in Legume* Rhizobium *Technology.* Honolulu: NifTAL Project, University of Hawaii.

Stanfield, S.W. Ielpi, L., O'Brochta, D., Helinski, D.R. & Ditta, G.S. 1988 The *ndvA* gene product of *Rhizobium meliloti is* required for *f-(1* -> 2) glucan production and has homology to the ATP-binding export protein H1yB. *Journal of Bacteriology* 170, 3523-3530.

Vandenbosch, K., Noel, K.D., Kaneko, Y. & Newcomb, E.H. 1985 Nodulo initiation elicited by noninfective mutants of *Rhizobium phaseoli. Journal of Bacteriology* 162, 950-959.

Vargas, C., Martinez, L.J., Megias, M. & Quinto, C. 1990 Identification and cloning of nodulation genes and host specificity determinants of the broad host-range *Rhizobium leguminosarum* bv. *phaseoli* strain CIAT 899. *Molecular Microbiology* 4, 18991910.

Vazquez, M., Davalos, A., De Las Penas, A., Sanchez, F. & Quinto, C. 1991 Novel organization of the common nodulation genes in *Rhizobium leguminosarum* bv. *phaseoli* strains. *Journal of Bacteriology* 173, 1250-1258.

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Table 1. Bacterial strains and plasmids.

Strain/plasmid	Relevant characteristics	Reference
Rhizobium		
TAL182	*R.* etli wild-type strain	George et al. (1992) George et al. (1992)
MLC640	Nal', Kan', Tn5-insertion mutant of TAL182 defective in nodule development	This study
SP909	Rif', Kan', Tn5-insertion mutant of TAL182 defective in nodule development	This study
SP922	Rif', Kan', Tn5-insertion mutant of TAL182 defective in nodule development	This study
SP950	Rif', Kan', Tn5-insertion mutant of TAL182 defective in nodule	
Escherichia coli		
DHSaMCR	Used for transformation	Bethesda Research Laboratories Leong et al. (1982) Merrick et al. (1978)
C2110	Nal', polA mutation	
Plasmids		
pRK2073	Sp', pRK2013::Tn7, Used for mobilizing cosmids	Leong et al. (1982) Beringer et al. (1978)
pPHIJI	Gm', P1 group plasmid used to eliminate pLAFR3-based cosmid from *Rhizobium* strains	Ditta et al. (1985) W. J. Buikema'
pRK404	Wide host range P1-group cloning vector	George et al. (1992)
pRK404A	Derivative of pRK404 from which the second EcoRI site was removed, keeping the one in the multiple cloning site	This study This study
pUHR80 and PUHR85	Tet', two cosmids with overlapping DNA fragments from *R.* etli strain TAL182 cloned in wide-host-range cloning vector pLAFRI	
pUHR122	3.6-kb EcoRI fragment of pUHR80 cloned in pUC19	This study This study
pUHR130 and pUHR136	Tet', the 3.6-kb EcoRI fragment from pUHR122 was cloned in either	

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sion in bean split-root systems (George *et al.* 1992). We report here the characterization of a gene for nodule development, contained in pUHR80, which was inactivated by the Tn5 insertion in the mutant MLC640.

Materials and Methods

Bacterial Strains and Plasmids

The *Rhizobium* and *Escherichia coli* strains and plasmids used are listed with their sources and relevant properties in Table 1. *Rhizobium* strains were grown at 28ºC in TY (Beringer 1974) or YEM (Bohlool & Schmidt 1970) media. For the isolation of indigenous plasmids or total genomic DNA, *Rhizobium* strains were grown in PA medium (Hirsch *et al.* 1980). *Escherichia coli* strains were grown at 37°C in LB medium (Miller 1972). When selective conditions were required the media were supplemented with antibiotics to yield the following concentrations (ug ml^{-1}): kanamycin, 50; rifampicin, 50; spectinomycin, 50; tetracycline, 10; ampicillin, 100; or nalidixic acic, 50. Transfer of plasmids from *E.* coli to *Rhizobium* was carried out by patch cross (Johnston *et al.* 1978) or on membrane filters (Beringer & Hopwood 1976) using pRK2073 (Leong *et al.* 1982) as helper.

DNA Isolation and Manipulation

Genomic DNA from *Rhizobium* strains was prepared by modification of standard procedures (Ausubel *et al.* 1989). PA medium (30 ml) was inoculated with 1 ml of stationary phase *Rhizobium* culture and grown overnight. The cells were harvested and washed once with 20 ml 1 m NaCI and twice with 20 ml TES buffer (10 mm Tris, pH 8, 25 mm EDTA, 15 mm NaCl). The cells were resuspended in 4 ml TE_{ZS} buffer (10 mm Tris, pH 8, 25 mm EDTA) and treated with 0.5 ml lysozyme (2 mg ml^{-1}) at 37°C for 10 min. The cells were then lysed with 0.5 ml 20% Sarkosyl and 0.5 ml Pronase (5 mg ml^{-1}) which was pre-incubated for 1 h. at 37°C. The tube was then incubated at 37°C for 2 to 16 h for complete digestion of the proteins. The viscous lysate was extracted three to four times with an equal volume of phenol and once with an equal volume of chloroform. The DNA was precipitated with 0.1 volume of 3 tea sodium acetate and 0.6 volume of propan-2-ol. The DNA thread was spooled with a hooked glass rod, washed with 70% ethanol, dried and resuspended in 1 ml of TE buffer (10 mm Tris, pH 8.0, 1 mm EDTA). Indigenous *Rhizobium* plasmids were isolated and visualized on horizontal agarose gels according to Hirsch *et al.* (1980). Standard procedures (Sambrook *et al.* 1989) were used for all DNA manipulations, including plasmid DNA extractions, restriction mapping and molecular cloning. Restriction enzymes and lygase were purchased from United States Biochemical, Cleveland, Ohio.