Isolation and Characterization of a Competition-Defective 
Bradyrhizobium japonicum Mutant

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Tn5 mutagenesis was coupled with a competition assay to isolate mutants of Bradyrhizobium japonicum 
defective in competitive nodulation. A double selection procedure was used, screening first for altered 
external cell polysaccharide production (nonmucoid colony morphology) and then for decreased competitive 
ability. One mutant, which was examined in detail, was deficient in acidic polysaccharide and lipopolysaccharide 
production. The wild-type DNA region corresponding to the Tn5 insertion was isolated, mapped, and 
cloned. A 3.6-kb region, not identified previously as functioning in symbiosis, contained the gene(s) necessary 
for complementation of the mutation. The mutant was motile, grew normally on minimal medium, and formed 
nodules on Glycine max cv. Williams when coinoculated with a competing strain. We isolated four mutants with 
decreased ability for competitive nodulation (Com−), and we describe mutant strain A3. A preliminary report of this work 
has appeared (2).

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. B. japonicum USDA110 was from the USDA Agricultural Research 
Service National Rhizobium Culture Collection, Beltsville, Md. MC617 is a Nod+ Nif+ derivative of B. japonicum 
USDA 110ARS (resistant to 10 μg of azide, 500 μg of rifampin, and 200 μg of streptomycin per ml), and it was a gift 
from L. D. Kuykendall of this laboratory. Strains NAD168 and NAD174 are slow-to-nodulate Tn5 mutants of 
B. japonicum USDA110 (9) and were obtained from G. Stacey (University of Tennessee). The B. japonicum strains 
were maintained and grown on minimal medium containing HM salts plus arabinose and gluconate (6). When appropri 
ate, media were supplemented with antibiotics. Escherichia coli strains were grown on LB medium (43).

Random Tn5 mutagenesis. Introduction of Tn5 into B. japonicum USDA110 was accomplished by conjugative 
transfer of plasmid pBLK1-2 (25). Tn5 encodes resistance to kanamycin and streptomycin (200 μg/ml each) in Bradyrhizobium 
spp. These antibiotics were used to select for strains with Tn5 insertions, and chloramphenicol (25 μg/ml) was 
added to counterselect against the E. coli donor. B. japonicum USDA110 is naturally resistant to chloramphenicol 
at this concentration. Resistant colonies were purified by re 
planting, and after 8 to 10 days of growth, colonies with altered morphology were selected.

Nodulation studies. Plant assays were done either with 
modified Leonard jar assemblies (51) or in a Monmouth fine 
sandy loam soil (7) in plastic pots (17.5-cm diameter). This soil 
contains less than 10 B. japonicum cells per g of soil (24). 
G. max cv. Williams seeds were surface sterilized (51) and 
soaked. Inoculation was performed with 1 ml of stationary 
phase cells of appropriate bradyrhizobia (approximately 10^8 
cells per ml). For paired competition studies, cell suspensions 
from two strains were mixed at predetermined ratios 
before inoculation, and seeds were inoculated at a final cell

* Corresponding author.
density of $3 \times 10^6$ cells per Leonard jar assembly. About a 1-cm layer of sterile gravel was placed on the surface. Plants were grown in a greenhouse with natural sunlight, supplemented with high-intensity light supplied by low-pressure sodium vapor lamps for a 14-h light: 10-h dark period. Plants were watered with nitrogen-free nutrient solution and harvested 25 to 27 days after inoculation. Nodule occupancy was determined from surface-sterilized nodules (51) on selective media containing the appropriate antibiotic(s). Nitrogen fixation rates were estimated by acetylene reduction assays as described previously (50).

**Pouch inoculation procedure.** Surface-sterilized soybean seeds were germinated on water agar plates for 3 days at 28°C in the dark (4). Seedlings were transferred aseptically to growth pouches (Northrup King Seed Co.) pretreated with nitrogen-free nutrient solution (9 ml per pouch). After roots had growth of 5 to 7 cm (2 days), plants were inoculated with the appropriate *B. japonicum* strain ($10^7$ cells per plant). The position of the root tip was marked on the overlaying plastic of the growth pouch at the time of inoculation (3, 4). The nodules were scored 8 to 10 days after inoculation.

**Assay for competitive nodulation.** An assay described by McLaughlin et al. (36) was adapted as follows: each mutant strain was challenged with a *Nod* $^+$ *NiF* $^-$ derivative of *B. japonicum* USDA I-1100ARS (strain MC617) at a ratio of 10:1 (Tn5 mutant/MC617). At this inoculum ratio, the plants are healthy and green if the Tn5 mutant is Fix $^+$ and competitive, while nitrogen-starved plants (pale green or yellow) indicate that the Tn5 mutant is defective in either nodulation or competitive ability. *Nod* $^-$ and Fix $^-$ mutants were distinguished from competition-defective (Com $^-$) mutants by single inoculation on plants. Com $^-$ mutants are Fix $^+$ and gave green plants when inoculated singly, while plants inoculated with Fix $^-$ or *Nod* $^-$ mutants were nitrogen starved and yellow.

**DNA biochemistry.** Total genomic DNA from *B. japonicum* was isolated as described by Sadowsky et al. (42). Restriction enzymes were purchased from United States Biochemical Corporation and T4 DNA ligase was from New England BioLabs. Southern and colony blot hybridizations, plasmid DNA isolation, ligation, and transformation of frozen competent cells of *E. coli* DH5$\alpha$ were performed by standard protocols (43). The *B. japonicum* USDA110 gene library was in cosmid vector pLAFR1 (11); prepared by B. Chelm). *B. japonicum* and *E. coli* matings were performed by the procedure of Ditta (11) on minimal medium supplemented with 0.1% yeast extract. *B. japonicum* transconjugants containing pLAFR1 cosmid constructs were selected on minimal medium containing 100 μg of tetracycline per ml.

**Polysaccharide analysis.** LPS was isolated by hot phenol-water extraction of cell homogenates by the method of Johnson and Perry (23). LPS was electrophoresed through 4% stacking and 12.5% separating gels, using the buffer system of Laemmli (30) with the following modifications: 4 M urea was incorporated into the gels; and the sodium dodecyl sulfate (SDS) concentration was increased from 0.1 to 1% in the gels and in the tank buffer. These modifications were reported to reduce LPS aggregation (39, 40). The gel was stained with the silver stain procedure for LPS (49).

*B. japonicum* cells were centrifuged for 15 min at 12,000 × g. Total CPS from mid-log-phase cells were isolated from the pelletted cells as described by Mort and Bauer (37) and estimated by the anthrone-sulfuric acid assay (1).

**Determination of motility and growth rate.** Motility was measured in minimal medium containing 0.4% agar. The bacteria were spot inoculated at equal cell densities, and the growth pattern was monitored over a period of 7 days. Motility studies in 0.4% agar medium in glass tubing were performed as described by Krieg and Gerhardt (28). Growth rate measurements were performed in liquid minimal medium (6), and cell number was determined by viable cell count.

**RESULTS**

Tn5 mutagenesis and screening for competition-defective mutants. *B. japonicum* USDA110 was mutagenized with Tn5, and colonies with appropriate antibiotic resistance were selected, purified, and replated. After 8 to 10 days of growth, mutants were observed for their colony morphology. From approximately 10,000 Tn5 insertions, about 115 mutants were selected for their relatively nonmucoid (Exo $^-$) colony type. These mutants were subjected to the paired competition assay described in Materials and Methods in which each mutant strain was challenged with a *Nod* $^+$ *NiF* $^-$ derivative of *B. japonicum* USDA I-1100ARS (strain MC617) at a ratio of 10:1 (Tn5 mutant/MC617). The results of the initial screening procedure, which was based on plant color alone, were confirmed by the analysis of nodule occupancy. The paired competition assays were performed in Leonard jars as well as in plastic pots containing unsterilized soil. The results presented in Table 1 indicate the different phenotypic groups of Tn5 mutants isolated. A large number of mutants were found to be "normal" in symbiosis (group I), although they were EPS deficient, indicating that the lack of EPS per se may not be the primary cause for a Com $^-$ phenotype. Group III mutants formed tiny nodules, and the plants were distinctly nitrogen starved. Group IV mutants were also nitrogen starved, although some C$_2$H$_2$ reduction activity was detected. Group II mutants were defective in the ability to occupy host nodules in the presence of a competing strain. The results of experiment 1 (Table 2) show that USDA110 (wild type) and the *NiF* $^-$ mutant (MC617) each form about 50% of the nodules when inoculated at a 1:1 ratio while, at a 10:1 ratio (USDA110/MC617), USDA110 forms 73% of the nodules (experiment 2). Experiments 3, 4, and 5 show that mutants A3 and A137 form very few nodules even at a 10:1 ratio in their favor. Similar results were obtained with mutant strains A115 and A145. All of these

<table>
<thead>
<tr>
<th>Strain</th>
<th>Colony morphology</th>
<th>Nodule formation</th>
<th>N$_2$ fixation</th>
<th>Competitive ability</th>
</tr>
</thead>
<tbody>
<tr>
<td>USDA110</td>
<td>Exo$^+$</td>
<td>G</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Group I (A101, A120, A152, A114)</td>
<td>Exo$^+$</td>
<td>G</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Group II (A3, A137, A145, A115)</td>
<td>Exo$^-$</td>
<td>G</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Group III (A111, A129, A135)</td>
<td>Exo$^-$</td>
<td>Y</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Group IV (A54, A118)</td>
<td>Exo$^-$</td>
<td>Y</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Group V (A146, A147)</td>
<td>Exo$^{+\circ}$</td>
<td>Y</td>
<td>-</td>
<td>ND</td>
</tr>
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</table>

* Pair competitive assays with strain MC617 in Leonard jar assemblies. Strains occupying <20% nodules at the inoculum ratio (Tn5 mutant/MC617) of 10:1 were determined to be competition defective (−). See Table 2 for quantitative data. ND, not determined.

* G, green; Y, yellow.

* Mutants formed colonies with excess polysaccharide.
TABLE 2. Competitive nodule occupancy by Com* mutants and transconjugants in paired inoculation experiments

<table>
<thead>
<tr>
<th>Expt</th>
<th>Coinoculated organisms</th>
<th>Nodules (%) formed by:</th>
<th>Ratio A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>USDA110</td>
<td>MC617a</td>
<td>10:1</td>
</tr>
<tr>
<td>2</td>
<td>USDA110</td>
<td>MC617</td>
<td>1:1</td>
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<td>A3</td>
<td>MC617</td>
<td>10:1</td>
</tr>
<tr>
<td>4</td>
<td>A3</td>
<td>MC617</td>
<td>10:1</td>
</tr>
<tr>
<td>5</td>
<td>A137*</td>
<td>MC617</td>
<td>10:1</td>
</tr>
<tr>
<td>6</td>
<td>A3</td>
<td>USDA110</td>
<td>10:1</td>
</tr>
<tr>
<td>7</td>
<td>A3(pAAB8.0)</td>
<td>MC617</td>
<td>10:1</td>
</tr>
<tr>
<td>8</td>
<td>A3(pAAB8.1)</td>
<td>MC617</td>
<td>10:1</td>
</tr>
<tr>
<td>9</td>
<td>A3(pAAB8.2)</td>
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<tr>
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</tr>
<tr>
<td>11</td>
<td>NAD168</td>
<td>MC617</td>
<td>10:1</td>
</tr>
<tr>
<td>12</td>
<td>NAD174</td>
<td>MC617</td>
<td>10:1</td>
</tr>
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</table>

a  Studies were performed in sterile vermiculite in Leonard jars unless otherwise indicated. A minimum of 96 nodules were used for nodule occupancy determination. The results are typical of three replicate experiments.

b  Nodules were visible occupancy.

A  MC617 is a Nif* Nod* mutant of B. japonicum USDA I-110ARS.

d  ND, not determined.

e  Performed in B. japonicum-free nonsterile soil.

FIG. 1. Region of B. japonicum USDA110 genome which complements the Exo and Com defects of mutant A3. Tn5 insertion A3 and the flanking DNA region were cloned in pUC18 (A) and used as a probe to isolate pAAB8.0 from a B. japonicum gene library (B). The 9.6-kb insert DNA was subcloned at EcoRI sites to get pAAB8.1 and pAAB8.2. E. EcoRI; X, XhoI; K, KpnI. No sites were observed for HindIII and BgIII. The arrowhead indicates the position of Tn5 in strain A3.

FIG. 2. Complementation of the nonmucoid colony phenotype (Exo*) of strain A3 by pAAB8.0. B. japonicum USDA110, Tn5 mutant A3, and the mutant carrying pAAB8.0 were streaked on minimal medium and incubated for 8 days at 28°C.
pouches (4) to examine for differences in early nodulation, which are difficult to monitor in Leonard jar assemblies. Pregermminated soybean seedlings in growth pouches were inoculated at a cell density of 10^6 cells per plant. The proportions of plants nodulating above the root tip (marked on the pouch at the time of inoculation) were 33 of 46 (71.7%) and 38 of 58 (65.6%) for USDA110 and A3, respectively. The numbers of nodules per plant formed above the position of the root tip at the time of inoculation were 2.5 ± 1.8 (USDA110) and 1.6 ± 1.1 (A3), and the average distances of the uppermost nodule from the root tip mark were 8.9 ± 5.4 mm for strain A3 and 11.3 ± 6.6 mm for USDA110. These results indicate that there is some decrease in the efficiency of nodulation by strain A3. In this context, we examined the competitive ability of two slow-to-nodulate Tn5 mutants of USDA110 (9). Mutant strains NAD168 and NAD174 retained their competitive ability under our assay conditions on soybean (Table 2) and other host plants (e.g., *Vigna unguiculata* cv. California Black-eye [cowpea], *V. radiata* cv. Berken 78 [mung bean], and *Macroptilium atropurpureum* cv. 296.959 [siratro]; data not shown), although they had a 2 (NAD168)- to 8 (NAD174)-day delay in nodulation and 20% (NAD168) to 50% (NAD174) reduction of nodule number per plant (9).

**DISCUSSION**

Tn5-induced mutants of *B. japonicum* USDA110 were screened for nonmucoid colony morphology (Exo⁺ phenotype) on minimal medium, and several Exo⁺ mutants were selected for further analysis of competitive nodulation ability. We adapted a competition assay (36) for these studies in which one of the competing strains (MC617) was Nif⁺. The lack of a functional nitrogenase system apparently does not reduce competitiveness of some *B. japonicum* strains (20), and our studies support this finding, since the wild-type parental strain USDA110 and its Nif⁻ derivative MC617 were found to be equally competitive (Table 2, experiment 1). Therefore, this is a very effective assay for screening for competition defects in the greenhouse or growth chamber. Several nonmucoid Tn5 mutants were isolated that nodulated normally in competition with MC617 (Table 1). Thus, reduced synthesis of the acidic EPS is not necessarily associated with a reduced competitive ability. Mutant strain A3 was defective in competition with USDA110 as well as with MC617 when tested in both sterile Leonard jar assays and nonsterile soil. Mutant strain A3 was motile, grew normally in minimal liquid medium, formed nitrogen-fixing nodules on soybean plants when inoculated singly, and contributed almost as much fixed nitrogen as the wild-type parent. However, the strain formed a somewhat reduced number of total nodules per plant when grown in Leonard jars as compared with the wild-type strain. The initiation of nodulation, examined with pregerminated seedlings in growth pouch experiments, indicated a slightly lower efficiency of nodulation for strain A3 as compared with the wild type. These differences do not appear to be a significant cause for the competition defect as other types of mutants, namely, NAD168 and NAD174, which have a much greater alteration in the rate of nodule initiation (9), were not altered in competition. One possible explanation is that strain A3 may be unable to sustain "competence" in the rhizosphere as well as the wild-type strain does. This will be explored in future studies.

Mutant A3 appears to be impaired but not totally deficient in EPS and LPS synthesis. Insertion of Tn5 in the 3.6-kb...
EcoRI fragment significantly altered the colony morphology and reduced the amount of EPS and LPS synthesized (Fig. 2 and 3). At this point it is not conclusive whether the EPS or the LPS deficiency is most responsible for the Com phenotype. We have complemented the Com− defect by the addition of small amounts (1.0 μg/ml of inoculum) of a crude EPS preparation from the wild-type strain (data not shown). These preliminary experiments are suggestive that the Com− defect results from a deficiency or structural alteration in the EPS.

The 9.6-kb DNA fragment and its subclone carrying a 3.6-kb region from the wild-type parent strain USDA110 appear to complement all defects in A3 at both the biochemical (EPS, CPS, and LPS production) and symbiotic (competitive nodulation) levels. Southern blot hybridization data, size of the DNA fragments, and restriction endonuclease analysis indicate that the insert DNA in plasmids pAA8.0 and pAA8.1 contains the wild-type gene(s) corresponding to Tn5 insertion A3. By comparing the restriction map with the available data on Nod and Fix regions of B. japonicum (18, 41), it appears that the 3.6-kb DNA region does not correspond to other previously identified regions and may represent a new symbiotic locus.

ACKNOWLEDGMENT

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REFERENCES