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# Isolation and Characterization of a Gene for Nodule Development Linked to the *ndvA* and *ndvB* Genes in *Rhizobium* sp. Strain TAL1145

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## Abstract

Six overlapping cosmid clones containing genes for nodule development were isolated from *Rhizobium* sp. strain TAL1145 that nodulates both *Phaseolus* bean and the tree legume leucaena. These clones complemented a *Rhizobium etli* mutant, MLC640, for defects in nodule development and motility. Two of these clones hybridized with chromosomal fragments containing the *ndvA* gene from *R. meliloti* and the *ndvB* gene from *R. fredii*, required for nodule development in alfalfa and soybean, respectively. A Tn*5*-insertion mutant, SP571, was constructed from TAL1145 by site-directed mutagenesis using one of these clones. SP571 formed nitrogen-fixing nodules on leucaena but non-fixing nodules on beans. The Tn*5* insertion in this mutant was located in a 2.1-kb *Eco*RI fragment, approximately 15 kb away from the fragment that hybridized with the *ndvA* and *ndvB* genes. The 2.1-kb fragment, cloned in a wide-host-range vector, complemented the mutant SP571 for nitrogen fixation on beans.

Keywords: *Rhizobium*, nodulation, nitrogen fixation, *ndv* gene

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## 1. Introduction

In addition to the plasmid-borne genes for nodulation and nitrogen fixation, fast-growing *Rhizobium* species require other chromosomal genes for nodule development and nitrogen fixation. The chromosomal genes *ndvA* and *ndvB* of *R. meliloti* are required for nodule development and nitrogen fixation in alfalfa (Dylan et al., 1986). Pooyan et al. (1994) characterized an *R. etli* chromosomal gene required for nodule development on beans (*Phaseolus vulgaris*). Similarly, *R. meliloti* genes *exoC, exoD, exoR* and *exoS*, involved in production of acidic exopolysaccharide (EPS) and also required for nitrogen fixation in alfalfa, are located on the chromosome (Doherty et al., 1988). Genes involved in lipopolysaccharide (LPS) synthesis and required for nitrogen fixation are located on the chromosome in *R. leguminosarum* bv. viciae (Priefer, 1989) and *R. leguminosarum* bv. phaseoli (Noel et al., 1986). In *R. leguminosarum* bv. phaseoli, several chromosomal mutants which formed ineffective nodules on beans have been reported (Vandenbosch et al., 1985). Similarly, in the wide-host-range *Rhizobium* strain NGR234, Chen et al. (1985) isolated several chromosomal mutants which are defective in EPS synthesis and nitrogen fixation. One of these mutants, ANU2840, formed ineffective callus on roots of leucaena, although it formed nitrogen-fixing nodules on siratro (Djordjevic et al., 1987).

*Rhizobium* sp. strain TAL1145 nodulates tree legumes of the genera *Leucaena*, *Calliandra* and *Gliricidia* very effectively (Turk and Keyser, 1992). It has shown superior competitiveness and effectiveness in nodulation of leucaena in both field and greenhouse studies (Moawad and Bohlool, 1984; Somasegaran and Martin, 1986). It also forms nitrogen-fixing nodules on *Phaseolus vulgaris* (George et al., 1994). TAL1145 has three indigenous plasmids and the genes for nodulation and nitrogen fixation are located on the largest of these, which is 230 Mdal in size (George et al., 1994). We describe here the isolation of a mutant from strain TAL1145 by insertion of the transposon Tn*5* into the chromosome. This mutation abolishes the ability of the strain to fix nitrogen on beans but not on leucaena. We also report here the isolation and characterization of the TAL1145 gene which complements this mutation.

## 2. Materials and Methods

### *Bacterial strains and plasmids*

*Rhizobium* and *Escherichia coli* strains and plasmids are listed with their sources and relevant properties in Table 1.

Table 1. Bacterial strains and plasmids

| Strain/plasmid | Relevant characteristics | Relevance |
|---|---|---|
| *Rhizobium* | | |
| TAL1145 | Leucaena-nodulating wild-type strain, $Rif^r$, $Str^r$ | George et al. (1994) |
| SP571 | Tn*5*-insertion mutant of TAL1145, defective in nodule development, $Rif^r$, $Str^r$, $Kan^r$ | This study |
| TAL182 | *R. etli* wild-type strain | George et al. (1992) |
| MLC640 | Tn*5*-insertion mutant of TAL182, defective in nodule development, $Nal^r$, $Kan^r$ | George et al. (1992) |
| *Escherichia coli* | | |
| DH5αMCR | Used for transformation | Bethesda Research Laboratories |
| C2110 | *polA* mutation, $Nal^r$ | Leong et al. (1982) |
| UNF510 | $Kan^r$, Tn*5* integrated in the chromosome | Merrick et al. (1978) |
| *Plasmids* | | |
| pLAFR3 | Wide-host-range P1-group cosmid vector, $Tet^r$ | Staskawicz et al. (1987) |
| pRK2073 | pRK2013::Tn*7*, used for mobilizing cosmids, $Spc^r$ | Leong et al. (1982) |
| pPH1JI | P1 group plasmid used to eliminate pLAFR3-based cosmid from *Rhizobium* strains, $Gm^r$ | 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 multiple cloning site. | W.J. Buikema* |
| pUHR1<br>pUHR2<br>pUHR3<br>pUHR4<br>pUHR5<br>pUHR6 | Six overlapping cosmid clones each containing approximately 25-kb chromosomal DNA of TAL1145 cloned in PLAFR3. | This study |
| pUHR5:: Tn*5*-571 | Derivative of pUHR5 containing a Tn*5* insertion in the 2.1-kb *Eco*RI a fragment, $Tet^r$, $Kan^r$ | This study |

Table 1 (Continued)

| | | |
|---|---|---|
| pUHR130 | 3.6-kb *Eco*RI fragment of TAL182 chromosomal DNA cloned in pRK404A, $Tet^r$ | Pooyan et al. (1994) |
| pUHR144<br>pUHR145 | 2.1-kb *Eco*RI $\alpha$ fragment of pUHR5 cloned in pRK404A in both orientations, $Tet^r$ | This study |
| pDOB13 | Contains the entire *ndvA* gene of *R. meliloti* in a 2.7 kb *Sal*I fragment | Stanfield et al. (1988) |
| pNDV101 | Contains a part of *ndvB* of *R. fredii* in a 2.0-kb *Eco*RI fragment subcloned from plasmid p300 | Bhagwat et al. (1992) |

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*Microbiological techniques*

*Rhizobium* strains were grown at 29°C in TY (Beringer, 1974) or YEM (Bohlool and Schmidt, 1970) medium. For the isolation indigenous plasmids or total genomic DNA, *Rhizobium* strains were grown in PA medium (Hirsch et al., 1980). *E. 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 (in $\mu$g per ml): kanamycin, 50; rifampicin, 50; spectinomycin, 50; tetracycline, 10; ampicillin, 100; nalidixic acid, 50. Transfer of plasmids from *E. coli* to *Rhizobium* was carried out by patch crosses (Johnston et al., 1978) or on membrane filters (Beringer and Hopwood, 1976) using pRK2073 (Leong et al., 1982) as the helper plasmid.

*DNA isolation and manipulation*

Genomic DNA from *Rhizobium* strains was prepared by the procedure described by Pooyan et al. (1994). Endogeous *Rhizobium* plasmids were isolated and visualized on vertical agarose gels by the method of Eckhardt (1978). Standard procedures (Sambrook et al., 1989) were used for all DNA manipulations, including plasmid DNA extraction, restriction mapping and molecular cloning. Restriction enzymes and ligase were purchased from United States Biochemical Corp., Cleveland, OH.

*Southern hybridization*

Total genomic DNA was cleaved by restriction endonucleases and electrophoresed overnight at 30 V through a 0.7% agarose gel. The DNA was transferred to a 'GeneScreen Plus' nylon membrane (Dupont NEN Research

Product) using an alkaline transfer procedure (Reed and Mann, 1985). Probes for hybridization were made by the random priming method (Feinberg and Vogelstein, 1983). In order to reuse the DNA-bound nylon membrane, they were washed with 0.4 M NaOH for 30 min, followed by washing with 2×SSC (1×SSC is 0.15 M NaCl, 15 mM sodium citrate), 1% SDS for 2 hr at room temperature.

*Tn5 mutagenesis of plasmid pUHR5 and isolation of site-directed mutants*

Tn*5* mutagenesis of the insert DNA in the plasmid pUHR5 was performed in *E. coli* strain UNF510 (Merrick et al., 1978), which contains a copy of the Tn*5* in the chromosome. Plasmid pUHR5 was used to transform UNF510. Tn*5* insertions on pUHR5 were isolated by selecting for joint transfer of resistances to kanomycin (specified by Tn*5*) and tetracycline (specified by pUHR5) in conjugal crosses into *E. coli* strain C2110. The pUHR5::Tn*5* derivatives were transferred to the wild-type *Rhizobium* strain TAL1145 and the Tn*5* was homogenotized by marker exchange (Ruvkun and Ausubel, 1981). The positions of the Tn*5* in the marker-exchanged mutants were verified by Southern analysis using pUHR130 as the probe.

*Plant inoculation tests*

*Leucaena leucocephala* (variety K8) was grown in plastic pouch bags as described by George et al. (1992). *Phaseolus vulgaris* (variety Brazil 2) was grown in 250 ml flasks containing horticultural vermiculite (Star Garden, Honolulu) as described by Pooyan et al. (1994). The ability of nodulated plants to fix nitrogen was measured by acetylene reduction assay (Hardy et al., 1968). Rhizobia were reisolated from the nodules as described by Somasegaran and Hoben (1985).

## 3. Results

*Identification and cloning of TAL1145 DNA that complements Fix⁻ R. etli mutant MLC640*

Previously we characterized the *R. etli* mutant MLC640 that showed decreased motility and formed only very small nodules in the roots of beans (George et al., 1992; Pooyan et al., 1994). MLC640 contains a Tn*5* insertion in the chromosome and was complemented by a cloned 3.6-kb *Eco*RI chromosomal DNA fragment of parental strain TAL182 (Pooyan et al., 1994). *R. etli* strain TAL182 nodulates beans but not leucaena. In order to isolate other genes for nodule development and determine their role in the symbiosis

with leucaena, we complemented the nodule-development defect of the mutant MLC640 with cloned DNA of the *Rhizobium* sp. strain TAL1145, which nodulates both beans and leucaena. George et al. (1994) prepared a clone library of TAL1145 by ligating partially-digested *Sau*IIIA genomic fragments, 20–30 kb in size, to the wide-host-range cosmid vector pLAFR3. The population of *E. coli* containing the library was mated *en masse* with strain MLC640. The mating mixture was used to inoculate beans. All plants inoculated with the mixture formed a few normal-size nodules and tetracycline resistant rhizobia (conferred by pLAFR3) were isolated from these nodules. Cosmid DNA from the rhizobia was isolated, used to transform *E. coli*, and then isolated from *E. coli* for restriction analysis.

Six overlapping cosmids that complemented the mutant MLC640 were isolated and designated as pUHR1 through pUHR6 (Fig. 1). The transconjugants of MLC640 containing these cosmids were used to inoculate beans. All plants inoculated with the transconjugants formed normal nitrogen-fixing nodules, whereas the plants inoculated with MLC640 formed only small swellings in the roots. Moreover, the motility defect of the mutant MLC640 was corrected by these plasmids in the transconjugants. These results confirmed that the cloned DNA in these six cosmids contained DNA which complemented the mutation in MLC640. The insert DNA in these cosmids spanned approximately 34 kb and a 8.4-kb region with three *Eco*RI fragments of 4.2 kb and 2.1 kb (doublet) was common in all six cosmids. An *Eco*RI-restriction map of this 34-kb region was constructed (Fig. 1).

*Homology of the 3.6-kb TAL182 DNA that complements the mutant MLC640 with the cloned DNA in cosmids pUHR1–pUHR6*

It was expected that there is a region in the cloned DNA in the cosmids pUHR1–pUHR6 which is homologous to the 3.6-kb fragment of pUHR130 that complements MLC640. Plasmid pUHR130 was used as a probe against the *Hin*dIII-digested DNA of these cosmids. As seen in Fig. 2, *Hin*dIII fragments of 3.3 kb and a 0.8 kb hybridized strongly with the probe, suggesting that the DNA homologous to the 3.6-kb *Eco*RI fragment of pUHR130 is located in these two fragments. When the same probe was used against the *Eco*RI-digested DNA of the plasmids pUHR1–pUHR6, it hybridized with a 2.1-kb fragment in all six plasmids and an additional 3.0-kb fragment in pUHR1, pUHR3, pUHR4, and pUHR6 (data not shown). The 2.1-kb and 3.0-kb *Eco*RI fragments are adjacent fragments in these cosmids and include the 0.8-kb and the 3.3-kb *Hin*dIII fragments that hybridized with the probe (Fig. 1). Since there are two 2.1-kb *Eco*RI fragments adjacent to each other in these cosmids,

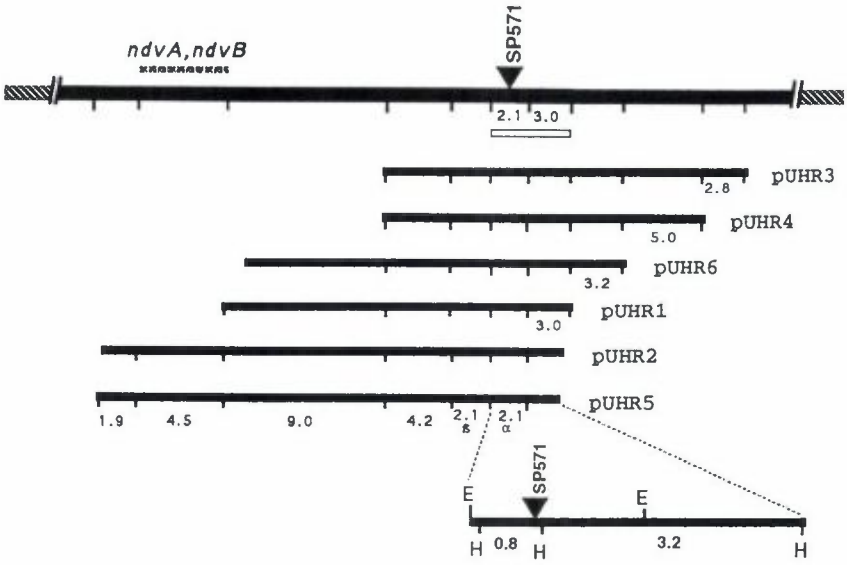


Figure 1. *Eco*RI-restriction map of a 34-kb region of chromosomal DNA from the *Rhizobium* sp. strain TAL1145 (thick line at the top). Six cosmid clones, pUHR1 through pUHR6, that contain overlapping DNA fragments representing the 34-kb chromosomal region of TAL1145 are shown. The 4.5-kb *Eco*RI fragment that hybridized with parts of *R. meliloti ndvA* and *R. fredii ndvB* genes is shown with a dashed line above the thick line (see also Fig. 3). The 2.1-kb and 3.0-kb *Eco*RI fragments that hybridized with the 3.6-kb fragment containing a *ndv* gene of TAL182 are shown with an open bar under the thick line (see text below). The location of the Tn*5* insertion in the mutant SP571 is shown as an inverted triangle. An expanded map of a 4.1 kb region of pUHR5 shows the position of the Tn*5* insertion in the mutant SP571 and *Hin*dIII (H) and *Eco*RI (E) sites. The numbers below various fragments indicate size in kb.

and these were subcloned, the 3.6-kb fragment of pUHR130 was used as the probe against these two subcloned 2.1-kb *Eco*RI fragments, designated as $\alpha$ and $\beta$ fragments (Fig. 1). Only the 2.1-kb $\alpha$ fragment hybridized with the probe (data not shown), suggesting that this fragment may contain the gene or genes that complement the mutation in MLC640. This 2.1-kb $\alpha$ fragment contained a 0.8-kb internal *Hin*dIII fragment which is the same as the 0.8-kb fragment that hybridized with pUHR130 (Fig. 2). The 2.1-kb $\alpha$ fragment was cloned into the broad-host-range vector pRK404A in both orientations and the resulting plasmids, pUHR144 and pUHR145, were transferred into MLC640. These transconjugants were used to inoculate beans. Neither of these two plasmids could complement MLC640 for nodulation and nitrogen

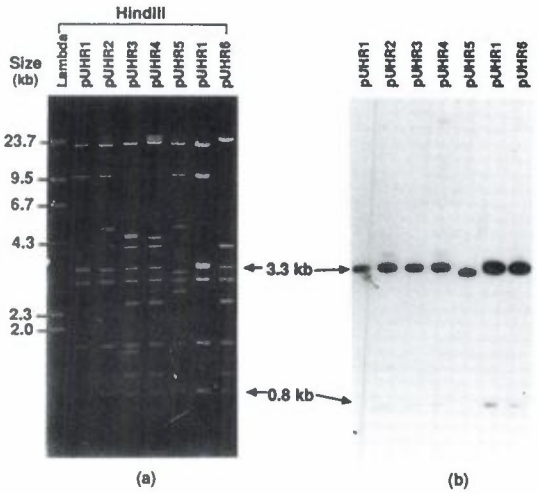


Figure 2. Hybridization of the 3.6-kb DNA fragment of TAL182 with cloned DNA of TAL1145 in cosmids pUHR1–pUHR6. The 3.2-kb fragment in pUHR5 is a part of the 3.3-kb fragment seen in other five cosmids (see also Fig. 1). The *Hin*dIII site at the right end of the cloned DNA in pUHR5 is from the cosmid vector pLAFR3.

fixation, suggesting that the 2.1-kb $\alpha$ fragment does not contain the entire sequence required to provide the missing functions of the gene(s) disrupted by the Tn*5* insertion in MLC640.

### ndvA *and* ndvB *genes are located in the cloned DNA in pUHR5*

Since the phenotype of the mutant MLC640 is similar to *ndvA* and *ndvB* mutants of *R. meliloti* (Dylan et al., 1986), we considered that the *ndvA* or *ndvB* gene may be interrupted by the Tn*5* insertion in MLC640 and that the plasmids pUHR1–pUHR6 that complement MLC640 may contain the *ndvA* and *ndvB* genes of TAL1145. In order to test this possibility, plasmids pUHR1–pUHR6 were probed with *ndvA* of *R. meliloti* (Dylan et al., 1986). The probe hybridized with a 4.7-kb *Eco*RI fragment of pUHR5 (Fig. 3). This fragment is located on the left end of the insert DNA in pUHR2 and pUHR5, and is not present in plasmids pUHR1, pUHR3, pUHR4 and pUHR6 (Fig. 1). The same 4.5-kb *Eco*RI fragment hybridized when the plasmid pNDV101 containing a part of *ndvB* of *R. fredii* in a 2.0-kb *Eco*RI fragment (Bhagwat et al., 1992) was used as the probe (data not shown). These results suggest that the gene(s) which is contained in the 2.1-kb $\alpha$ fragment of pUHR5, is located at least 15 kb apart from the *ndvA* and *ndvB* genes on the chromosome (Fig. 1).

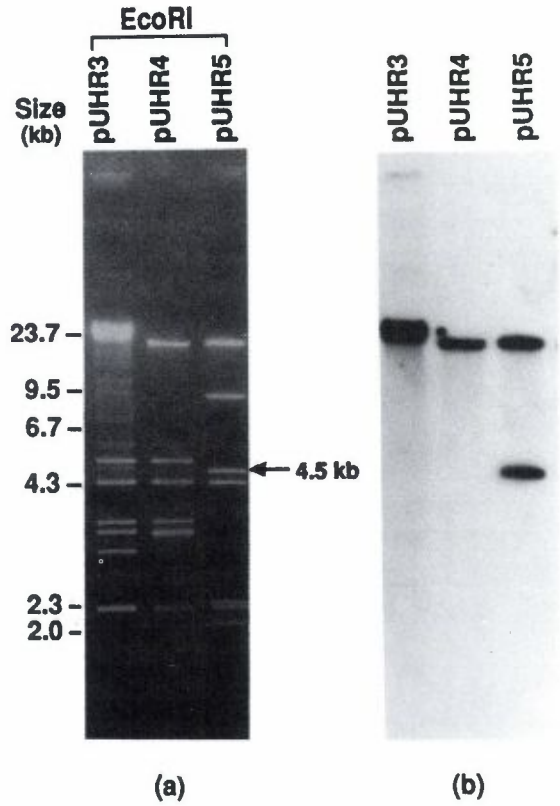


Figure 3. Hybridization of *ndvA* of *R. meliloti* with TAL1145 DNA cloned in pUHR5. Plasmid pDOB13 containing the entire *ndvA* gene in a 2.7-kb *Sal*I fragment was used as the probe. The same 4.5-kb *Eco*RI fragment of pUHR5 hybridized when the plasmid pNDV101 containing a part of *ndvB* of *R. fredii* in a 2.0-kb *Eco*RI fragment was used as the probe (data not shown). Cosmid pUHR2 also contains the same 4.5-kb *Eco*RI fragment (see Fig. 1).

*Site-directed Tn5 mutagenesis of pUHR5 and isolation of a TAL1145 mutant that cannot fix nitrogen in beans*

In order to determine if there are other genes in the 8.4-kb overlapping region of cosmids pUHR1–pUHR6, one cosmid, pUHR5, was mutagenized in *E. coli* with random Tn*5* insertions. These pUHR5::Tn*5* derivatives were transferred to *Rhizobium* strain TAL1145 and the Tn*5* insertions were marker-exchanged into the corresponding homologous positions in the chromosome. In this way 50 site-directed mutants of TAL1145 were isolated. These mutants were tested on both beans and leucaena for nodulation and nitrogen-fixing abilities. All

mutants formed normal nitrogen-fixing nodules in leucaena. However, one mutant SP571 was identified that failed to fix nitrogen in bean nodules as judged from the acetylene reduction assay and yellow color of the plants. The nodules made by SP571 were smaller than those made by TAL1145 and had a pale brown color compared to the pink brown color of the nodules formed by TAL1145. When a few of these nodules were dissected and observed under the microscope, no leghemoglobin was observed. From these nodules kanamycin resistant *Rhizobium* could be isolated.

Mutant SP571 could grow in *Rhizobium* minimal medium, indicating that this mutant does not contain an auxotrophic defect. This mutant was complemented by both pUHR3 and pUHR5, suggesting that the Tn*5* insertion may be located in any of the three *Eco*RI fragments, common to these plasmids. The *Eco*RI and *Hin*dIII fragments of the overlapping regions were subcloned in broad-host-range plasmid vector pRK404A and the resulting plasmids were transferred by conjugation to mutant SP571. These transconjugants were used to inoculate beans. Plasmids pUHR144 and pUHR145 containing the 2.1-kb *Eco*RI $\alpha$ fragment in either orientation enabled the mutant SP571 to form nitrogen-fixing nodules on beans. This 2.1-kb fragment contained the 0.8-kb *Hin*dIII fragment and a part of the 3.3-kb *Hin*dIII fragment that hybridized with the 3.6-kb DNA fragment of TAL182 (Fig. 2).

Plasmid pUHR130, containing the 3.6-kb *Eco*RI fragment of TAL182 which complements the mutant MLC640, was transferred to the mutant SP571 and the transconjugants were used to inoculate beans. These transconjugants formed nitrogen-fixing nodules on beans, suggesting that the 3.6-kb fragment from TAL182 contains the equivalent gene and therefore could complement the mutation in SP571.

From the results of subcloning and complementation experiments, it was expected that the Tn*5* in SP571 is located on the 2.1-kb *Eco*RI $\alpha$ fragment. When a 2.0-kb internal fragment of Tn*5* was used as the probe to the *Eco*RI-digested genomic DNA of SP571, the probe hybridized with a 7.8-kb *Eco*RI fragment (Tn*5* is 5.7 kb and does not contain an *Eco*RI site) suggesting that the Tn*5* in this mutant is located on a 2.1-kb *Eco*RI fragment. The same blot was used for hybridization with the 2.1-kb *Eco*RI $\alpha$ fragment. As expected, the 2.1-kb *Eco*RI fragment of TAL1145 was replaced by a 7.8-kb *Eco*RI fragment in the mutant SP571 (data not shown). Since the mutant SP571 was developed by marker exchange of the Tn*5* from the plasmid pUHR5::Tn*5*-571, the position of Tn*5* in SP571 can be determined from the position of Tn*5* in this plasmid also. When the *Hin*dIII-digested DNA of plasmids pUHR5 and pUHR5::Tn*5*-571 was separated on a 1% agarose gel and compared, the 0.8-kb *Hin*dIII fragment of pUHR5 was replaced by three *Hin*dIII fragments of 3.4 kb, 1.9 kb

and 1.3 kb in pUHR5::Tn*5*-571 (data not shown). The 3.4-kb band is the internal *Hin*dIII fragment of Tn*5*; both the 1.9-kb and the 1.3-kb fragments contain a 1.2-kb region of DNA from the two inverted repeats of Tn*5*. Based on the sizes of these fragments, the position of the Tn*5* was located on the 0.8-kb *Hin*dIII fragment inside the 2.1-kb *Eco*RI $\alpha$ fragment. The 7.8-kb *Eco*RI fragment containing the Tn*5* on the 2.1-kb *Eco*RI $\alpha$ fragment was subcloned in pUC18 from pUHR5::Tn*5*-571 and the position of the Tn*5* was determined as shown in Fig. 1.

## 4. Discussion

We have identified a gene(s) in the *Rhizobium* sp. strain TAL1145 required for nitrogen fixation in beans but not in leucaena. The Tn*5* insertion mutant SP571 forms non-fixing nodules on beans that are smaller than the nitrogen-fixing nodules formed by the wild-type strain TAL1145. Kanamycin resistant rhizobia could be isolated from the bean nodules formed by SP571, suggesting that it was blocked at a later stage of nodule development. The phenotype of mutant SP571 resembles those of *ndvA* and *ndvB* mutants of *R. meliloti* (Dylan et al., 1986), which are unable to synthesize cyclic $\beta$-1,2-glucan but produce normal amounts of exopolysaccharide and form small empty nodules.

Host-specific symbiotic defects of several *Rhizobium* mutations have been described previously. Borthakur et al. (1986) isolated *pss* mutants of *R. leguminosarum* bv. viciae that prevented nodulation of peas, a host that forms indeterminate nodules. The same *pss* mutations in *R. leguminosarum* bv. phaseoli did not affect the nodulation and nitrogen-fixing abilities on beans, a host that forms determinate nodules. In contrast, several EPS-defective mutants of *R. loti* PN184 isolated by Hotter and Scott (1991) formed effective nodules on *Lotus pedunculatus*, a determinate nodulating host, but small ineffective nodules on leucaena, an indeterminate nodulating host. In broad-host-range *Rhizobium* NGR234, Djordjevic et al. (1987) described several chromosomal mutant which had different symbiotic phenotypes on different legume hosts. One such mutant, AN2840, formed normal nitrogen-fixing nodules on siratro but formed only non-fixing callus on leucaena. Like ANU2840, mutant SP571 in the present study has lost the ability to establish effective symbiosis with one of its symbiotic hosts; however, SP571, unlike ANU2840 or the *pss* mutants, did not have any visible defect in EPS synthesis.

Several other classes of chromosomal mutants in different *Rhizobium* have been reported to have Fix$^-$ phenotypes. These include some *exo* mutants of *R. meliloti* (Leigh et al., 1985; Finan et al., 1985), EPS-defective mutants of *R. leguminosarum* bv. phaseoli (Noel et al., 1984; Vandenbosch et al., 1985)

and LPS-defective mutants of *R. leguminosarum* bv. viciae (Priefer, 1989) and *R. leguminosarum* bv. phaseoli (Noel et al., 1986). Mutant SP571 in the present study is indistinguishable from TAL1145 in colony morphology and fluorescence on TY agar containing a fluorescent dye, Calcofluor. SP571 did not autogglutinate like the LPS-defective mutant of *R. leguminosarum* bv. viciae (Priefer, 1989). The effect of the mutation in SP571 on its motility could not be determined since the parental strain TAL1145 is also nonmotile on 0.3% agar (George et al., 1994). We have not determined if SP571 produces cyclic $\beta$-1,2-glucan.

The mutated gene in SP571 is linked to *ndvA* and *ndvB* genes on the chromosome. The *ndvA* and *ndvB* genes of *R. meliloti* are homologous to *chvA* and *chvB* genes of *Agrobacterium tumefaciens* and are located on the chromosome (Dylan et al., 1986). Cloned DNA in pUHR5 did not hybridize with any of the three indigenous plasmids of TAL1145 in Southern hybridization, suggesting that it is a part of the chromosome (S. Pooyan, unpublished). The 3.6 kb *Eco*RI fragment of *R. etli* strain TAL182 that hybridizes with pUHR5 is also a part of the chromosome (Pooyan et al., 1994).

The 2.1 kb *Eco*RI $\alpha$ fragment that complemented SP571 did not complement the *R. etli* mutant MLC640. Moreover, plasmid pUHR5::Tn*5*-571, which was used to develop mutant SP571 by marker exchange, complemented mutant MLC640. This suggests that the genes interrupted by Tn*5* insertions in SP571 and MLC640 are different. This is also apparent from the fact that the mutants have different nodulation phenotypes; while MLC640 formed only swellings on beans, SP571 formed small non-fixing nodules on beans.

The nodules of beans and leucaena are structurally different. The bean nodules are determinate with spherical meristems, whereas leucaena nodules are indeterminate and elongated with apical nodule meristem. Brewin (1991) suggested that the symbiosis phenotypes of LPS-defective *Rhizobium* mutants differ, depending on whether the host plant gives rise to nodules with determinate or indeterminate morphology. LPS is involved in endocytosis (bacterial release) and is more important in determinate nodules in which endocytosis of rhizobia occurs at an early stage. LPS-defective mutants lacking O-antigen form empty nodules on determinate nodulating species *Phaseolus* and *Glycine*, (Puvanesarajah et al., 1987), but show impaired bacteroid release in peas (Priefer, 1989) and no observed phenotype in alfalfa (Clover et al., 1989), both of which produce indeterminate nodules. It is possible that the 2.1 kb $\alpha$ fragment in the present study contains a gene whose product is required for endocytosis of rhizobia or some other functions essential in determinate nodules.

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## REFERENCES

Beringer, J.E. 1974. R factor transfer in *Rhizobium leguminosarum*. *J. Gen. Microbiol.* **84:** 188–198.

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

Beringer, J.E., Beynon, J.L., Buchanan-Wollaston, A.V., and Johnston, A.W.B. 1978. Transfer of the drug-resistance transposon Tn*5* to *Rhizobium*. *Nature* **276:** 633–634.

Bhagwat, A.A., Tully, R.A., and Keister, D.L. 1992. Isolation and characterization of an *ndvB* locus from *Rhizobium fredii*. *Mol. Microbiol.* **6:** 2159–2165.

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

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

Brewin, N.J. 1991. Development of the legume root nodule. *Annu. Rev. Cell Biol.* **7:** 191–226.

Charles, T.C., Newcomb, W., and Finan, T.M. 1991. *ndvF*, a novel locus located on megaplasmid pRmeSU47b (pEXO) of *Rhizobium meliloti*, is required for normal nodule development. *J. Bacteriol.* **173:** 3981–3992.

Chen, H., Batley, M., Remond, J., and 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.

Clover, R.H., Kieber, J., and Signer, E.R. 1989. Lipopolysaccharide mutants of *Rhizobium meliloti* are not defective in symbiosis. *J. Bacteriol.* **171:** 3961–3967.

Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, X-W, Finlay, D.R., and 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.

Djordjevic, S.P., Chen, H., Batley, M., Redmond, J.W., and Rolfe, B.G. 1987. Nitrogen-fixing ability of exopolysaccharide synthesis mutants of *Rhizobium* sp. strains NGR234 and *Rhizobium trifolii* by the addition of homologous exopolysaccharide. *J. Bacteriol.* **169:** 53–60.

Doherty, D., Leigh, J.A., Glazebrook, J., and Walker, G.C. 1988. *Rhizobium meliloti* mutants that overproduce the *R. meliloti* acidic Calcofluor-binding exopolysaccharide. *J. Bacteriol.* **170:** 4249–4256.

Dylan, T., Ielpi, L., Stanfield, S., Kashyap, L., Douglas, C., Yanofsky, M., Nester, E., Helinski, D.R., and Ditta, G. 1986. *Rhizobium meliloti* genes required for nodule development are related to chromosomal virulence genes in *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. USA* **83:** 4403–4407.

Eckhardt, T. 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. *Plasmid* **1:** 584–588.

Feinberg, A.P. and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Annals Biochem.* **132:** 6–13.

Finan, T.M., Hirsch, A.M., Leigh, J.A., Johansen, E., Kuldau, G.A., Deegan, S., Walker, G.C., and 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., and Borthakur, D. 1992. Genetic analysis of *Rhizobium leguminosarum* bv. phaseoli mutants defective in nodulation and nodulation suppression. *Appl. Environ. Microbiol.* **58:** 1050–1053.

George, M.L.C., Young, J.W.P., and Borthakur, D. 1994. Genetic characterization of *Rhizobium* sp. strain TAL1145 that nodulates tree legumes. *Can. J. Microbiol.* **40:** 208–215.

Hardy, R.W.F., Holsten, R.D., Jackson, E.K., and Burns, R.C. 1968. The $C_2H_2$ assay for nitrogen fixation: laboratory and field evaluation. *Plant Physiol.* **43:** 1185–1207.

Hirsch, P.P., van Montagu, M., Johnston, A.W.B., Brewin, N.J., and Schell, J. 1980. Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of *Rhizobium leguminosarum*. *J. Gen. Microbiol.* **120:** 403–412.

Hotter, G.S. and Scott, D.B. 1991. Exopolysaccharide mutants of *Rhizobium loti* are fully effective on a determinate nodulating host but are ineffective on an indeterminate nodulating host. *J. Bacteriol.* **173:** 851–859.

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

Leigh, J.A., Signer, E.R., and Walker, G.C. 1985. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. *Proc. Natl. Acad. Sci. USA* **82:** 6231–6235.

Leong, S.A., Ditta, G.S., and Helinski, D.R. 1982. Heme biosynthesis in *Rhizobium*. Identification of cloned gene coding for $\alpha$-aminolevulinic acid synthetase from *Rhizobium meliloti*. *J. Biol. Chem.* **257:** 8724–8730.

Merrick, M., Filser, M., Kennedy, C., and Dixon, R. 1978. Polarity of mutations induced by insertion of transposons Tn*5*, Tn*7* and Tn*10* into the *nif* gene cluster of *Klebsiella pneumoniae*. *Mol. Gen. Genet.* **165:** 103–111.

Miller, J.H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, New York, pp. 6609–6613.

Moawad, H. and Bohlool, B.B. 1984. Competition among *Rhizobium* spp. for nodulation of *Leucaena leucocephala* in two tropical soils. *Appl. Environ. Microbiol.* **48:** 5–9.

Noel, K.D., Vandenbosch, K., and Kulpaca, B. 1986. Mutations in *Rhizobium phaseoli* that lead to arrested development of infection threads. *J. Bacteriol.* **168:** 1392–1401.

Pinero, D., Martinez, E., and Selander, R.K. 1988. Genetic diversity and relationships among isolates of *Rhizobium leguminosarum* biovar phaseoli. *Appl. Environ. Microbiol.* **54:** 2825–2832.

Pooyan, S., George, M.L.C., and Borthakur, D. 1994. Characterization of a *Rhizobium etli* chromosomal gene required for nodule development on *Phaseolus vulgaris* L. *World J. Microbiol. Biotechnol.* **10:** 583–589.

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

Puvanesarajah, V, Schell, F.M., Gerhold, D., and Stacey, G. 1987. Cell surface polysaccharides from *Bradyrhizobium japonicum* and a nonnodulating mutant. *J. Bacteriol.* **169:** 137–141.

Reed, K.C. and Mann, D.A. 1985. Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Res.* **13:** 7207–7221.

Ruvkun, G.B. and Ausubel, F.M. 1981. A general method for site-directed mutagenesis in prokaryotes. *Nature* **289:** 85–88.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Somasegaran, P. and Hoben, H.J. 1985. Methods in legume *Rhizobium* technology. NifTAL Project, University of Hawaii.

Somasegaran, P. and Martin, R.B. 1986. Symbiotic characteristics and *Rhizobium* requirements of a *Leucaena leucocephala X Leucaena diversifolia* hybrid and its parental genotypes. *Appl. Environ. Microbiol.* **52:** 1422–1424.

Stanfield, S.W., Ielpi, L., O'Brochta, D., Helinski, D.R., and Ditta, G.S. 1988. The *ndvA* gene product of *Rhizobium meliloti* is required for $\beta$-(1 $\rightarrow$ 2) glucan production and has homology to the ATP-binding export protein HlyB. *J. Bacteriol.* **170:** 3523–3530.

Staskawicz, B., Dahlbeck, D., Keen, N.T., and Napoli, C. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. glycinea. *J. Bacteriol.* **169:** 5789–5794.

Turk, D. and Keyser, H.H. 1992. Rhizobia that nodulate tree legumes: specificity of the host for nodulation and effectiveness. *Can. J. Microbiol.* **38:** 451–460.

Vandenbosch, K., Noel, K.D., Kaneko, Y., and Newcomb, E.H. 1985. Nodule initiation elicited by noninfective mutants of *Rhizobium phaseoli. J. Bacteriol.* **162:** 950–959.