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Host-Controlled Restriction of Nodulation by *Bradyrhizobium japonicum* Strains in Serogroup 110†

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We previously reported the identification of a soybean plant introduction (PI) genotype, PI 417566, which restricts nodulation by *Bradyrhizobium japonicum* MN1-1c (USDA 430), strains in serogroup 129, and USDA 110 (P. B. Cregan, H. H. Keyser, and M. J. Sadowsky, Appl. Environ. Microbiol. 55:2532–2536, 1989, and Crop Sci. 29:307–312, 1989). In this study, we further characterized nodulation restriction by PI 417566. Twenty-four serogroup 110 isolates were tested for restricted nodulation on PI 417566. Of the 24 strains examined, 62.5% were restricted in nodulation by the PI genotype. The remainder of the serogroup 110 strains tested (37.5%), however, formed significant numbers of nodules on PI 417566, suggesting that host-controlled restriction of nodulation by members of serogroup 110 is strain dependent. Analysis of allelic variation at seven enzyme-encoding loci by multilocus enzyme electrophoresis indicated that the serogroup 110 isolates can be divided into two major groups. The majority of serogroup 110 isolates which nodulated PI 417566 belonged to the same multilocus enzyme electrophoresis group. *B. japonicum* USDA 110 and USDA 123 were used as coinoculants in competition-for-nodulation studies using PI 417566. Over 98% of the nodules formed on PI 417566 contained USDA 123, whereas less than 2% contained USDA 110. We also report the isolation of a Tn*5* mutant of USDA 110 which has overcome nodulation restriction conditioned by PI 417566. This mutant, D4.2-5, contained a single Tn*5* insertion and nodulated PI 417566 to an extent equal to that seen with the unrestricted strain USDA 123. The host range of D4.2-5 on soybean plants and other legumes was unchanged relative to that of USDA 110, except that the mutant nodulated *Glycine max* cv. Hill more efficiently. While strain USDA 110 has the ability to block nodulation by D4.2-5 on PI 417566, the nodulation-blocking phenomenon was not seen unless strain USDA 110 was inoculated at a 100-fold greater concentration than the mutant strain.

Bradyrhizobium japonicum strains form nitrogen-fixing, root nodule symbioses with soybean plants (*Glycine max* (L.) Merr.). However, establishing *B. japonicum* strains in a significant proportion of the nodules of field-grown soybean plants (*G. max*) remains a critical problem. Faced with competition from less effective indigenous bradyrhizobial populations, even highly efficient nitrogen-fixing strains will form few nodules and have a minimal effect on plant productivity. One approach for increasing nodulation by desirable strains would be the identification of soybean genotypes in which nodulation by undesirable strains is eliminated or substantially reduced (4).

Previous studies have indicated that various soybean genotypes are differentially nodulated by strains of *B. japonicum* (4, 5, 7, 9, 12, 29, 31). Soybean genotypes restricting nodulation by specific strains or serogroups of *Bradyrhizobium* have been reported, and in several cases, the plant locus or loci responsible for nodulation restriction have been identified. The single dominant genes Rj_2, Rj_3, and Rj_4 condition restricted nodulation by strains in the 122 and c1 serogroups, USDA 33, and USDA 61, respectively (31). Recessive plant genes which restrict nodulation by all bradyrhizobia, *Rj1*, *Rj5*, and *Rj6*, have also been reported (18, 29).

Cregan and Keyser (4) identified several soybean genotypes (including plant introductions [PI] and cultivars [cv.]) which restricted nodulation by *B. japonicum* USDA 123. The PI genotypes PI 377578 and PI 371607 were shown to restrict nodulation by different *B. japonicum* serocluster 123 isolates (4–6, 20, 21, 23). Serogroup-specific nodulation restriction, however, appears to be relatively complex and has recently been shown to operate on strains in several *B. japonicum* serogroups. For example, PI 377578 also restricts nodulation by strain USDA 61 and soybean plants containing the *Rj4* allele can restrict nodulation by some serogroup 123 strains (20). More recently, we have identified an additional soybean genotype, PI 417566, which restricts nodulation and reduces the competitiveness of strain USDA 430 (5, 6). PI 417566, however, also inhibited nodulation by strains USDA 129 and USDA 110 (5, 6). Caldwell and Vest (3) and Ferrey et al. (9) also reported that *G. max* cv. Peking restricts nodulation by *B. japonicum* USDA 110.

In this study, we examined 24 *B. japonicum* serogroup 110 strains to determine the extent to which their nodule-forming ability was restricted by PI 417566. We report here that PI 417566 restricts nodulation by a majority of, but not all, *B. japonicum* serogroup 110 strains. We also report that the serogroup 110 isolates can be divided into two major groups on the basis of multilocus enzyme electrophoresis (MLEE) analysis and that most nodulation-unrestricted isolates belong to the same MLEE subgroup. In addition, we describe the isolation and initial characterization of a transposon Tn*5* mutant of

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strain USDA 110 which has the ability to overcome nodulation restriction conditioned by PI 417566.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The *B. japonicum* serogroup 110 strains, designated by two-letter postal code abbreviations (AR-91b, AR14-2a, AR7-4a, KS6-1a, IN76, IN77, IL17, IL19, IL25, IL46, MS2-4b, and MS5-7a), were originally isolated from soybean root nodules obtained from five U.S. states: Arkansas, Kansas, Indiana, Illinois, and Mississippi. The *B. japonicum* strains were isolated from nodules as described previously (30), and single-colony isolates were purified by streaking onto yeast extract-mannitol medium (30). The identity of all isolates was confirmed by using serogroup 110-specific fluorescent antibodies produced as described by Schmidt et al. (24). The 12 strains have since been deposited in the culture collection of the U.S. Department of Agriculture, Agricultural Research Service, Beltsville, Md., under accession numbers USDA 443, USDA 455, USDA 450, USDA 460, USDA 459, USDA 454, USDA 458, USDA 457, USDA 449, USDA 453, USDA 461, and USDA 446, respectively. Strains WI3054 (USDA 456), WI3728 (USDA 452), WI3306 (USDA 444), and WI3258 (USDA 462) were originally obtained from Barbara Kamicker, University of Wisconsin, Madison. The serogroup 110 isolates USDA 16, USDA 17, USDA 20, USDA 30, USDA 110, USDA 137, USDA 141, USDA 335, and USDA 451 were obtained from the culture collection of the U.S. Department of Agriculture, Beltsville, Md. All strains were isolated in the United States, except for strains USDA 17, USDA 141, and USDA 335, which were isolated in Yugoslavia, Thailand, and China, respectively. Three additional serogroup 110 isolates, isolates B3-110 and D10Z and isolate 7-110 ELS, were obtained from B. Bohlool (University of Hawaii) and E. Schmidt (University of Minnesota), respectively. Strain D10Z is synonymous with USDA 451. Strains D4, D4.2-1, D4.2-5, D4.2-12, D4.2-18, and D4.2-25 are Tn*5* mutants of strain USDA 110 whose nodulation is not restricted by *G. max* PI 417566. All *Bradyrhizobium* strains were grown at 28°C on arabinose-gluconate (AG) medium (23). Strains containing Tn*5* were grown on AG medium supplemented with 200 μg of kanamycin per ml and 100 μg of streptomycin per ml.

Plant assays. All plant assays were done in Leonard assemblies containing a 3:1 mixture of vermiculite and perlite and N-free plant nutrient solution as described elsewhere (12, 23). Sterilized Leonard jars were planted with three surface-sterilized (30) seeds of either *G. max* cv. Kasota, cv. Hill, cv. Peking, or PI 417566, and 3 days after emergence, seedlings were thinned to one to two of each genotype per Leonard jar. Plants were inoculated with 1.0 ml (about 10^8 cells) of AG-grown *B. japonicum* serogroup 110 cultures. Isolates were inoculated in triplicate. Uninoculated plants served as negative controls, and strain USDA 123 served as a positive control. Plants were incubated in a plant growth chamber with a photoperiod of 16 h and a constant temperature of 20°C. Plants were watered, alternately, with nitrogen-free nutrient solution (12) and tap water, as needed, and harvested 40 days after inoculation. Nodule numbers and nodule dry mass were measured and statistical significance was determined by using the analysis of variance and Duncan's new multiple range procedures of SAS.

Tn*5* mutagenesis. Random transposon Tn*5* mutagenesis was performed by patch mating *Escherichia coli*(pBLK1-2) (13) to *B. japonicum* USDA 110. Transconjugants were selected on AG minimal medium (21) containing 200 μg each of kanamycin and streptomycin ml^{-1}. Approximately 10,000 randomly selected kanamycin-resistant (Kan^r) and streptomycin-resistant (Str^r) transconjugants were pooled into groups of 50 colonies and tested for nodulation on *G. max* PI 417566. Pooled transconjugants were inoculated on PI 417566 in Leonard jar assemblies and incubated as described above. Nodules were counted after 5 weeks of plant growth. Nodules were picked from plants, surface sterilized (30), and crushed in 100 μl of sterile water in the wells of microtiter plates. The nodule homogenates were examined with fluorescent antibody specific for strain USDA 110, and those reacting with the antibody were purified by streaking on AG medium containing kanamycin and streptomycin. Presumptive nodulation-competent transconjugants were grown in liquid AG media containing kanamycin and streptomycin and reinoculated onto PI 417566 seeds, in triplicate, as described above.

MLEE. The preparation of cell extracts, starch gel electrophoresis, and selective staining of enzymes were done as described previously (17, 25). Twenty-seven *B. japonicum* strains were used for MLEE studies. The Tris-citrate (pH 8.0) electrophoresis buffer system was used for all enzymes. Seven enzymes were assayed: isocitrate dehydrogenase, NAD-malate dehydrogenase, hexokinase, phosphoglucomutase, pyruvate dehydrogenase, esterases, and glucose 6-phosphate dehydrogenase. Distinctive-mobility variants of each enzyme (electromorphic variants) were determined, and electromorphic profiles (electrophoretic types [ETs]) were equated with multilocus genotypes (25). Genetic diversity for an enzyme locus and genetic distance between each pair of ETs were calculated as described elsewhere (17). Clustering from a matrix of pairwise genetic distances was done by using the average-linkage method of Sneath and Sokal (26). The results of cluster analysis were displayed as a dendrogram.

Competition-for-nodulation studies. The competitive ability of strains USDA 110 and USDA 123 on PI 417566 was evaluated by using triplicate Leonard jar assemblies as described above. Treatments included USDA 110 alone, USDA 123 alone, and USDA 110 plus USDA 123. PI 417566 seeds were inoculated with 10^8 cells of each strain, and plants were grown for 5 weeks in a growth chamber under the conditions described above. The identity of nodule occupants was determined by using a spot blot immunoassay as described elsewhere (5). Antibodies to the somatic antigens of strains USDA 110 and USDA 123 were prepared as described previously (24), and cross-adsorbed, strain-specific antibodies (19) were used to minimize cross-reactivity between strains. All nodules from each treatment were examined with each antibody.

Nodulation-blocking analyses. To test whether strain USDA 110 was able to block nodulation by mutant D4.2-5, triplicate PI 417566 seedlings were inoculated with three different ratios of strains USDA 110 and D4.2-5 (10^6:10^6, 10^6:10^8, and 10^8:10^6 cells per seed). Plant growth conditions were as described above. Plants individually inoculated with strain USDA 110 or mutant D4.2-5 and uninoculated plants served as controls. Nodules were surface sterilized (30), and nodule occupants were determined by streaking aliquots of nodule homogenates on AG medium with or without 200 μg each of kanamycin and streptomycin per ml.

Host range for nodulation analyses. To determine if mutant D4.2-5 had an altered host range for nodulation of several soybean cultivars and for plants in other legume genera, triplicate Leonard jars containing *G. max* cv. Hill, Kasota, Peking, Williams, or PI 417566 and *Vigna unguiculata*, *Phaseolus vulgaris*, *Macroptillium atropurpureum*, *Trifollium repens*, and *Medicago sativa* seedlings were individually inoculated at 10^8 cells per seed with AG-grown cultures of *B. japonicum* D4.2-5, USDA 110, or USDA 123. Uninoculated plants served as negative controls. Plants were grown for 5 weeks in a plant growth chamber, and nodule numbers were determined as described above.

RESULTS AND DISCUSSION

Nodulation of PI 417566 by serogroup 110 strains. Of the 24 *B. japonicum* serogroup 110 isolates examined in plant infection studies, 15 (62.5%) were restricted for nodulation by PI 417566 (Table 1). These isolates produced nodule numbers and masses on PI 417566 that were not significantly different from those on the uninoculated control plants or plants inoculated with strain USDA 110. The nodulation-restricted strains produced an average of 2.3 (mean range = 0 to 6.7) nodules on the PI genotype. On the other hand, 9 of 24 serogroup 110 strains (37.5%) were not restricted for nodulation by PI 417566. The nodulation-unrestricted serogroup 110 isolates produced mean nodule numbers ranging from 19.3 to 44.3 nodules per plant and mean nodule weights of 39.7 to 65.0 mg (per nodule). The majority of serogroup 110 strains that were not restricted for nodulation of PI 417566 produced nodule numbers on the PI genotype that were not significantly different, or were greater than, the number produced by the nodulation-unrestricted strain USDA 123. There was no direct relationship between the isolation origin of the serogroup 110 strains and their ability to nodulate PI 417566, although 8 of the 15 isolates which were restricted for nodulation were isolated from Illinois, Indiana, or Iowa and all 3 of the serogroup 110 strains that were isolated in Arkansas were not restricted by the PI genotype. All the *B. japonicum* serogroup 110 strains produced abundant nodule numbers and masses on *G. max* cv. Evans (Table 1), indicating that the strains have the capacity to form effective symbioses with a standard North American soybean cv.

Taken together, results in Table 1 show that PI 417566 has the ability to restrict the nodulation of only a subset of serogroup 110 strains. These results are in agreement with results from our previous studies which showed that restriction of nodulation of serocluster 123 strains by *G. max* PI 377578 is also strain dependent (12, 20, 23). Consequently, these and our previous results indicate that several genes controlling nodulation restriction in soybean plants may need to be combined into a single plant genotype in order to effectively eliminate nodulation by strains constituting a specific serological group.

The reason why only certain serogroup members are restricted for nodulation is currently unknown. It may be, however, that the nodulation-nonrestricted strains have a gene(s), similar in function to *nolA* (21), that allows them to nodulate

TABLE 1. Nodulation responses of *B. japonicum* serogroup 110 strains on *G. max* genotype PI 417566 and cv. Evans

Strain	Nodulation response on *G. max* genotype[a]:			
	PI 417566		Evans	
	No. of nodules	Nodule wt (mg)	No. of nodules	Nodule wt (mg)
WI 3054 (USDA 456)	44.3 A	65.0 A	27.0 E	32.3 FG
AR7-4a (USDA 450)	38.0 AB	63.0 A	43.0 CDE	30.6 G
USDA 335	32.7 BC	51.3 ABC	44.0 CDE	37.3 EFG
USDA 123	32.0 BC	65.0 A	47.0 BCDE	40.3 DEFG
MS5-7a (USDA 446)	31.0 BC	59.0 AB	48.7 BCDE	42.3 CDEFG
AR14-2a (USDA 455)	27.7 BCD	58.3 AB	53.7 BCD	44.6 CDEFG
WI 3728 (USDA 452)	27.0 CD	48.7 ABC	59.0 BCD	39.3 DEFG
AR9-1b (USDA 448)	25.0 CD	39.7 C	70.0 AB	46.3 CDEFG
D10Z (USDA 451)	22.0 CD	52.7 ABC	43.3 CDE	36.0 FG
USDA 20	19.3 D	43.7 BC	43.3 CDE	44.3 CDEFG
USDA 141	6.7 E	9.7 D	41.3 CDE	42.3 CDEFG
KS6-1a (USDA 460)	6.3 E	7.3 D	40.0 CDE	44.0 CDEFG
IL-46 (USDA 453)	5.3 E	12.7 D	49.0 BCDE	47.7 BCDEF
MS4-2b (USDA 461)	5.3 E	9.7 C	57.3 BCD	52.0 ABCDEF
USDA 30	3.3 E	3.0 D	47.0 BCDE	47.7 BCDEF
USDA 110	3.3 E	2.7 D	62.7 ABC	45.7 CDEFG
USDA 16	2.0 E	9.0 D	69.7 AB	57.0 ABCDE
USDA 17	2.0 E	2.3 D	63.7 ABC	46.3 CDEFG
IL-17 (USDA 458)	0.7 E	2.3 D	85.0 A	70.3 A
IN-77 (USDA 454)	0.0 E	0.0 D	61.3 BCD	59.7 ABCD
WI 3306 (USDA 444)	0.0 E	0.0 D	69.3 AB	58.0 ABCD
USDA 137	0.0 E	0.0 D	37.3 DE	34.6 FG
IL-25 (USDA 449)	0.0 E	0.0 D	63.7 ABC	57.3 ABCDE
IN-76 (USDA 459)	0.0 E	0.0 D	85.0 A	66.3 AB
IL-19 (USDA 457)	0.0 E	0.0 D	68.0 AB	61.3 ABC
None (uninoculated control)	0.0 E	0.0 D	0.0 F	0.0 H

[a] Values are means for triplicate samples. Numbers in a column not followed by the same letter are significantly different at $P = 0.05$.

PI 417566. Alternatively, the PI genotype may include a specific resistance (*Rj*) gene which prevents only certain strains from nodulating the host genotype. Answers to these questions must await results from further plant breeding and molecular biological studies.

Competition for nodulation on restrictive and nonrestrictive soybean hosts. Results from the competition-for-nodulation experiment are shown in Table 2. When PI 417566 was inoculated with equal numbers of *B. japonicum* USDA 110 and USDA 123, more than 98% of the nodules were found to be occupied by USDA 123. Strain USDA 110 occupied an average of 1.2% of nodules on the PI genotype. On the other hand, just the reverse situation was seen on soybean cv. Kasota. When USDA 110 was coinoculated with USDA 123 on soybean cv. Kasota, strain USDA 110 occupied the majority of nodules (37.6%). Only 17.6% of cv. Kasota nodules were occupied by strain USDA 123. It should be noted that while no nodules on the PI genotype were occupied by both strains, the incidence of double infections increased to 44.6% on soybean cv. Kasota. The high number of doubly infected nodules on cv. Kasota may, in part, be due to the high inoculation rates and the artificial growth system used in this study. Others have reported that 12 to 32% of soybean nodules are infected by more than one strain (15). Inoculation of PI 417566 with equal numbers of the two strains did not significantly change the total number of nodules produced on a per-plant basis. Plants inoculated with strain USDA 123 alone or USDA 123 plus USDA 110 had means of 33.7 and 28.3 nodules per plant, respectively. However, plants inoculated with strain USDA 110 alone had an average of 0.67 nodules per plant. All cv. Kasota plants were well nodulated, irrespective of the inoculum used. These results indicate that in addition to restricting nodulation, PI 417566 also significantly affects the competitiveness of strain USDA 110. PI 417566 has also been reported to reduce the nodulation ability and competitiveness of a *B. japonicum* serogroup 127 strain, USDA 430 (previously MN1-1c) (5, 6). The reduced competitiveness of USDA 110 and USDA 430 (and presumably other nodulation-restricted strains) on the PI genotype is most likely due to the host plant's direct inhibition of nodulation by one of the competitor strains. However, strain-strain interactions cannot be ignored as being an important part of the competition process.

TABLE 2. Competition for nodulation between strains of *B. japonicum* on two soybean genotypes

Soybean genotype	% Nodule occupancy by USDA strain(s)[a]:		
	110	123	110 + 123
Kasota	37.6 A	17.6 B	44.6 A
PI 417566	1.2 B	98.8 A	0.0 B

[a] Values are means for at least 35 nodules. Means within a column not followed by the same letter differ significantly at $P = 0.05$ as tested by Duncan's new multiple range test.

MLEE analysis of *B. japonicum* serogroup 110 strains. We used MLEE analyses of 27 *B. japonicum* serogroup 110 isolates in an attempt to correlate nodulation restriction on PI 417566 with the genetic relatedness of the serogroup 110 strains. Three ETs were identified from analysis of the seven enzymes (Table 3). The esterases identified two alleles (*EST1* and *EST2*). The serogroup 110 isolates were remarkably similar at six of the seven enzyme loci tested, and the two esterase alleles showed diversity among the isolates. Results from cluster analysis of the MLEE data (Fig. 1) indicated that the serogroup

TABLE 3. Allelic profiles of *B. japonicum* serogroup 110 strains

ET	No. of isolates	Allelic variant at enzyme locus[a]:							
		HEX	*IDH*	*G6P*	*PGM*	*MDH*	*PDH*	*EST1*	*EST2*
1	17	2	2	2	2	2	2	1	5
2	8	2	2	2	2	2	2	2	2
3	2	2	2	2	2	2	2	1	2

[a] Abbreviations: IDH, isocitrate dehydrogenase; MDH, NAD-malate dehydrogenase; HEX, hexokinase; PGM, phosphoglucomutase; PDH, pyruvate dehydrogenase; EST, esterases; G6P, glucose 6-phosphate dehydrogenase.

110 isolates could be divided into two closely related major groups. The majority of isolates, 62.9%, were found to cluster in group 2, whereas 37.1% of the isolates fell into group 1. The group 1 isolates consisted of two subgroups, a and b, which were very similar to each other and to the group 2 isolates. Overall, the data in Table 3 and Fig. 1 indicate that all of the serogroup 110 isolates were very similar and the group 1 and group 2 isolates were highly related to each other at a similarity value of 0.85 or greater. The majority of group 1 strains, 80%, were restricted for nodulation on PI 417566, whereas 64% of the group 2 strains failed to nodulate PI 417566. Our division of the 110 serogroup strains into specific groups by MLEE analysis is similar to what has been reported by others using *nod* gene hybridization analysis (27). However, the serogroup strains were more tightly clustered by MLEE analysis (divergence in similarity was at 0.85%) than they were by hybridization studies, in which strains were found to diverge at 65%. Interestingly, the two group 1a serogroup 110 isolates that were not restricted for nodulation on the PI genotype, USDA 452 (WI 3278) and USDA 456 (WI 3054), also fall into a distinctive group on the basis of *nod* probe hybridization analysis (27).

Isolation of a USDA 110 mutant with the ability to nodulate PI 417566. *B. japonicum* mutant D4 (USDA 110::Tn*5*) was isolated from one of 26 large nodules produced on nitrogen-fixing PI 417566 plants inoculated with the pooled Tn*5*-containing USDA 110 transconjugants. All putative nodulation-competent transconjugants isolated from the 20 nodules grew on medium containing 200 μg each of kanamycin and streptomycin per ml, suggesting they all contained Tn*5*. Moreover, all bacteria isolated from nodules reacted strongly with fluorescent antibodies specific for strain USDA 110. Strain D4 was reinoculated onto soybean genotype PI 417566 and incubated for 5 weeks, and bacteria were recovered from several of the 20 nodules produced. Results from a comparative nodulation study involving six of the new isolates from PI 417566 nodules (D4, D4.2-1, D4.2-5, D4.2-12, D4.2-18, and D4.2-25) indicated that Tn*5* mutants of strain USDA 110 nodulated PI 417566 to

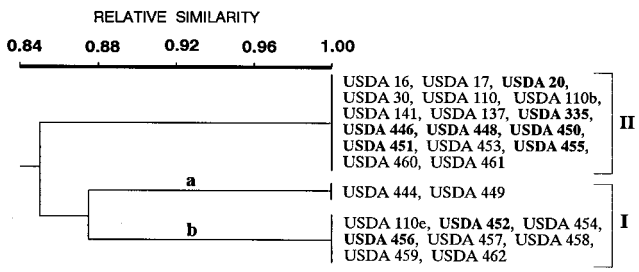

FIG. 1. Dendrogram of relatedness of *B. japonicum* serogroup 110 strains based on MLEE analysis. Strains shown in boldface type are not restricted for nodulation on soybean genotype PI 417566.

TABLE 4. Nodulation responses of USDA 110 mutant strains on *G. max* genotype PI 417566 and cv. Williams and Evans

Inoculant	No. of nodules produced on *G. max* cv.[a]:		
	PI 417566	Williams	Evans
USDA 123	27.0 A	24.5 A	48.3 A
D4	21.8 A	24.0 A	28.7 C
D4.2-1	31.0 A	43.3 A	34.3 BC
D4.2-5	27.3 A	42.3 A	42.7 AB
D4.2-12	29.8 A	29.3 A	44.0 AB
D4.2-18	20.5 A	39.3 A	35.7 BC
D4.2-25	28.3 A	34.8 A	40.7 AB
USDA 110	3.5 B	24.0 A	39.3 ABC
None	0.0 B	0.0 B	0.0 D

[a] Values are means for triplicate samples. Numbers in a column not followed by the same letter are significantly different at $P = 0.05$.

an extent equal to that observed with the unrestricted strain USDA 123 (Table 4). All of the mutants retained the ability to effectively nodulate soybean cv. Evans. However, while the Tn*5*-containing mutant strains nodulated PI 417566 and produced what appeared to be nitrogen-fixing nodules (i.e., leghemoglobin was present), the PI 417566 plants appeared more chlorotic than those inoculated with strain USDA 123, indicating an impaired symbiotic association on PI 417566. When one of the mutants, D4.2-5, was tested with other *G. max* genotypes, all plants appeared healthy with no observable chlorosis. It should be noted, however, that host range extension mutants of *B. japonicum* USDA 438 and *Rhizobium fredii* USDA 257 have been reported to produce effective symbioses on soybean plants with nodulation-restricting genotypes (1, 10, 11). It would seem, therefore, that while the D4 mutants have gained the ability to nodulate PI 417566, they possess some defect(s) which affects their symbiotic capability. A similar result was observed when strain SD61c(pMJS12) was inoculated on PI 377578 (21).

To determine if the Tn*5* mutants recovered from nodules contained a single copy of transposon Tn*5*, genomic DNAs from six of the mutants and USDA 110 were hybridized to a Tn*5* gene probe. Southern hybridization analysis (Fig. 2) revealed that the Tn*5* mutants all contained a single copy of Tn*5*

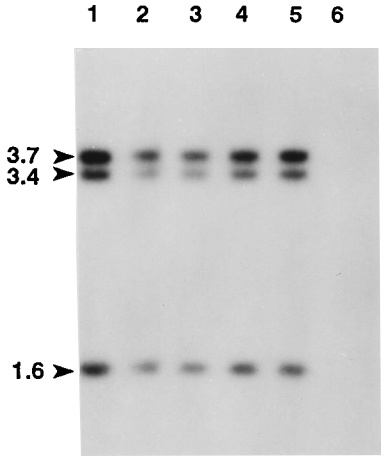

FIG. 2. Southern hybridization of *Hin*dIII-digested genomic DNAs from several host range extension mutants of *B. japonicum* USDA 110 to ^{32}P-labelled Tn*5* gene probe. Lanes: 1, mutant D4; 2, mutant D4.2-1; 3, mutant D4.2-5; 4, mutant D4.2-12; 5, mutant D4.2-18; and 6, wild-type USDA 110. Values on the left are sizes in kilobase pairs.

TABLE 5. Nodulation of five *G. max* genotypes by USDA 110 and its host range extension mutant D4.2-5[a]

Inoculant	No. of nodules produced on soybean genotype:				
	PI 417566	Hill	Kasota	Peking	Williams
USDA 110	1.0 B	26.5 B	26.0 A	10.0 A	24.0 A
D4.2-5	26.7 A	42.0 A	19.2 A	25.7 A	42.3 A

[a] Values are means for triplicate samples. Numbers in a column followed by a different letter are significantly different at $P = 0.05$.

and that all of the mutants contained Tn*5*-hybridizing fragments of the same size, suggesting that the nodule-recovered mutants were siblings. Moreover, when the genomic DNAs were hybridized to a nodulation gene probe, pR32, which contained many of the common, host-specific and genotype-specific nodulation genes (21), the wild-type and mutant DNAs were found to have the same hybridization pattern (data not shown), indicating that the Tn*5* insertion was not in most of the known nodulation loci. Due caution, however, must be exercised in the interpretation of results obtained from Tn*5* mutagenesis. In order to verify that the Tn*5* insertion is the cause of the observed change in phenotype, the mutation must be reconstructed in the wild-type strain with the same observable change in phenotype. In a study by Judd et al. (11), a reconstructed mutant of USDA 438 failed to give the same phenotype as the original host range extension mutant. We have localized the site of Tn*5* insertion to a 5.2-kb *Eco*RI fragment and are currently analyzing independent Tn*5* insertions obtained by saturation mutagenesis and reconstructing these mutations in strain USDA 110.

Host range for nodulation. To determine if the D4 mutants have an altered nodulation phenotype on several commercial soybean varieties, we inoculated mutant D4.2-5 and wild-type USDA 110 onto five *G. max* genotypes and several other legume species. Table 5 shows that there was no significant difference in nodulation by *B. japonicum* strains USDA 110 and mutant D4.2-5 on *G. max* cv. Williams, Peking, and Kasota. The mutant, however, did produce significantly greater nodule numbers on *G. max* cv. Hill than did the wild-type strain. Moreover, the mutant did not lose the ability to nodulate *M. atropurpureum* and *V. unguiculata* or gain the ability to nodulate pea, bean, or alfalfa plants (data not shown). Taken together, our results indicate that a mutated genetic determinant(s) in mutant D4.2-5 specifically controls nodulation on PI 417566. This result is similar to what was previously reported for a Tn*5*-containing mutant of *B. japonicum* USDA 438 which had specifically gained the ability to overcome nodulation restriction by PI 377578 and by plants containing the soybean *Rj4* allele (11).

Competitive nodulation blocking of strain D4.2-5 by strain USDA 110. Nodulation of PI 417566 by the mutant is subject to competitive nodulation blocking by cells of wild-type USDA 110 (Table 6). Blocking was observed, however, only when strain USDA 110 was present in 100-fold excess over mutant D4.2-5. When strain USDA 110 was added to PI 417566 with mutant D4.2-5 at an equal or a 10-fold-lesser concentration, the mutant nodulated the soybean plant to the extent seen when the plant was inoculated with the mutant alone. While the concentration of wild-type strain required to block nodulation by the host range extension mutant is considerably higher than that reported by Balatti and Pueppke (1) for a USDA 257 mutant, our results indicate that the same generalized phenomenon is occurring in the two soybean systems. One system, however, involves a strain of *R. fredii*, and the other involves a *B. japonicum* strain. The nodulation-blocking phenomenon has also been reported for the interaction of *Rhizobium leguminosarum* bv. viceae strains and *Pisum sativum* (8, 14). While the actual mechanism(s) involved in nodulation blocking, and competition for nodulation, for that matter, in our system and those reported by others remains unknown, several explanations can be forwarded to account for the phenomenon. These include a generalized resistance of the host to further invasion by rhizobia, the blockage of nodule initiation sites (2), and saturation of the nodulation capacity of the host (1). Since USDA 110 has the ability to curl root hairs, initiate infection thread formation, and induce nodule primordia on PI 417566 (22), our results suggest that host-strain incompatibility for nodulation and nodulation blocking is most likely governed by an autoregulatory feedback mechanism which operates after the initiation of the infection process (16, 27).

TABLE 6. Competitive nodulation blocking of strain D4.2-5 by USDA 110 on *G. max* genotype PI 417566

Inoculant	No. of nodules[a]
None	0.0 B
USDA 110	1.0 B
D4.2-5	25.5 A
USDA 110 + D4.2-5 (10^6:10^6)	33.8 A
USDA 110 + D4.2-5 (10^6:10^8)	24.5 A
USDA 110 + D4.2-5 (10^8:10^6)	3.0 B

[a] Values are means for triplicate samples. Numbers in a column not followed by the same letter differ significantly at $P = 0.05$.

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