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Note

Transposon Tn*5* Mutagenesis of *Bradyrhizobium japonicum*: A Histidine Auxotrophic Mutant of *B. japonicum* Shows Defective Nodulation Phenotype on Soybean

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Transposon Tn*5* was used to induce random insertional mutations in *Bradyrhizobium japonicum*, a soybean endosymbiont. By genomic Southern blot analysis, transposition events were found to have occurred randomly throughout the *B. japonicum* genome. After screening 3,626 mutants by auxotrophy test, a histidine auxotroph was isolated. Upon plant infection test, the His^- mutant showed a 3~4 day delay in nodule formation.

Bradyrhizobium japonicum is a Gram-negative soil bacterium which induces root nodules in soybean. Nodule development is a complex and stepwise process that requires intimate cell-to-cell interactions between infecting bacteria and the host plant. A number of bacterial genes essential for the development of the symbiosis have been identified to consist of *nif*, *nod* and *fix* genes. *Nif* genes are responsible for the construction of nitrogenase and *fix* genes are essential for nitrogen fixation. Nod genes are those involved in the formation of nodules. *B. japonicum nif* and *fix* genes have been studied in some detail (4, 6) and recently *nod* genes have also been studied (14, 15).

However, little is known about the effects of general metabolic mutations on the symbiotic functions of the *B. japonicum*. One reason for this is that several mutagenesis procedures have failed to induce a range of defined mutations including metabolic and symbiotic deficiencies. Several tryptophane requiring mutants of *B. japonicum* have been isolated following nitrous acid-mutagenesis and were reported to be non-infective (17). More recently, symbiotically defective histidine auxotrophs have also been reported (10). Characterization of a variety of metabolic mutants may allow a dissection of the role that general metabolism plays in symbiotic functions. In this study, we report the random transposon mutagenesis and subsequent isolation of a histidine auxotrophic mutant of *B. japonicum*. The mutant showed a defective nodulation phenotype.

Bacterial Growth and DNA Manipulations

Bacterial strains and plasmids used are listed in Table 1. *B. japonicum* was grown at 30°C on YEM medium (16). For auxotrophic tests *B. japonicum* was grown in Bishop's minimal medium (1). *Escherichia coli* strains were grown at 37°C in LB medium (2). Antibiotics were added to the medium to the following final concentrations: *B. japonicum*, 100 μg/ml kanamycin and 200 μg/ml streptomycin: *E. coli*, 50 μg/ml ampicillin, 15 μg/ml tetracycline, 30 μg/ml chloramphenicol and 50 μg/ml kanamycin.

Total genomic DNA of *B. japonicum* was obtained by the method described previously (14). Plasmid DNA isolation, restriction enzyme digestion of DNA samples, agarose gel electrophoresis, preparation of radiolabeled DNA and Southern blot hybridization were carried out essentially as described by Sambrook et al. (11).

Random Tn*5* Mutagenesis

Introduction of Tn*5* into *B. japonicum* was accomplished by conjugative transfer of the plasmid pSUP1011 (13) which can be mobilized from *E. coli* to a *B. japonicum* recipient with the help of the *tra functions* provided *in trans* by the helper plasmid, pRK2073 (7). In this study, mobilization was achieved using the triparental mating system of Ditta et al. (3), where the *E. coli* donor strain was HB101 and transfer functions were provided by pRK2073 (Fig. 1). The *E. coli* donor strain was grown to a density of 10^9 cells/ml in LB and the *B. japonicum* recipient strain to a density of 10^8 cells/ml in YEM. *B. japonicum* cells were concentrated to a density of 10^9 cells/ml by centrifugation. Equal volumes (0.1 ml) of donor and recipient cells were mixed and suspensions were spread onto YEM agar. The plates were dried and incubated at 30°C for 4 days. Bacteria from the plates were then suspended in 4 ml YEM broth

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containing 0.01% Tween 80. Suspensions were vortexed until the cell aggregates were removed and 0.1 ml aliquots were spread onto YEM agar. Streptomycin and kanamycin were added to select *B. japonicum* transconjugants containing Tn5. Tn5 encodes resistance to kanamycin and streptomycin in various species of *Rhizobium* (12) and this is also the case with *Bradyrhizobium*. 30 μg/ml of chloramphenicol was added to counterselect against *E. coli* donor and helper strains because at this concentration, *B. japonicum* is naturally resistant to chloramphenicol. In order to determine the frequency at which kanamycin and streptomycin (Km/Sm)-resistant *rhizobia* arise, a portion of the mating mixtures was serially diluted in YEM, plated onto YEM agar containing chloramphenicol and incubated at 30°C for 8~10 days. Colonies were counted, and the putative transposition frequency of Tn5 was determined in terms of the number of Km/Sm-resistant cells per recipient.

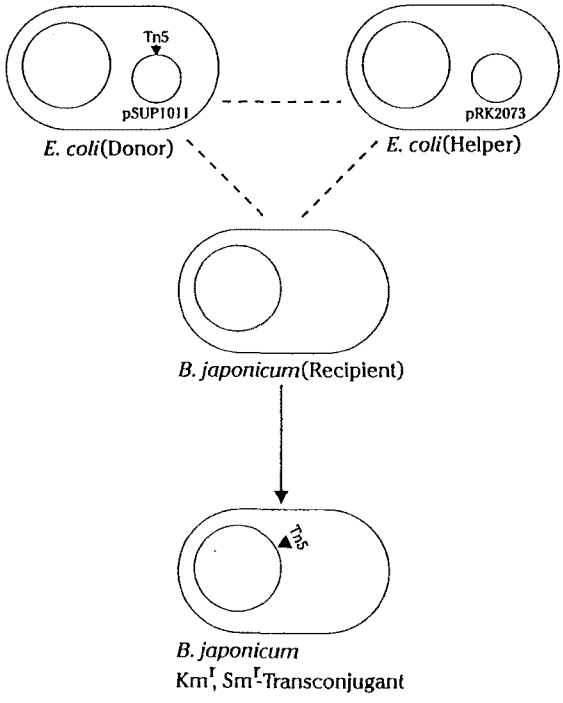

Fig 1. Random insertional mutagenesis scheme
Tn5 insertions are symbolized by arrowheads. Large circles represent the chromosome of bacteria. Small circles are plasmids. Tn5 carried on the suicide plasmid pSUP1011 was mobilized into the recipient *B. japonicum* with help of *tra* functions provided from a helper plasmid pRK3073. In the *B. japonicum* background, the suicide plasmid pSUP 1011 is degraded because it cannot replicate. At the same frequency, Tn5 on the introduced plasmid hops onto the chromosome of *B. japonicum* resulting in an insertion mutation selectable by resistance to kanamycin and streptomycin. The resulting mutants were tested individually to determine their auxotrophy.

The frequency at which Km/Sm-resistant *B. japonicum* transconjugants were obtained after conjugation with pSUP1011 in the presence of helper plasmid pRK2073 was 4.8×10^{-7}. The frequency of spontaneous kanamycin and streptomycin resistance in *B. japonicum* was less than 10^{-10}. The transposon Tn5 has been used to generate mutants in a number of *Bradyrhizobium* species (8, 10) and the resulting mutants were used to clone the DNA sequences essential for nodulation (14, 15). Since Tn5 encodes resistance to both kanamycin and streptomycin in *Bradyrhizobium*, a double selection for resistance to these antibiotics can be used as an indicator for Tn5 transposition. Southern analysis of the isolated Km/Sm-resistant *B. japonicum* transconjugants indicated the presence of Tn5 within their genome. The frequency of Km/Sm-resistant colonies per recipient (ca. 10^{-7}) is similar to the values obtained in other studies (8).

Verification that the Km/Sm-resistant *B. japonicum* isolates contained Tn5 within their genome was obtained by the genomic Southern blot analysis with nick translated Tn5 probe (i.e., pSUP1011). The results obtained with 8 Km/Sm-resistant transconjugants chosen randomly confirmed the presence of Tn5 (Fig. 2). Restriction endonuclease *Bgl*II cleaves Tn5 at two positions (one in each of its inverted repeat sequences) which are separated by a distance of approximately 2.7 kb (5). Thus, any *B. japonicum* transconjugant containing Tn5 should have a 2.7 kb fragment that would hybridize to the Tn5 probe. In addition, insertion of Tn5 into the genome of *B. japonicum* should result in the presence of two additional junction fragments showing homology to both the right and left ends of Tn5.

Auxotrophy and Plant Infection Test.

Auxotrophy tests were performed as described by Davis et al. (2). Soybean seeds were surface sterilized and germinated as described (9). Nodulation and ace-

Table 1. Bacterial strains and plasmids used.

Strains & plasmids	Relevant characteristics	Reference
USDA110	Wild-type	(14)
JS721	His$^-$ Nod$^-$ Tn5-mutant	This study
HB101	F$^-$ *hsdR hsdM recA13 thi-1 leuB6 proA2 lacZ4 supE44 tonA21 Str^r*	(2)
pRK2073	tra$^+$(RK2:ColEI)	(7)
pSUP1011	Km^r Nm^r (by Tn5) Cm^r *ori*T(RP4)	(13)

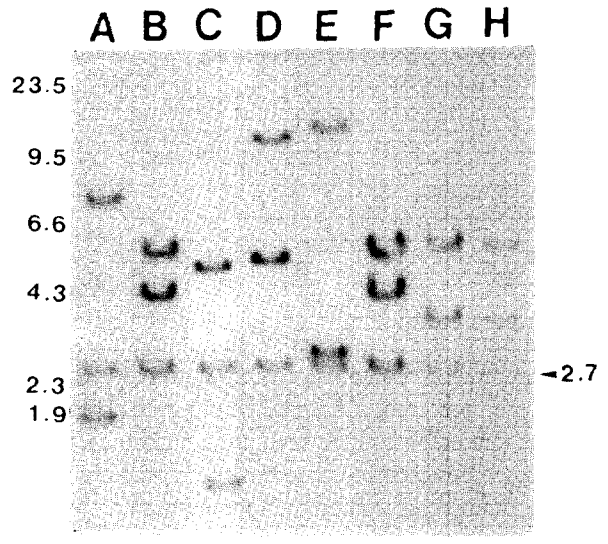

Fig 2. Genomic Southern blot analysis of randomly chosen Tn5 mutants

Southern hybridization analysis of *Bgl*II digested genomic DNA from 8 Km/Sm-resistant transconjugants. DNAs were digested with *Bgl*II, Southern blotted onto a nitrocellulose filter and probed with the nick translated pSUP1011 DNA. Size markers on left are in kb. The 2.7 kb internal *Bgl*II fragment of Tn5 are marked with an arrowhead on right.

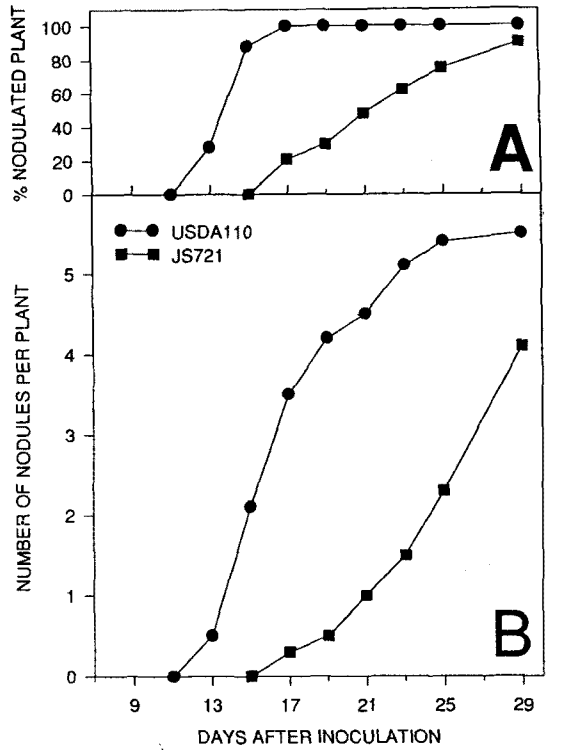

Fig 3. Nodulation phenotype of the His mutant.

Wild type strain (USDA110) and His mutant (JS721) were tested on *Glycine max* for their nodulation phenotype. Nodulation kinetics are shown as percentage of plants nodulated (A), and as the average number of nodules per plant (B).

tylene reduction ability were assayed as previously described (16). A total of 3,626 Km/Sm-resistant colonies were examined for auxotrophy. One was found to require L-histidine (50 μg/ml) for growth on minimal medium and named as JS721. The mutant strain JS721 grew in the presence of L-histidine and L-histidinol, but failed to grow when L-histidinol phosphate was supplied. To examine the symbiotic properties of the His$^-$ mutant, the mutant was inoculated onto soybean seedlings. Wild type strain began to induce visible nodules 11 days after inoculation whereas the mutant strain induced nodule formation after 15 days (Fig. 3B). Even after 29 days of cultivation, only 90% of the inoculated plants were nodulated by the mutant (Fig. 3A). When the acetylene reduction activity (indirect assay method for nitrogen fixation activity) was measured for nodules formed, both nodules were able to reduce acetylene to ethylene, indicating that the nodules formed were occupied by differentiated form of rhizobia. Furthermore, by genomic Southern blot analysis, it was revealed that the nodules formed by the mutant were occupied by the original inoculants and not by the revertant (data not shown).

However, since it has been known in several cases that the insertion of transposon could affect the the expression of genes located in the vicinity of the insertion sites, the possibility cannot be ruled out that the inserted Tn5 exerts its effect on unknown nodulation genes located close to the His locus. Further studies are underway to isolate the His locus by cloning the Tn5 flanking DNA fragment from the JS721. Once the mutated sequence is obtained, corresponding wild type sequence will be isolated from a wild type gene library. Subsequent investigation of the wild-type sequence will provide more information on the mechanism of how histidine requirement links to nodulation process in *B. japonicum*.

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References

1. Bishop, P.E., J.G. Guvara, J.A. Engelke, and H.J. Evans. 1976. Relation between glutamine synthetase and nitrogenase activities in the symbiotic association between *Rhizobium japonicum* and *Glycine max*. *Plant Physiol.* **57**: 542-546.
2. Davis, R.W., D. Botstein, and J.R. Roth. 1980. *Advanced bacterial genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
3. Ditta G., S. Stanfield, D. Gorbin, and D.R. Helinski. 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc. Natl. Acad. Sci.* USA **77**: 7347-7351.

4. Hahn, M.L., L. Meyer, D. Studer, B. Rosenburger, and H. Hennecke. 1984. Insertion and deletion mutations within the *nif* region of *Rhizobium japonicum*. *Plant Mol. Biol.* **3**: 159-168.
5. Jorgensen, R.A., S.J. Rothstein, and W.S. Reznikoff. 1979. A restriction enzyme map of Tn5 and location of a region encoding neomycin resistance. *Mol. Gen. Genet.* **177**: 65-72.
6. Kaluza, K., M. Hahn, and H. Hennecke. 1985. Repeated sequences similar to insertion elements clustered around the *nif* region of the *Rhizobium japonicum* genome. *J. Bacteriol.* **162**: 535-542.
7. Leong, S.A., G.S. Ditta, and D.R. Helinski. 1982. Heme biosynthesis in *Rhizobium*. Identification of a cloned gene coding for deltaamiolevulinic aid synthesis from *Rhizobium meliloti*. *J. Biol. Chem.* **257**: 8724-8730.
8. Rostas, K., P.R. Sista, J. Stanley, and D.P.S. Verma. 1984. Transposon mutagenesis of *Rhizobium japonicum*. *Mol. Gen. Genet.* **197**: 230-235.
9. Russell, P., M.G. Schell, K.K. Nelson, L.J. Halverson, K.M. Sirotkin, and G. Stacey. 1985. Isolation and characterization of the DNA region encoding nodulation function in *Bradyrhizobium japonicum*. *J. Bacteriol.* **164**: 1301-1308.
10. Sadowsky, M.J., K. Rostas, P.R. Sista, H. Bussy, and D.P.S. Verma. 1986. Symbioticaly defective histidine auxotrophs of *Bradyrhizobium japonicum*. *Arch. Microbiol.* **144**: 33 4-339.
11. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
12. Selvaraj, G., and V.N. Iyer. 1984. Transposon Tn5 specifies streptomycin resistance in *Rhizobium* spp. *J. Bacteriol.* **158**: 580-589.
13. Simon, R., U. Priefer, and A. Puhler. 1983. A broad host range mobilization system for *in vitro* genetic engineering: Transposon mutagenesis in gram negative bacteria. *Biotechnol.* **1**:784-791.
14. So, J.-S., A.L.M. Hodgson, R. Haugland, M. Leavitt, Z. Banfalvi, A.J. Nieuwkoop, and G. Stacey. 1986. Transposon-induced symbiotic mutants of *Bradyrhizobium japonicum*: Isolation of two gene regions essential for nodulation. *Mol. Gen. Genet.* **207**: 15-23.
15. So, J.-S. 1991. Molecular cloning of a gene region from *Bradyrhizobium japonicum* essential for lipopolysaccharide synthesis. *FEMS Microbiol. Lett.* **83**: 299-304.
16. Wacek, T.J., and W.J. Brill. 1976. Simple, rapid assay for screening nitrogen-fixing ability in soybean. *Crop Sci.* **15**: 519-523.
17. Wells, S.E., and D.L. Kuykendall. 1983. Tryptophane auxotrophs of *Rhizobium japonicum*. *J. Bacteriaol.* **156**: 1356-1358.

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