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NOTES

Isolation of Competition-Defective Mutants of *Rhizobium fredii*†

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We coupled Tn*5* mutagenesis with a competition assay to isolate mutants of *Rhizobium fredii* USDA 257 that are defective in competition for nodulation of soybeans. Two mutants with single Tn*5* inserts in the chromosome showed reduced competitiveness in vermiculite but were identical to the wild-type strain in symbiotic properties when inoculated alone. Recombination of Tn*5* and flanking genomic regions cloned from the mutants into the parent strain showed that Tn*5* was responsible for the mutant phenotype.

Competition between *Rhizobium* strains for nodulation of the legume host plant is of major agronomic importance, as it may determine whether or not nodules are occupied by an effective strain. This competition is highlighted in soils of the midwestern United States, where inoculation with strains of *Bradyrhizobium japonicum* efficient at nitrogen fixation rarely results in increased soybean yields, owing to the inability of the introduced strains to compete with the resident population (7, 13).

When a *Rhizobium* inoculum is added to soil, it is exposed to a complex environment. The mechanisms that confer saprophytic competence and competitiveness on a *Rhizobium* strain in such an environment are poorly understood (12, 18, 20, 23, 30). In this study we used a competition assay to isolate Tn*5*-induced mutants of *Rhizobium fredii* whose sole known defect is reduced competitiveness. Characterization of these mutants may provide a clue to the basis of the competitiveness of parent strain USDA 257 (19).

Strain EA213 is a Fix$^-$ mutant of strain 191 *str-1* (2) with Tn*5* in *nifD* (1); strain 257 *spc-2* is a spontaneous mutant of strain USDA 257 resistant to 250 μg of spectinomycin per ml (19). The growth media were tryptone-yeast extract broth (5) and tryptone-yeast extract plus 1.5% agar for *R. fredii* and L broth (21) for *Escherichia coli*. Plasmid pKS4 (22), containing an internal *Hin*dIII-*Bam*HI fragment of Tn*5*, was from D. Sutton, Agrigenetics Advanced Science Co., Madison, Wis. Plasmid pSUP202 and strain SM10(pSUP1011) were from R. Simon, Universität Bielefeld, Bielefeld, Federal Republic of Germany (28).

Transposon Tn*5* (4) was introduced into *R. fredii* 257 *spc-2* by using SM10(pSUP1011) as described by Simon et al. (28). Transconjugants were selected on tryptone-yeast extract agar containing spectinomycin and kanamycin (50 μg/ml). After single-colony purification, 600 randomly chosen isolates were tested for their competitive ability (Fig. 1).

R. fredii USDA 257 and USDA 191 are Fix$^+$ on soybean cultivar Peking (15). Strains 257 *spc-2* and EA213 were of equal competitiveness in vermiculite (Table 1). When inoculated at a 1:1 ratio, approximately 50% of the nodules were formed by each strain, and the plants were green. At a 10:1 ratio in favor of EA213, approximately 90% of the nodules were formed by EA213, and the resulting plants were yellow and stunted due to nitrogen deficiency. This color assessment was used to isolate competition-deficient mutants (Fig. 1).

Soybean seedlings (*Glycine max* cv. Peking) were prepared for nodulation as described previously (2). Competition studies were performed in a modified Leonard jar assembly (E. Appelbaum, personal communication) containing vermiculite and a nitrogen-free nutrient solution (9). Inoculation was done by mixing strains 257 *spc-2*::Tn*5* and EA213 at a 10:1 ratio in favor of the Tn*5* mutant in sterile distilled water and applying the suspension immediately to the plants, to yield approximately 10^8 cells per pot. The plants were placed in a growth chamber for 35 days at 22 and 20°C (day and night temperatures, respectively) and watered as required with a nitrogen-free solution (9). The plants were examined visually for signs of nitrogen starvation. When the

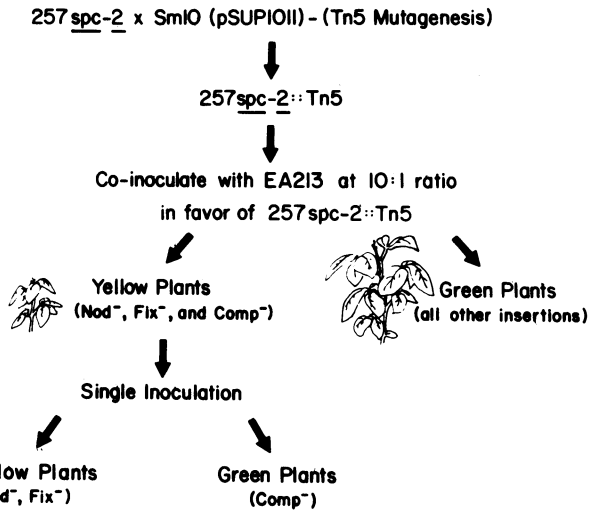

FIG. 1. Competition assays were done by coinoculation with a strain ratio of approximately 10:1 (USDA 257 *spc-2*::Tn*5* to EA213). Plant color was scored after approximately 4 weeks of growth. Yellow plants resulted when the Fix$^-$ strain EA213 formed the majority of the nodules because of the inability of the 257 *spc-2*::Tn*5* derivative to compete for nodulation. Yellow plants also arose when the 257 *spc-2* derivative was itself defective in nodulation or nitrogen fixation. When inoculated alone, Comp$^-$ mutants produced green plants, while Nod$^-$ and Fix$^-$ mutants produced yellow plants.

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TABLE 1. Competition studies with Comp$^-$ mutants and transconjugants

Strains[a]		Input ratio (strain A/strain B)	Nodules formed (%) by strain(s)[b]:			Plant color	Significance[c]
A	B		A	B	A + B		
257 *spc-2*	EA213	1:1	54	45	2	Green	NS
		10:1	93	7	0	Green	NS
		1:10	14	86	0	Yellow	NS
TML90	EA213	1:1	8	91	1	Yellow	***
		10:1	37	62	1	Yellow	***
		1:10	0	100	0	Yellow	***
TML90-11	EA213	1:1	6	86	8	Yellow	***
		10:1	25	70	5	Yellow	***
TML54	EA213	1:1	0	100	0	Yellow	***
		10:1	0	96	4	Yellow	***
		1:10	0	100	0	Yellow	***
TML54-15	EA213	1:1	0	100	0	Yellow	***
		10:1	0	96	4	Yellow	***
TML125	EA213	1:1	5	95	0	Yellow	***
		10:1	13	87	0	Yellow	***
		1:10	0	100	0	Yellow	***
TML41	EA213	1:1	0	100	0	Yellow	***
		10:1	38	56	6	Yellow	***
		1:10	2	98	0	Yellow	***

[a] TML90-11 and TML54-15 are recombinants in which the Tn*5*-bearing fragment from the corresponding mutants has replaced the wild-type fragment in 257 *spc-2*.

[b] Nodule occupancy was determined for 80 nodules from five plants for each treatment.

[c] A chi-square analysis was used to test the deviation of the results from the expected ratio (1:1, 10:1, and 1:10) for the single-strain/single-strain nodules (df = 1), NS, Not significant; ***, $P \geq 0.005$.

Tn*5*-harboring strain was competitive with EA213, it formed the majority of the nodules and a green plant resulted. When a competition-defective mutant resulted from Tn*5* insertion, EA213 formed the majority of the nodules and the plant appeared yellow. Yellow plants also arose when the Tn*5* mutant was symbiotically defective.

Nodule occupancy was determined after surface-sterilized nodules were crushed (19) in sterile phosphate-buffered saline and the nodule contents were transferred to tryptone-yeast extract agar with the appropriate antibiotics. Strain EA213 was sensitive to spectinomycin and resistant to high levels of streptomycin (1,000 μg/ml). The 257 *spc-2* derivatives were resistant to spectinomycin and sensitive to high levels of streptomycin.

Seven transconjugants were isolated that gave a yellow plant in competition with EA213. Serotyping and analysis of plasmid content confirmed that these transconjugants were derivatives of strain 257 *spc-2*. Three of the seven strains were found to be symbiotically defective (Fix$^-$) when inoculated alone, while four were Fix$^+$. When coinoculated with EA213 at a 10:1 ratio in their favor, the four Fix$^+$ mutants (TML41, TML54, TML90, and TML125) produced yellow plants and occupied a significantly lower percentage of nodules than did the wild-type strain 257 *spc-2* (Table 1). Strains TML90 and TML54 were also competition defective against parent strain 257 *spc-2*, occupying less than 10% of the nodules when inoculated at a 1:1 ratio.

When these four mutants were inoculated alone, no significant differences were detected between them and the wild-type strain in nodule mass, nodule numbers, acetylene reduction activity (14), or plant dry weight of 10 replicates after 35 days of growth. Other characteristics including motility, growth on minimal medium [containing 0.2% succinate, 0.1% $(NH_4)_2SO_4$, and 0.1% monosodium glutamate; 6], and number of days to nodule formation were also measured. No significant differences were detected between the mutants and the wild-type strain, except in the case of TML125, which was delayed in nodulation by 2 days. Growth rates in the rhizosphere, measured as described by Brewin et al. (8), were not significantly different between strains TML90 and TML89 (257 *spc-2*::Tn*5*, normal compet-

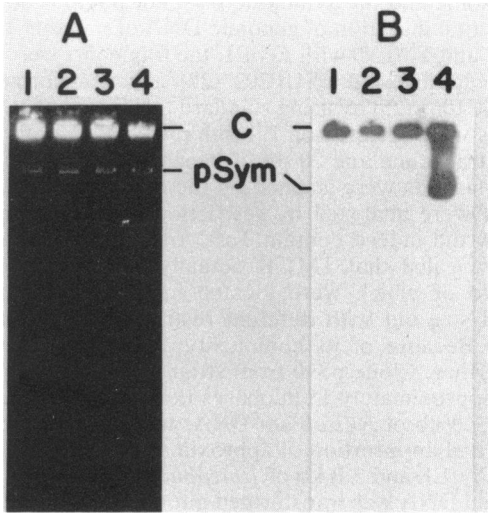

FIG. 2. Plasmid profile (A) and hybridization analysis (B) of mutants. The hybridization probe was pKS4 (22). Lanes: 1, TML125; 2, TML90; 3, TML54; 4, TML41. C, Chromosome.

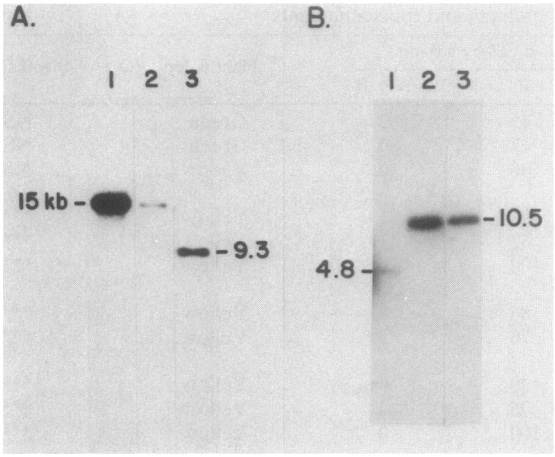

FIG. 3. (A) Southern blot hybridization analysis of transconjugants from mating of S17-1(pS90) with 257 *spc-2* with pS90 as a probe. Lanes: 1, TML90; 2, TML90-11; and 3, 257 *spc-2*. Transconjugant TML90-11 was identical to TML90, indicating that a double homologous recombination event occurred. (B) Southern blot hybridization of transconjugants from mating of S17-1(pS54) with 257 *spc-2* with pS54 as a probe. Lanes: 1, 257 *spc-2*; 2, TML54-15; 3, TmL54. Transconjugant TML54-15 was identical to TML54.

itiveness) when each was mixed with EA213 at 1:1, 10:1, and 1:10 input levels (data not shown). Thus, three mutants defective in competiton for nodulation of soybeans (cv. Peking) were isolated.

Total genomic DNA from the mutants was prepared by the method of Scott et al. (24). Southern blot analysis (29) of *Eco*RI-digested genomic DNA with the Tn*5*-specific probe pKS4 (22) conducted under conditions described by Appelbaum et al. (2) revealed that TML90 and TML54 had a single Tn*5* insertion, while TML41 showed two hydridizing fragments (data not shown). Judging from the size of the restriction fragments into which Tn*5* was inserted, we concluded that each mutation was distinct. Tn*5* was found to be in a chromosomal location in mutants TML90 and TML54 after Eckhardt gel electrophoresis (11, 26) and hybridization with pKS4 (Fig. 2) . TML41 had Tn*5* sequences in both the chromosome and the symbiotic plasmid, pSym (Fig. 2).

After total digestion of genomic DNA of mutants TML41, TML54, and TML90 with *Eco*RI, the fragments were ligated with *Eco*RI-digested pSUP202 (27) and transformed into strain MC1061 (25) by using standard techniques (17). Transformants were selected on L broth plates (21) containing 10 μg of tetracycline and 20 μg of kanamycin per ml. Recombinant plasmids were isolated by the method of Klein et al. (16) and were analyzed by restriction mapping to confirm that they did indeed contain Tn*5*. Analysis of the resulting clones revealed that TML41 actually had three copies of Tn*5*, two of which were located in fragments of nearly identical size but with different restriction maps (data not shown). Because of its complexity, TML41 was not analyzed further. Clone pS90 from strain TML90 had an insertion of approximately 15 kilobases (kb) with Tn*5* flanked by 1.5 and 7.8 kb of *Rhizobium* DNA, while pS54 from strain TML54 had an insertion of approximately 10.5 kb with Tn*5* flanked by 1.8 and 3.0 kb of *Rhizobium* DNA.

Plasmid DNA was transformed into *E. coli* S17-1 (27) with selection for Kan^r Tet^r colonies. To prove that Tn*5* was responsible for the mutant phenotype, pS90 and pS54 were transferred back into strain 257 *spc-2* from S17-1, with selection for transconjugants that were resistant to spectinomycin and kanamycin on minimal medium (6). To identify transconjugants in which Tn*5* had recombined into the 257 *spc-2* genome as the result of a double homologous recombination event, tetracycline-sensitive transconjugants were identified. Total genomic DNA was isolated from these strains and subjected to Southern hybridization analysis (29) (Fig. 3). The majority of the tetracycline-sensitive transconjugants (≈95%) had suffered transposition of Tn*5* to another location in the genome. However, from each mating a single recombinant with the desired genotype was obtained (TML54-15 and TML90-11). These recombinants containing Tn*5* in the desired location were also competition defective, thus proving that Tn*5* was responsible for the mutant phenotype in TML54 and TML90 (Table 1).

Strain USDA 257 has a single plasmid (pSym) that contains both *nod* and *nif* genes (2). The two Comp$^-$ mutants with single Tn*5* insertions had Tn*5* in the chromosome rather than in this plasmid (Fig. 2), suggesting that the genes involved may be distinct from *nod* and *nif*. It was not determined which Tn*5* insertion was responsible for the mutant phenotype of TML41.

The screening procedure described above allowed the isolation competition-defective mutants on the basis of plant color. Yellow plants obtained in the first level of this assay could result from a mutation in a number of different genes (e.g., *nif*, *nod*, *fix*, or competition genes). When inoculated alone, these Fix$^-$ and Nod$^-$ mutants produced yellow plants, whereas Comp$^-$ mutants produced green plants. In this assay, competition-defective and symbiotic mutants were screened simultaneously. Indeed, three mutants defective in nitrogen fixation were also identified during this study. In addtion, strain TML125 was found to be delayed in nodulation.

To isolate Comp$^-$ mutants in *Rhizobium* species, a simple, rapid assay is essential. It would be possible to isolate such mutants by using mixed inocula and nodule typing, but this procedure would be tedious. Preliminary reports of other plant assays that could be adapted to allow the isolation of competition-defective mutants have been published (3, 10).

With this screening assay, we have at our disposal for the first time a method of isolating competition mutants in *Rhizobium* species and three mutants whose sole known defect is reduced competitiveness. By characterizing the genes that are involved in competition, we may soon understand why one strain is more competitive than another in forming nodules. We are presently involved in cloning the wild-type genes correspondng to strains TML54 and TML90 and inserting them into noncompetitive strains in the hope of increasing the competitive ability of more efficient nitrogen-fixing strains.

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