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Genetic Diversity in *Bradyrhizobium japonicum* Serogroup 123 and Its Relation to Genotype-Specific Nodulation of Soybean

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The genetic diversity among 20 field isolates of *Bradyrhizobium japonicum* serogroup 123 was examined by using restriction endonuclease digestions, one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis of total cell proteins, Southern hybridization analysis of *nif* and *nod* genes, and intrinsic antibiotic resistance profiles. All of the isolates were previously separated into three broad nodulation classes (low, medium, and high) based on their ability to form symbioses with specific soybean genotypes. Results of our studies indicate that there is a relationship between these three genotype-specific nodulation classes and groupings that have been made based on genomic DNA digestion patterns, sodium dodecyl sulfate-protein profiles, and Southern hybridizations to a *nifHD* gene probe. Intrinsic antibiotic resistance profiles and *nodAB* gene hybridizations were not useful in determining interrelationships between isolates and nodulation classes. Southern hybridizations revealed that two of the isolates had reiterated *nod* genes; however, there was no correlation between the presence of extra *nodAB* genes and the nodulation classes or symbiotic performance on permissive soybean genotypes. Hybridizations with the *nif* gene probe indicated that there is a relationship among serogroup, nodulation class, and the physical organization of the genome.

Members of the genus *Bradyrhizobium* are slow-growing, gram-negative soil bacteria which invade and form nitrogen-fixing nodules on the roots of specific legumes (14, 31). *Bradyrhizobium japonicum* is the root nodule microsymbiont of soybean (*Glycine max* (L.) Merr.). Isolates of *B. japonicum* exhibit a high degree of serological specificity and can be divided into serological groups (serogroups) based on their surface somatic antigens (1, 5, 8, 21, 27, 28, 32). In the majority of soils in the midwestern United States, members of *B. japonicum* serogroup 123 are the most competitive and have been reported to occupy 60 to 80% of the nodules formed on field-grown soybeans (3, 4, 10, 17, 19, 23).

Recently, Cregan and Keyser (3) identified several soybean genotypes, including plant introductions (PIs), which restrict the nodulation of *B. japonicum* USDA 123. Two of the soybean genotypes were examined in more detail and were found to exclude strain USDA 123 in favor of other inoculant strains. In all cases, less than 10% of the nodules that formed on the PIs contained USDA 123 (3). In a subsequent study (16), 20 field isolates of serogroup 123, from diverse geographical regions of the United States, were evaluated for their ability to nodulate the USDA 123-restrictive PIs. These 20 isolates were separated into three broad nodulation classes (low, medium, and high) based on the nodule mass produced with the restrictive soybean genotypes. The high nodulation class isolates were those with nodule masses not significantly different than the top-ranked strain, and the low nodulation class isolates had nodule masses less than that of USDA 123 (on average, about 9% of the mass produced by the high nodulation class). The medium nodulation class contained isolates that produced nodule masses between isolates in the other two classes (about 57% of the nodule masses produced by the high nodulation class isolates).

Results from several studies have indicated that there is considerable physiological (13, 22, 24, 25), symbiotic (17), serological (5, 6, 27, 32), and genetic (9, 11, 29) diversity among strains and serogroups of *B. japonicum*. Little information is available, however, about the relationship between serological and genetic properties and symbiotic characteristics.

Members of *B. japonicum* serogroup 123 have been defined as those isolates which cross-react with antiserum prepared against USDA 123 (5). Results of immunoabsorption studies (5, 7, 27) have shown that serogroup 123 consists of three distinct serotypes: 123, 127, and 129. In this study, the genetic diversity among 20 serogroup 123 isolates was examined by using DNA restriction endonuclease fragment analysis, Southern hybridizations to known symbiotic genes, intrinsic antibiotic resistance patterns, and total cell protein profiles on sodium dodecyl sulfate (SDS)-polyacrylamide gels. We found that there is a correlation between the three genotype-specific nodulation classes and groupings that were made based on some of these characteristics. Our results suggest that there is a relationship between serological and genetic properties and nodulation characteristics on specific soybean genotypes.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The 17 *B. japonicum* strains, designated by the two-letter state postal code abbreviations (AK1-3a, AK9-3b, DE3-1a, IA3H2-6, IA3H2-8, IA3H2-17, KS6-3b, KS5-2c, MN1-1c, MS6-4a, MN6-1b, MN5-4a, NC3-1a, NJ1-4c, NJ2-1a, SC2-3c, and SD6-1c), were previously isolated from 10 U.S. states (18). Isolates from the same state were obtained from different locations. *B. japonicum* USDA 162, USDA 185, and USDA 228 were isolated in 1979 from soybean nodules obtained from the People's Republic of China. The serotype strains USDA 123, USDA 127, and USDA 129 were from the culture collection of the U.S. Department of Agriculture, Beltsville, Md. All of the isolates reacted strongly with fluorescent antibodies (FAs) against strain USDA 123 that were prepared by the method described by Schmidt et al.

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(27). Serological relationships among these isolates and strains USDA 123, USDA 127, and USDA 129 were determined previously (16) by using cross-adsorbed FAs, as described (5, 7, 27). The field isolates were divided into three nodulation classes (low, medium, and high) (16) based on the mass of nodules produced on restrictive (PI 371607 and PI 377578) and permissive (*G. max* cv. Williams) soybean genotypes (3). Isolates were grown in arabinose-gluconate (AG) medium (HM salts medium [2] supplemented with 0.1% yeast extract, 0.1% L-arabinose, and 0.1% sodium gluconate) at 28°C and maintained on AG agar slants.

Genomic DNA and plasmid isolation, restriction enzyme analysis, and Southern hybridizations. For total genomic DNA isolation, cultures of *B. japonicum* were grown for 2 days at 28°C in AG medium. Cells were centrifuged at 10,000 × *g* for 10 min at 4°C, washed once in TEN buffer (50 mM Tris, 10 mM disodium EDTA, 50 mM NaCl) (pH 8.0), and suspended in 16 ml of TEN buffer. Lysozyme (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 0.5 mg/ml, and cell suspensions were incubated at 37°C for 30 min. Predigested protease solution (2 ml; 5 mg of protease [Type X; Sigma] per ml in TEN buffer heated at 37°C for 1 h) was added, and the suspensions were incubated at 37°C for 30 min. A 2-ml fraction of 20% (wt/vol) Sarkosyl (*N*-lauroylsarcosine; Sigma) was added, and the mixtures were incubated at 37°C for 1 h. CsCl (31 g), 7.5 ml of TEN buffer, and 1.6 ml of ethidium bromide solution (10 mg/ml) were added to the cell lysates; and the mixtures were centrifuged at 40,000 rpm for 48 h at 20°C in a fixed-angle rotor (60 Ti; Beckman Instruments, Inc., Fullerton, Calif.). The high-molecular-weight DNA band was removed, and the DNA was repurified by ethidium bromide equilibrium density centrifugation, as described above.

Genomic DNAs were digested with the restriction endonucleases *Eco*RI, *Hin*dIII, and *Pst*I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), as specified by the manufacturer. Restriction fragments were separated by horizontal electrophoresis on 0.7% agarose gels in Tris-EDTA-borate buffer (20), stained in ethidium bromide (0.5 μg/ml), and photographed under UV light.

For hybridizations, DNA was transferred to Gene Screen (New England Nuclear Corp., Boston, Mass.), and ^{32}P-labeled probes were prepared and hybridized to the filters as described previously (20). The *B. japonicum* nodulation gene probe was a 1.7-kilobase (kb) *Eco*RI fragment from pRJUT10 (26) that was cloned into plasmid pHC79, which contained both *nodA* and *nodB* genes (kindly provided by Gary Stacey, University of Tennessee, Knoxville). The nitrogenase structural gene probe (*nifH* and *nifD*) was obtained from Choong-Hyun Kim (our laboratory) and contained a 3.7-kb *Eco*RI fragment from *Rhizobium meliloti* 102F34 cloned into plasmid pUC9.

SDS-polyacrylamide gel electrophoresis. Bacterial strains were grown in 5 ml of AG medium to the early stationary phase at 30°C with shaking. Cultures were adjusted to an A_{660} of 0.9, and 1.5-ml fractions were centrifuged at 12,000 × *g* for 5 min. The cells were washed once in 12 mM MOPSO buffer [3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; pH 6.9] and suspended in 1 ml of cell treatment buffer (62.5 mM Tris, 2.0% [wt/vol] SDS, 10.0% [vol/vol] glycerol, and 5.0% [vol/vol] 2-mercaptoethanol [pH 6.8]). The suspensions were incubated at 100°C for 10 min and centrifuged at 12,000 × *g* for 5 min, and 250-μl fractions of the resulting supernatants and 25 μl of phenol red solution (0.02%) were added to 250 μl of treatment buffer. Samples were subjected to discontinuous slab-gel electrophoresis in SDS-Tris-glycine buffer (24) with a Protean electrophoresis system (Bio-Rad Laboratories, Richmond, Calif.). The gel measured 16 cm by 15 cm by 1.5 mm. Gels consisted of a 1-cm, 4.0% acrylamide stacking gel and a 13-cm, 10% acrylamide resolving gel layer. A 35-μl sample was loaded onto each well of the gel. Electrophoresis was done at a constant current of 60 mA for 4 to 5 h. After electrophoresis, the gels were stained by the modified silver staining method described by Hitchcock and Brown (12). Although this method was originally developed for staining lipopolysaccharides, we found it to be quite sensitive for staining pro-teins.

Intrinsic resistance to antibiotics. Resistance to low concentrations of antibiotics was determined essentially as described by Josey et al. (15). Two-day-old AG-grown cultures of the isolates were streaked onto the surface of AG agar plates containing the following antibiotics: streptomycin sulfate (20 μg/ml), spectinomycin sulfate (10 μg/ml), kanamycin sulfate (20 μg/ml), tetracycline hydrochloride (30 μg/ml), nalidixic acid (10 μg/ml), erythromycin sulfate (30 μg/ml); and rifampin (1 μg/ml). Plates were incubated at 28°C for 7 days and scored for visible growth.

Plant assays. Plant assays were done in soybean rhizobia-free Monmouth fine sandy loam soil limed to a neutral pH with dolomite. Plants were grown in 17.5-cm, surface-sterilized, plastic pots (3) containing 2.4 kg of soil. *G. max* cv. Williams seeds were surface sterilized (3) before they were planted. Seeds were inoculated with 1.0 ml of stationary-phase *B. japonicum* cultures (about 10^9 cells per ml), and a 1-cm layer of sterile gravel was placed on the soil surface. Plants were grown in the greenhouse with natural sunlight, with incandescent light supplemented to extend the photoperiod to 18 h and were harvested 42 days after inoculation. Nodule mass and total nitrogen was determined as described previously (3).

RESULTS

Restriction enzyme digestion patterns. The *Eco*RI digestion patterns of genomic DNAs from the 20 isolates of serogroup 123 are shown in Fig. 1. Subjective evaluation of the banding patterns allowed for the separation of the isolates into three broad restriction enzyme groups. With few exceptions, the enzyme digestion groups closely correlated with the nodulation classes. In general, group 1 (lanes 1 to 5) consisted of those isolates belonging to the high nodulation class, group 2 (lanes 6 to 13) consisted of isolates belonging to the medium nodulation class, and group 3 (lanes 14 to 20) consisted of isolates with a low or restricted nodulation phenotype.

While in most cases there was good agreement between the groupings made based on nodulation classes and restriction enzyme digestion profiles, there were some exceptions (Table 1). Two of the isolates previously classified as having high nodule mass on the two *G. max* PIs, AK1-3a (lane 6) and NC3-1a (lane 8), had digestion patterns more similar to those of the isolates in the medium nodulation class; and isolate MS6-4a (lane 4), a medium nodulation class isolate, had a DNA fingerprint similar to those of some of the isolates in restriction enzyme group 1. In addition, isolates SC2-3c and MN5-4a (lanes 15 and 19, respectively), which belonged to the medium nodulation class, had *Eco*RI digestion patterns similar to those of isolates in restriction enzyme group 3. The isolates in restriction enzyme group 1 (high nodulation class) appeared to be genetically diverse since their digestion patterns were heterogeneous. Two types of group 1 isolates could be identified. Digestion patterns of three of the group 1 isolates, USDA 185 (lane 2), MS6-4a (lane 4),

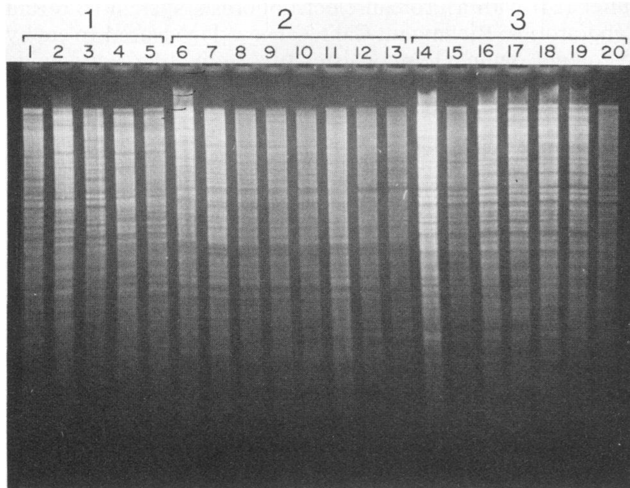

FIG. 1. Restriction enzyme *Eco*RI digestion patterns of genomic DNA from 19 *B. japonicum* serogroup 123 field isolates. Lanes: 1, MN1-1c (H); 2, USDA 185 (H); 3, DE3-1a (H); 4, MS6-4a (M); 5, USDA 228 (H); 6, AK1-3a (H); 7, AK9-3a (M); 8, NC3-1a (H); 9, IA3H2-17 (M); 10, IA3H2-8 (M); 11, KS6-3b (M); 12, NJ1-4c (M); 13, NJ2-1a (M); 14, USDA 162 (L); 15, SC2-3c (M); 16, SD6-1c (L), 17, KS5-2c (L); 18, MN6-1b (L); and 19, MN5-4a (M). Lane 20 contains DNA from the serotype strain USDA 123 (L). Letters in parentheses following the strain designations refer to low (L), medium (M), and high (H) nodulation classes. Indicator bars at the top of the figure refer to restriction enzyme groups.

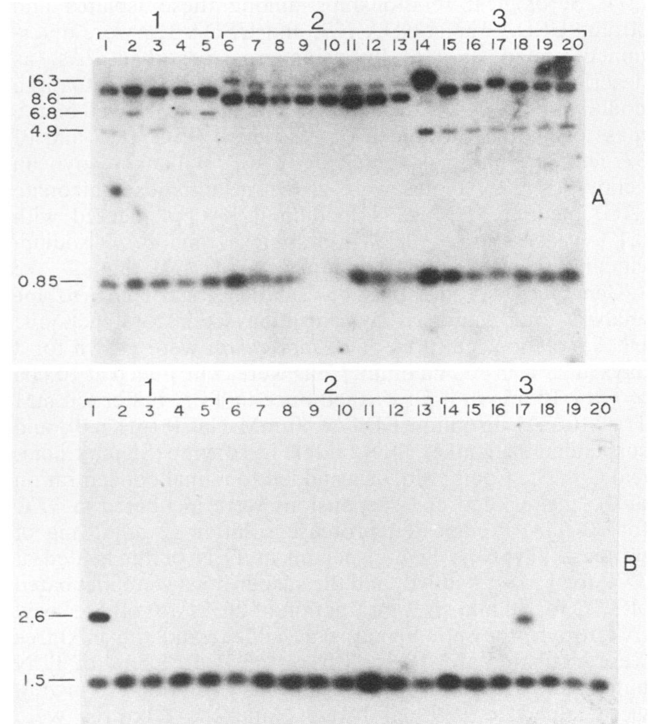

FIG. 2. Southern hybridization of genomic DNA from 19 field isolates in *B. japonicum* serogroup 123. DNAs were digested with restriction enzyme *Hin*dIII and hybridized to a ^{32}P-labeled *nifHD* gene probe (A) and digested with *Eco*RI and hybridized to a ^{32}P-labeled *nodAB* gene probe (B). Lanes: 1, MN1-1c; 2, USDA 185; 3, DE3-1a; 4, MS6-4a; 5, USDA 228; 6, AK1-3a; 7, AK9-3a; 8, NC3-1a; 9, IA3H2-17; 10, IA3H2-8; 11, KS6-3b; 12, NJ1-4c; 13, NJ2-1a; 14, USDA 162; 15, SC2-3c; 16, SD6-1c; 17, KS5-2c; 18, MN6-1b; 19, MN5-4a; and 20, USDA 123. Nodulation class designations are as given in the legend to Fig. 1. Molecular size values (to the left of the gels) are in kilobases. Indicator bars at the top of the figure refer to hybridization groups. The low-molecular-weight fragments missing from lanes 9 and 10 in panel A are an artifact of the Southern transfer.

and USDA 228 (lane 5), were nearly identical. Interestingly, isolates MN1-1c and DE3-1a (lanes 1 and 3, respectively) had DNA fingerprints which appeared to be more similar to those of the group 3 isolates than to those of the other isolates in group 1 or to those of isolates in group 2. The digestion patterns of these two isolates were sufficiently different from those in restriction enzyme group 3, however, to warrant separate group status. Digestion of the genomic DNAs from the 20 serogroup isolates with the restriction enzymes *Hin*dIII and *Pst*I similarly grouped the isolates into the same three restriction enzyme groups (data not shown).

TABLE 1. Relationship between nodulation classes and the serological, hybridization, DNA restriction, and protein groupings of 20 *B. japonicum* serogroup 123 field isolates

Isolate(s)	Nodulation class[a]	Serotype	DNA restriction, *nif* hybridization, and protein groups
MN1-1c, USDA 185, DE3-1a, USDA 228[b]	H	127	1
AK1-3a	H	129	2
NC3-1a	H	129	2
IA3H2-17, NJ2-1A, IA3H2-8, KS6-3b, NJ1-4c, AK9-3a	M	129	2
MS6-4a[b]	M		1
SC2-3c	M	127	3
MN5-4a	M	123	3
USDA 162, SD6-1c, KS5-2c[b], MN6-1b	L	123	3
USDA 123	L	123	3

[a] Based on the nodule mass produced on *G. max* PIs 371607 and 377578.
[b] These isolates failed to react with FAs specific for the serotype strains USDA 123, USDA 127, or USDA 129.

While all 20 of the field isolates reacted strongly with FAs prepared against strain USDA 123, they have recently been separated into their respective serotypes by using FAs specific for USDA 123, USDA 127, and USDA 129 (16). Our results indicate that there is generally a close relationship between the groups based on DNA digestion patterns (and hence nodulation classes) and serotype (Table 1). While isolates in *Eco*RI restriction enzyme group 3, with the exception of isolates KS5-2c, SC2-3c, and MN5-4a, were members of serotype 123, isolates within restriction enzyme group 2 (mostly the medium nodulation class isolates) belonged to serotype 129. Isolates within restriction enzyme group 1 were members of serotype 127, with the exceptions of isolates MS6-4a and USDA 228 (serotype undefined).

Hybridization of the *R. meliloti nifHD* gene region. Genomic DNAs from the 20 field isolates were digested with the restriction enzyme *Hin*dIII and hybridized to a ^{32}P-labeled *nifHD* gene probe. The results presented in Fig. 2A indicate that each of the isolates had three hybridizing fragments. These *nif* hybridization patterns fell into three markedly different groups (*nif* groups 1, 2, and 3) which correlated well with the restriction enzyme groups (Fig. 1) and the nodulation classes. Both the faintly and strongly hybridizing frag-

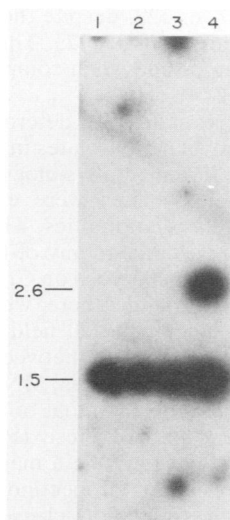

FIG. 3. Southern hybridization of *Eco*RI-digested genomic DNA from the *B. japonicum* serotype strains USDA 123 (lane 1), USDA 127 (lane 2), and USDA 129 (lane 3) and from the serogroup 123 field isolate MN1-1c (lane 4) to a ^{32}P-labeled *nodAB* gene probe. Molecular size values (to the left of the gels) are in kilobases.

ments showed the relationship between the hybridization and restriction enzyme groups. Hybridization group 3 consisted of those isolates in the severely reduced (low) nodulation class (with the exception of isolates MN5-4a and SC2-3c, which were medium nodulators), and most of the isolates had similar *nifHD* hybridizing fragments with estimated sizes of 10.3, 5.2, and 0.85 kb. However, two of the isolates, KS5-2c (lane 17) and USDA 162 (lane 14), had different hybridization profiles. In isolate KS5-2c, pCHK12 hybridized to *Hin*dIII fragments of 12.7, 5.2, and 0.85 kb; and in USDA 162, pCHK12 hybridized to fragments of 16.3, 5.2, and 0.85 kb. The isolates in hybridization group 2 (mostly of the medium nodulation class) appeared to be similar and had hybridizing fragments of 14.4, 8.6, and 0.85 kb. The one exception in this group was isolate AK1-3a (lane 6), which had a larger *nif* hybridizing fragment of 16.3 kb.

The results shown in Fig. 2A indicate that hybridization group 1 (high nodulation class) consists of isolates with two distinct *nif* hybridization patterns. Isolates USDA 185 (lane 2), MS6-4a (lane 4), and USDA 228 (lane 5) had identical hybridization patterns with *nif* hybridizing fragments of 9.3, 6.8, and 0.85 kb. Likewise, isolates MN1-1c (lane 1) and DE3-1a (lane 3) had the same hybridization profiles with fragments of 9.3, 4.9, and 0.85 kb. As indicated previously for the restriction enzyme digestion patterns, the hybridization patterns of DE3-1a and MN1-1c were more similar to those of the group 3 isolates than to those of the other two group 1 isolates. Interestingly, the hybridization patterns of these two isolates were quite similar to those in hybridization group 3. As before, there was a relationship between groups that could be made based on *nif* hybridization patterns and serotype for the low and medium nodulation class isolates (Table 1).

Conservation of *nodAB* genes in serogroup 123. Genomic Southern blots of *Eco*RI-digested DNAs from the 20 field isolates were also hybridized to a ^{32}P-labeled *nodAB* gene probe from *B. japonicum* I110. Results (Fig. 2B) indicate that, with the exception of isolates MN1-1c (lane 1) and KS5-2c (lane 17), all of the isolates had a single hybridizing fragment of 1.5 kb. In addition, isolates MN1-1c and KS5-2c had a second, larger (2.6-kb) fragment. These data suggest that the *nodAB* genes are reiterated in these two isolates. There was no relationship between the *nod* hybridization patterns and nodulation classes or the groupings based on *nif* gene hybridizations or *Eco*RI digestion patterns. In addition, it should be noted that there was no relationship between reiteration and nodulation class since MN1-1c and KS5-2c were in the high and low nodulation classes, respectively.

To determine if there was a relationship between the presence of reiterated *nodAB* genes and serotype, we hybridized the ^{32}P-labeled *nod* gene probe to *Eco*RI-digested genomic DNAs from the serotype strains USDA 123, USDA 127, and USDA 129 (Fig. 3). The results indicate that the reiteration of *nodAB* genes in the field isolates MN1-1c and KS5-2c is not related to serogroup, since each of the serotype strains had a single 1.5-kb *Eco*RI hybridizing fragment, while only isolate MN1-1c had the 2.6-kb hybridizing fragment. It should be noted, however, that while isolate MN1-1c is a member of serogroup 127, the serogroup status of KS5-2c has not been determined, since it failed to react with FAs specific for USDA 127 or USDA 129 (16).

To determine if the extra copies of the *nodAB* genes enhanced the symbiotic performance of the isolates on a nonrestrictive soybean host, we inoculated *G. max* cv. Williams seedlings with strains that had single and multiple copies of *nod* genes. The results (Table 2) indicate that there was no significant difference in nodule mass or nitrogen accumulation (total nitrogen) in plants inoculated with isolates that differed in the number of *nodAB* copies.

Sequence relatedness in serogroup 123 field isolates. While only a limited number of isolates and restriction enzymes was used, we nevertheless obtained an estimate of the relatedness of the isolates (9, 29) by comparing the fraction of conserved restriction fragments in the *nif* hybridizations in Fig. 2A. This was done by using the method described by Upholt (30), which assumes that comigrating fragments correspond to similar regions of the chromosome and that sequence divergence is due only to base substitutions in and around the probed sequence. We did pairwise comparisons between the fraction of comigrating fragments in USDA 123 versus those of the other serogroup 123 field isolates, and our results indicate that isolates in the low nodulation class are more closely related to USDA 123 than to the isolates in the other nodulation classes. The low nodulation class isolates MN6-1b, SD6-1c, and USDA 162 had base substitution values of 0% when compared with that of USDA 123. The one isolate with reiterated *nod* genes, KS5-2c, had an estimated percent base substitution value of 2.3%. Isolates in the medium (group 2) and high (group 1) nodulation

TABLE 2. Relationship between reiteration of *nodAB* genes and symbiotic performance of serogroup 123 isolates on the soybean *G. max* cv. Williams

Isolate	No. of common *nod* genes	mg/plant[a]		Nodule class[b]
		Nodule dry wt	Nitrogen accumulation	
USDA 123	1	295a	81a	L
KS5-2c	2	268a	46a	L
MN1-1c	2	256a	84a	H
AK1-3a	1	241a	78a	H

[a] Values are means of four replicates. Values within a column not followed by the same letter differ significantly at $P = 0.05$ as tested by Duncan's new multiple range test.

[b] Based on nodule mass produced on *G. max* PIs 371607 and PI 377578 (16). Abbreviations: L, low; H, high.

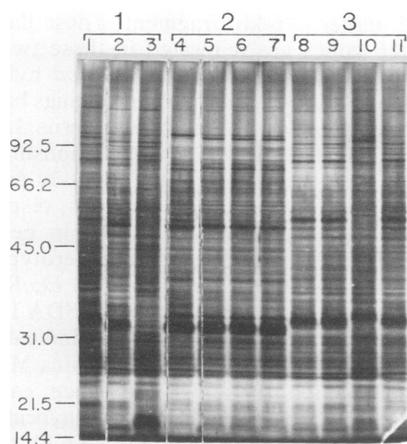

FIG. 4. SDS-polyacrylamide gel electrophoresis of total proteins from selected *B. japonicum* serogroup 123 field isolates. Lanes: 1, MN1-1c; 2, DE3-1a; 3, USDA 228; 4, AK1-3a; 5, AK9-3a; 6, NC3-1a; 7, IA3H2-8; 8, SD6-1c; 9, MN6-1b; 10, KS5-2c; and 11, USDA 123. Values in the margin are in kilodaltons. Indicator bars at the top of the figure refer to protein groups.

classes all had base substitutions of 16.6% when compared with that of the serotype strain USDA 123, and thus were less closely related to isolates within the low nodulation class (group 3).

Isolate analysis by using SDS-polyacrylamide gel electrophoresis of total proteins. Eleven of the serogroup 123 field isolates were also analyzed by SDS-polyacrylamide gel electrophoresis to determine if the groupings based on restriction enzyme and *nif* hybridization patterns extended to the protein level. The results (Fig. 4) indicate that with few exceptions, the protein patterns placed the isolates in the same groups as were determined by using restriction or hybridization data. Three protein profile groups can be identified in Fig. 4. Group 1 (lanes 1 to 3) consisted of isolates MN1-1c, DE3-1a, and USDA 288, respectively. As determined by the other identification methods, the number of differences in the protein banding patterns indicates that isolates in this group are genetically heterogeneous. Protein fingerprints indicated that there was no apparent relationship among any of the isolates in protein group 1. In addition, one isolate, USDA 228 (lane 3) had a protein profile that was dramatically different from thoseof all other isolates tested. Protein group 2 (lanes 4 to7) consisted of isolates AK1-3a, AK9-3a, NC3-1a, and IA3H2-8, respectively. These isolates were also placed in group 2 based on restriction enzyme and *nif* hybridization data and appeared to have identical protein profiles. Interestingly, these isolates all belonged to serogroup 129 and had major protein bands of approximately 100, 79, 55, 42, 35, and 33 kilodaltons (kDa). It should be noted that AK1-3a and NC3-1a were originally placed into the high nodulation class based on the nodule mass produced on the restrictive soybean genotypes. The group 3 isolates (belonging to the low nodulation class) in lanes 8 to 11 were SD6-1c, MN6-1b, KS5-2c, and USDA 123, respectively. Three of these isolates (SD6-1c, MN6-1b, and USDA 123) had identical protein patterns with major bands of 96, 83, 56, and 34 kDa.

Interestingly, by the protein profile, isolate KS5-2c appeared to be different from the rest of the group 3 isolates in that it lacked the 56-kDa protein band and had a 36-kDa band instead of the 34-kDa species that was present in the others. As was previously noted, however, KS5-2c is not a member of serogroup 123, 127, or 129, despite the fact that it reacts with FA prepared against USDA 123. This may account for its variation in protein profile when compared with those of the other serogroup 123 isolates.

Intrinsic antibiotic resistance. To determine whether there was a relationship between the isolates in a given nodulation class and an easily determined physiological phenotype, the 20 serogroup 123 field isolates were examined for their resistance to low levels of antibiotics. There was no direct correlation between resistance to any of the tested antibiotics and their nodulation phenotypes on *G. max* PI 371607 and PI 377578. In addition, while there were 10 patterns of antibiotic resistance among the 20 field isolates (data not shown), there was no relationship between the patterns and nodulation class. Two of the isolates, MN1-1c and IA3H2-6, had resistance patterns similar to that of the serotype strain USDA 123; however, both of these isolates belonged to separate nodulation classes. While a majority of the tested field isolates were resistant to spectinomycin, only some members of the high nodulation class were resistant to nalidixic acid. In general, isolates of the medium nodulation class, with the exception of strains IA3H2-6 and SC3-2a, were sensitive to the majority of the tested antibiotics.

DISCUSSION

We examined the diversity among field isolates of *B. japonicum* serogroup 123 to determine if there was a relationship between their genetic, physiological, and serological properties and their symbiotic characteristics. Our results show that there is a relationship between the nodulation phenotype on restrictive soybean genotypes and groups based on restriction enzyme digestion patterns, *nif* gene hybridization profiles, SDS-protein patterns, and serological reaction with serotype-specific FAs. This relationship was strongest in those isolates that belonged to the low and medium nodulation classes. Field isolates that were determined to be restricted for nodulation on soybean genotypes PI 371607 and PI 377578 (group 3) had nearly identical DNA fingerprints and, with few exceptions, similar size fragments that hybridized to a *nifHD* gene probe. With the exception of one isolate, KS5-2c, isolates in this nodulation class had identical protein profiles. Isolate KS5-2c, however, differed from other serogroup 123 isolates in that it had reiterated *nod* genes. While the uniqueness of this strain was not reflected in the *nif* hybridization patterns, it was reflected in its protein profile and serological reaction. By most criteria, the medium nodulation class isolates (protein and *nif* group 2) were nearly identical to each other. On the other hand, isolates within the high nodulation class were relatively heterogeneous in all of the determined characteristics and therefore represented genetically diverse organisms.

The restriction enzyme patterns (and subsequent *nif* and protein profiles) of the 20 field isolates indicated that there are exceptions to the class designations based on nodulation phenotypes. These exceptions were isolates NC3-1a and AK1-3a, both of which were in the high nodulation class, which had DNA digestion profiles similar to those of the medium nodulation class isolates. In addition, isolate SC2-3c, of the medium nodulation class, had characteristics that were more similar to those of the isolates in the low nodulation class. It may be that groupings based on these criteria are more sensitive to small genetic differences than are those made based on gross phenotypic characteristics such as relative nodulation ability and antibiotic resistance. Both DNA and protein fingerprinting previously have been

shown (9, 24) to be sensitive methods for detecting small differences among isolates and strains of rhizobia.

The conservation of the genomic arrangement of common nodulation genes in our isolates suggests that genetic loci other than this region of the genome are responsible for genotype-specific nodulation. Such conservation in *nod* genes has been noted by Stanley et al. (29) in their examination of several *Bradyrhizobium* serotype strains. Our hybridizations with a *nod* gene probe indicated that two of the isolates, MN1-1c and KS5-2c, had reiterated *nodAB* genes. There was no correlation, however, between the presence of extra *nod* genes and nodulation class on restricted soybean genotypes or with symbiotic performance on a permissive host. It may be that either higher doses of these gene products are insufficient for increased nodulation without a corresponding increase in *nod* activator genes (such as *nodD*) or that other genetic loci are required to bring about a higher nodulation rate.

The *nif* hybridization data (Fig. 2A) remarkably separated the isolates into their respective nodulation classes (with the exceptions noted above). While the *nif* genes themselves are most likely not involved in genotype-specific nodulation, the hybridization data nevertheless reflect the genomic organization of symbiotic genes (and therefore the genetic similarity among strains). Our data also indicate that there is less conservation in the arrangement of DNA regions within and adjacent to *nifDH* genes in our isolates than has previously been reported by others (9, 29).

While Stanley et al. (29) showed nearly identical *nif* gene hybridizations in the seven *B. japonicum* strains that they examined (representing at least four distinct serogroups), our serogroup 123 field isolates (consisting of three serotypes) had vastly different hybridization patterns. This suggests that more isolates within a given serogroup need to be examined before a clear understanding of genomic relationships can be ascertained. The *nif* hybridization profiles presented in Fig. 2A and our estimate of sequence divergence within and around the *nif* genes, however, suggest that isolates within a given nodulation class are more closely related to each other than to isolates in other nodulation classes.

Our results indicate that there is no relationship between the geographical origin of the isolates and the groups based on any of the tested characteristics. This suggests that serologically related bradyrhizobia within a given population may be phenotypically distinct, and also that geographically separate regions may contain bacteria with similar nodulation and genetic characteristics.

Recently, Schmidt et al. (27) extended previous studies (5, 7) and found that serogroup 123 consists of the closely related, immunologically cross-reactive serotypes 123, 127, and 129. They proposed the term serocluster to reflect the interrelationship that is observed among the serogroups. Our data and those of Keyser and Cregan (16) suggest that, with few exceptions, isolates within a given nodulation class (and hence restriction enzyme, hybridization, and protein groups) are members of the same serotype. That is, isolates within the low (group 3) nodulation class are all members of serotype 123, with the exception of isolate KS5-2c (serotype undetermined); and isolates in the medium nodulation class (group 2), with the exception of MN5-4a (serotype 123), SC2-3c (serotype 127), and MS6-4a (serotype undetermined), all belong to serotype 129. The high nodulation class, on the other hand, contains isolates in both serotypes 127 and 129 and most likely reflects the genetic diversity within this subgroup. Aside from serological relationships, the diversity within the group 1 isolates was also noted by using restriction enzyme digestion patterns, SDS-protein profiles, and *nif* hybridizations. Our results also indicate that some of the group 1 isolates had restriction enzyme digestion patterns and *nif* hybridizing sequences which appeared to be more similar to those of some of the group 3 isolates than to those of the other isolates in group 1 or to those in group 2. These results and those of Schmidt et al. (27) suggest that some of *B. japonicum* serotype 127 isolates appear to be genetically more related to serotype 123 isolates than to isolates belonging to serotype 129.

In summary, our results suggest that, at least within the restricted and partially restricted nodulation classes, there is a relationship between the serological, genetical, and physiological properties of an isolate and its nodulation characteristics. While there is generally good agreement between the class designations based on nodulation and the groups made based on the criteria listed above, however, the exceptions that have been noted point to the genetic diversity within this group of isolates and indicate that a larger sample of serogroup 123 isolates need to be examined before a complete understanding of the relationships between these characteristics and nodulation can be ascertained. In addition, as more *nod* and host specificity genes are identified, they could be used as probes to provide for a finer subdivision of the isolates within a given nodulation class.

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